MiT Family of Transcription Factors Regulates B cell Development and Immune Tolerance

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II. KEYWORDS AND ABBREVIATIONS

Keywords:

Naïve: not having being exposed previously to an antigen.

Autoimmunity: caused by autoantibodies or T cells that attack molecules, cells, or tissues of the organism producing them.

Abbreviations:

Ab, Antibody

AID, Activation Induced Deaminase

APCs, Antigen Presenting Cells

B, B cell

BAFF, B cell-activating factor

BCR, B Cell Receptor

b-HLH-Zip, basic helix-loop-helix/Leucine zipper

BR, basic region

B/T, B and T cell

CD, Cluster of Differentiation

CD40L, CD40 Ligand

CSR, Class Switch Recombination

Fas, Fas receptor

FADD, Fas-associated protein with death domain

FDC, Follicular Dendritic Cells

FasL, Fas Ligand

FO, Follicular

GALT, gut-associated lymphoid tissue

GC, Germinal Center

Heavy chain, H-chain

HSC, Hematopoietic Stem Cell

Ig/IgH/IgL, Immunoglobulin/ Immunoglobulin Heavy Chain/ Immunoglobulin Light Chain

IgM-IC, IgM-immune complexes

IFN-α, interferon-alpha

Lpr, lymphoproliferative

LPS, Lipopolysaccharides

LZ, Leucine zipper

MZ, Marginal Zone

MiT, Microphtalmia Family of Transcription Factors

Mitf, Microphtalmia associated Transcription Factor

Mi, microphtalmia

MS, Multiple Sclerosis

NLS, nuclear localization signal

Ns, not significant

Optimal Cutting Temperature, OCT

PALS, peri-arteriolar lymphoid sheath

PAS, Periodic Acid Schiff

PAMPs, pathogen-associated molecular patterns

PC, Plasma cell

PerC, Peritoneal cavity

PE, Phycoerythrin

PP, Peyer's patch

preBCR, pre-B Cell Receptor

RA, Rheumatoid arthritis

RBC, Red Blood Cell

RF, Rheumatoid Factor

SHM, Somatic Hypermutation

SLC, Surrogate Light Chain

SLE, Systemic Lupus Erythematosus

Spf, specific pathogen free

T-1/T-2, Transitional-1 and Transitional-2

T, T cell

TCR, T cell receptor

T_H, T Helper cell

TD/ TI, Thymus-dependent/ Thymus-independent

TDN, Trans dominant negative

TdT, terminal deoxynucleotidyl transferase

TLR, Toll-like receptor

TNF, Tumor necrosis factor

VDJ, Variable, Diversity and Joining

VEGF, Vascular Endothelial Growth Factor

III. ABSTRACT

B cells are the sole producers of antibodies that are essential for protective immunity, but when directed against self cause severe autoimmune disease. In the current work, I analyzed the mechanism of regulation of B cell development, function, and tolerance to self by the MiT family of transcription factors by studying how their functional absence in mice affects these B cell properties. Previous studies on Mitf, a MiT member, suggested its importance for maintaining a sufficient threshold of B cell activation needed to curb benign autoantibody production. However, the experimental design of those studies left open the questions of to what extent this was B cell intrinsic and whether other MiT family members were involved. To address these issues, I developed new models of MiT family inactivation designed to reveal B lymphocyte-specific functions of the MiT family. Among these are transgenic mice that express in lymphocytes a recombinant trans dominant negative (TDN) protein that inhibits all MiT family proteins by forming inactive heterodimers. Additionally, I used mouse lines with genetic deficiencies in Mitf, TFE3 and/or TFEB. Using these complementary mouse genetic approaches of MiT family inactivation, I determined 1) that Marginal Zone B cells, critical for antibody responses against encapsulated bacteria, and peritoneal B-1a B cells, a major source of immunoregulatory natural IgM antibody, both depend on TFEB and TFE3; 2) that simultaneous inactivation of Mitf, TFE3, and TFEB in B cells leads to T helper dependent splenomegaly, follicular dysplasia, elevated spontaneous plasma cell development, elevated IgG, and benign autoantibody production, and that 3) MiT inactivation in B cells dramatically accelerates and exacerbates lupus-like autoimmune disease caused by the *lpr* Fas mutation. My studies reveal the critical importance of the MiT family and individual MiT members to B cell development and tolerance. These findings identify new pathways and possible therapeutic target candidates that may be used to control pathological B cell responses.

IV. INTRODUCTION

Early B cell development

B cells, the source of antibodies (Ab), are among the most well characterized of hematopoietic cells, and their extensive study has help shape research on the development of other blood cell lineages. Hematopoietic Stem Cells (HSCs) are the ancestors of all blood cell lineages, which include lymphocytes (B and T cells), myeloid cells (including the monocytes, dendritic cells and macrophages) and erythroid cells (Red Blood Cells: RBCs). HSCs reside primarily in fetal liver during embryogenesis and in bone marrow following birth. In mammals, B cells develop from them in the fetal liver and then adult bone marrow. In these "primary" lymphoid organs, B cells begin to differentiate and rearrange their antigen receptor loci. The B cell antigen receptor genes - those encoding the immunoglobulin (Ig) heavy and light chains (IgH and IgL, respectively) - are generated in a step-wise manner. First, the Variable (V) Diversity (D) and Joining (J) gene segments are joined in the IgH locus. During this process, so-called N-region nucleotides, non-templated nucleotides that contribute to the diversity, are added at the junctions of the gene segments by the template-independent DNA polymerase terminal deoxynucleotidyl transferase (TdT), giving the assembled V regions of IgH genes additional diversity in the amino acid sequences that will bind to antigen (1).

A mechanistic dilemma is that, although millions of diverse IgH-chain genes are assembled, most are not going to encode functional proteins, either from out-of-frame assembly or structural defects. This is resolved developmentally by undergoing a functionality test: once the IgH gene rearrangement is complete, the IgH protein, if made, must pair with a L-chain-like molecule, known as the Surrogate Light Chain (SLC), to create the pre- B cell receptor (pre-BCR). Signaling through the preBCR activates proliferation, promotes survival, and propels subsequent developmental steps. Pro-B cells that synthesize no H-chain or H-chains that fail to associate with the SLC will not progress and are eliminated due to the lack of the preBCR signal.

The SLC is an invariant IgL-like molecule that serves as a IgL-chain template, screening IgH-chains for future IgL compatibility, and through its role as part of the preBCR, helps shape the IgH repertoire to insure its functionality (2, 3). As mentioned above, assembly and function of the preBCR is a critical checkpoint for the success of the V(D)J recombination process during antigen-independent B cell development (development in the absence of antigen) as it is required for pre-B cell differentiation (1). The preBCR signal promotes differentiation in part by repressing SLC gene expression and enhancing IgL-chain rearrangements; IgL-chain recombination proceeds by the VJ recombination process (L-chains do not possess D regions) until an IgH-compatible IgL is synthesized, resulting in the mature B Cell Receptor (BCR) (4). The BCR signal, if not self-reactive (see below), terminates IgL-chain rearrangement and activates subsequent steps in maturation, including the egress of cells from the bone marrow to secondary lymphoid organs such as the spleen and lymph nodes. While some V (D) J rearrangements occur more frequently than others, in general, the emerging B Cell Receptor (BCR) repertoire contains a quasi-random assembly of V region exons, and the end product is a diverse anticipatory repertoire of Igs that collectively can recognize antigens from innumerable possible pathogens (5).

The antigen binding identity of newly created BCRs must also be functionally tested because the VDJ assembly process does not “know or design” the antigen (Ag) specificity of Ab genes created. Rather, a built in risk of the quasi-random assembly process is that by chance a high frequency of BCRs will bind self-antigens. It has been estimated that up to 50% of newly formed immature B cells in the bone marrow express BCRs that are strongly self-reactive (4, 5, 6). Under normal circumstances, mechanisms of self-tolerance eliminate these BCRs from the B cell repertoire while still in the bone marrow. To achieve self-tolerance, autoreactive B cells are programmed to undergo *receptor editing*, in which self-antigen binding, through BCR signaling, reactivates rearrangement of IgL segments primarily, resulting in a change of the VL region and thus a change in specificity of the BCR. Alternatively, *clonal deletion* can occur in which BCR signaling results in apoptosis. This occurs if the B cell cannot successfully edit or if interactions with self-Ag are very strong. Cells that successfully edit its BCR, and are only weakly or non-self-reactive, mature further. The non-autoreactive immature B cells are then allowed to exit the bone marrow and travel to peripheral organs such as the spleen, attracted by a gradient of chemokines that helps lymphocytes home to secondary lymphoid organs.

The development of B-1 and B-2 B cells

Mature and immunologically naïve B cells are IgM^+ - the first form of the BCR – and are comprised of several different subsets: the B-1 cells, made up of B-1a and B-1b cells, and the B-2 cells, which are follicular (FO) and marginal zone (MZ) cells (6). In part, the subsets reflect the different anatomical locations where these B cell reside, the general antigen binding properties of the BCRs they express, and thus the different immunological purpose each subset is thought to have. The spleen is a good example of this because it is unique among lymphoid organs in that its architecture merges its function in RBC homeostasis with white blood cell immunological surveillance of the blood. The spleen is arranged in well-organized compartments that include the red pulp, where RBC destruction takes place; the white pulp, where most lymphocytes reside and are activated; and the marginal sinus and the marginal zone areas, which are in direct contact with the circulatory system and where macrophages and other B cell subsets reside (Fig 1).

Fig. 1:

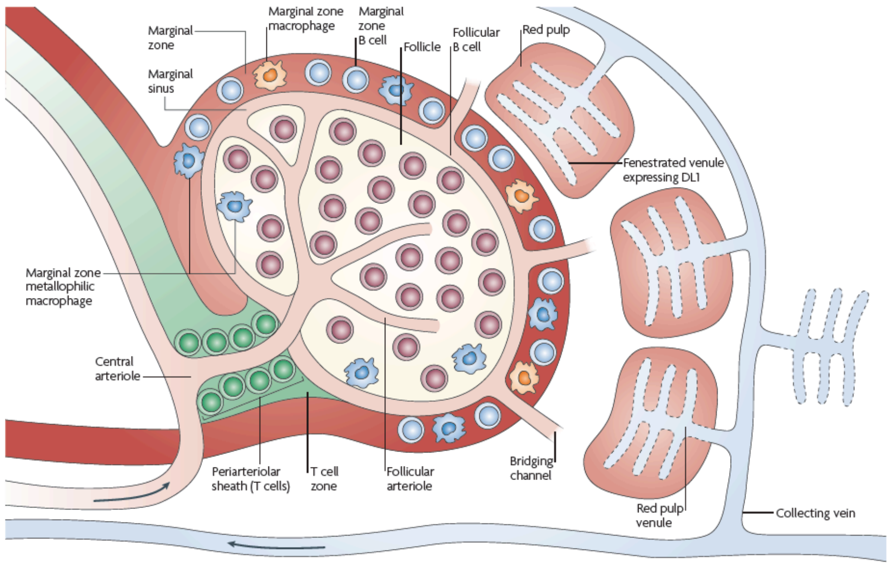


Fig. 1: **Anatomy of the Spleen.** The functions of the spleen are centered on the systemic circulation. The red pulp is a blood filter that removes foreign material and damaged erythrocytes. The spleen also initiates immune responses to blood-borne antigens. This function is charged to the white pulp, which surrounds the central arterioles. The white pulp is composed of three sub-compartments: the periarteriolar lymphoid sheath (PALS, green), the follicle (center and off-white), and the marginal zone (surrounding follicle, dark red) and marginal sinus (in between follicle and MZ, beige) (6).

Immature B-2 cells newly emigrated from the bone marrow that enter the spleen go through subsequent "Transitional" B cell stages (T-1 and T-2) that shape their developmental fates into at least one of two different outcomes: a FO B cell, or a MZ B cell (6). These subsets are characterized by the expression of different levels of surface receptors: FO are $CD21^{low}CD23^{high}$ and MZ are $CD21^{high}CD23^{low}$. These are the complement receptor and the low affinity IgE receptor, respectively. Differentiation into these lineages depends on the properties and strength of their BCR signaling, and is also influenced by environmental signals communicated by the transcription factors Notch-2 and NF-κB, as well as the B cell cytokine BAFF (B cell-activating factor) (6).

The FO B-2 cells are commonly activated during a vaccine immune response. FO B-2 cells, which are located in the follicles, are largely responsible for Ab responses to protein antigens, and can either become Ab secreting PCs, or can undergo an alternative differentiation program into memory B cells. In doing so, they switch their BCR heavy chain (H-chain) from IgM to IgG, IgA, or IgE, depending on the instructions received from the activating T cell (7). This dependency of memory B cell development on T cells is referred to as thymus-dependent (TD): in order to become fully activated and differentiate into plasma cells (PCs) and memory B cells, the mature B cell stimulated by antigen must also obtain help from helper CD4 T (T_H) cells that matured in the thymus and that must also be activated during the antibody response. The molecular signals that helper T cells provide molecular signals to FO B cells are through CD40-CD40L interactions, cytokines and interleukins. After FO B cells are activated, they proliferate into germinal center (GC) B cells, which subsequently differentiate into PCs and memory B cells. This is the goal of vaccine administration, to generate memory B cells and plasma cells (7).

In contrast, B cells of the Marginal Zone (MZ), an area in direct contact with the circulatory system, are the first responders against blood-borne pathogens and often respond to a special subset of immunologically important antigens like bacterial polysaccharides; as such they are recruited in early "innate phase" responses (8, 9). The importance of this subset is evident in individuals who have lost their spleens, because they become particularly susceptible to life threatening infection by encapsulated bacteria. These responses are considered thymus-independent because activation occurs in the absence of MHC-II dependent T cell help; appropriately since polysaccharides cannot be processed through the MHC presentation system. Instead, innate immune receptors such as Toll-like receptors (TLRs), that recognize pathogen-associated molecular patterns (PAMPs), are activated by pathogenic molecules such as bacterial polysaccharides and nucleotides, serving as mitogenic stimuli for MZ B cells. In mice, MZ B cells are non-recirculating cells that seem to have a lower threshold than recirculating or immature B cells for activation, proliferation and differentiation into plasma cells making them an important potential target for regulatory and effector roles (9).

The last remaining subset of B cells is called B-1. B-1 B cells develop from fetal liver precursor cells and the splanchnopleura region during fetal hematopoiesis (10). B-1 cells can be found in humans and in mice and are divided into two sub-types: B-1a cells, which express CD5 and are involved in innate-like immune responses, and B-1b cells, which do not express CD5, comprise a smaller proportion of B-1 cells, and are critical for clearing bacteria. B-1b cells also undergo clonal expansion upon antigen exposure, and like B-1a cells, B-1b cells are also independent of T cell help (11). They are maintained by self-renewal with limited proliferation since there is no evidence that they arise from any precursor after birth. B-1 are mainly abundant in the peritoneal and pleural cavities, but also reside in the spleen, although there is evidence to suggest that B-1a cells from the spleen and B-1a cells from the peritoneal cavity (PerC) come from different progenitors (9, 11). B-1a cells also reside in the Peyer's patches (aggregated lymphoid follicles surrounded by epithelium in the gut) and are responsible for most of the IgA-producing plasma cells in the gut lamina propria. IgA is critically important for protection against enteric pathogens.

Like any other B cell, antigen specificity is crucial for B-1 cells' development and function. B-1 cells secrete IgM antibodies that tend to be polyreactive and can react weakly against self-antigens, though are of low affinity but higher avidity. Examples of common specificities are oxidized lipids and antigens such as Annexin IV and phosphatidylcholine expressed by apoptotic cells (12). They have a low activation threshold and have been reported to spontaneously secrete antibodies (12). They are generated in the absence of foreign antigen, and this feature is why these polyreactive antibodies are termed the so-called "natural antibody" IgM repertoire. It is the secretion of these natural antibodies by B-1 cells that also aid in the maintenance of tissue homeostasis. For example, they bind to altered self-antigens, such as oxidized lipids and those expressed by apoptotic cells, and the action of these natural IgM in clearing the damaged cells preventing inflammation, that could lead to autoimmune reactions (12). The binding of these self-antigens helps shape the BCR repertoire of B-1 cells, serving as a template for evolutionary 'useful' antibody specificities (13). In this way, IgM works as an immunomodulatory molecule, preventing inflammatory responses. Thus, not only do they have a protective role against pathogens, but B-1a cells also play an important role in preventing autoimmunity by the clearance of apoptotic cells with their regulatory antibodies (e.g. natural Igs) (13). B-1 cells not only produce natural antibodies, but some cells also make specific antibodies against a variety of pathogenic molecules such as phosphorylcholine of gram positive bacteria, lipopolysaccharides (LPS) of gram negative bacteria, and various viral and parasite antigens, making these cells important in the first line of defense (9, 11).

Tolerance, Autoimmunity, and the role of B-1a and MZ cells

In any organism, the immune system is designed to protect against invading pathogens, and to also exclude reaction to self-molecules. In general, an organism is tolerant to self- antigenic substances, and the loss of this self-tolerance leads to the development of autoimmunity. An autoimmune disease can start when self-reactive lymphocytes evade tolerance mechanisms, such as negative selection, and are activated. Tolerance is developmentally divided into two components: *Central Tolerance* and *Peripheral Tolerance*. In Central Tolerance, immature lymphocytes that recognize self-antigens are eliminated by apoptosis or editing in primary lymphoid organs (bone marrow and thymus); and in Peripheral Tolerance, mature self-reactive lymphocytes, upon meeting self-antigens in the peripheral lymphoid organs, are either eliminated or rendered anergic (silenced) (14). When these tolerance mechanisms fail, autoimmune disease can ensue. Examples of autoimmune diseases are systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). These diseases are characterized by the production of pro-inflammatory autoantibodies, such as anti-nuclear antibodies against DNA and RNA and rheumatoid factors (RF), which are antibodies against the Fc portion of IgG.

Since the vast majority of cells that exhibit self-reactive BCRs are eliminated, edited, or anergized by negative selection, how is it possible that B-1 cells with weakly autoreactive BCRs are permitted during B cell development? It is believed that the low affinity signal for self-antigens from B-1 cell BCRs might be counterbalanced by the negative regulator of BCR signaling, CD5, allowing for evasion of negative selection (12, 13). Moreover, there is evidence showing that auto-antibody producing B-1 cells are in fact positively selected based on the weak self-reactivity of their BCR. B-1 cells contain BCRs that lack TdT-dependent N nucleotides in their CDR3 regions and thus are generally able to preserve germline encoded specificity for certain self-antigens. These self-reactive B-1 cells may be selected by specificity for self, but also have a protective role against foreign pathogens (15). For example, anti-Th-1 (glycoprotein) autoreactive B-1 cells express V_H3609 and the other autoreactive B-1 cells, specific for phosphatidylcholine (PtC) antibodies express V_H11 and V_H12 (15, 16). Both of these antibodies are protective against bacterial pathogens (15, 16).

However, it may be exactly because of these properties of producing poly-reactive natural antibodies capable of acting in a TI manner, that MZ and B-1a cells may become involved in autoimmunity (17, 18, 19, 20). As mentioned above, MZ and B-1a B cells are known to become plasma cells in a TI manner upon stimulation through their BCR and/or TLRs. Signaling through these receptors is critical to develop an Ab response. Like B-1 cells, MZ B cells' positive selection involves a BCR signal to generate and maintain this subset (9, 11). However, rather than being anergized by negative selection, some, such as murine autoreactive anti-Smith (Sm) B cells, can be positively selected into the MZ B cell and B-1 cell pool, becoming a potential danger (19). In summary, B1a cells and MZ B cells can make autoreactive natural IgM antibodies that not only have regulatory properties that protect against bacterial infection, but also recognize self-material and facilitate its "safe" clearance, thus preventing the development of autoreactivity that leads to an autoimmune disease.

Dynamics of B cell activation

In order to make specific antibodies and memory against pathogens, B cells need to be activated by T cells and undergo a germinal center (GC) reaction. This is primarily the function of FO B cells. The germinal centers are microenvironments where B cells are actively proliferating, undergoing somatic hypermutation (SHM) and class switch recombination (CSR) and eventually generating both Ab secreting PCs and memory B cells (21). SHM is the process that introduces point mutations to the Ig V regions of the BCR genes during an immune response with the goal of creating higher affinity Abs. In CSR, DNA recombination results in isotype class switching, in which the H-Chain class of the Abs produced by an activated B cell switches from IgM to IgG, IgA or IgE. Both SHM and CSR require the action of activation induced deaminase (AID), an enzyme which catalyzes deamination of deoxycytidine residues resulting in the conversion of C:G pairs to U:G pairs. This is followed by repair of the U:G mismatch, generating different mutational outcomes. If any of these mutational processes result in a B cell with a lower affinity or non-functional BCR, the cell undergoes apoptosis. The end result of activity in the GC is a population of B cells that expresses high affinity class switched Abs (21, 22). These can become PCs or memory B cells that remain in the body as long-lived cells in a resting state. They can be re-activated to become plasma cells in future infections when stimulated by the same or immunologically related antigens (known as "antigen-retrieved" or "recall" responses).

How does the memory B cell differentiation program during the GC reaction get activated in a TD response? The GC reaction starts by stimulating the B cells to divide in response to its specific antigen along with other environmental signals (22). First, naïve B cells just stimulated by cognate antigen migrate from the follicle border into the inter follicular zone, an area where T cells and B cells meet, driven by the upregulation of chemokine receptors in response to their antigenic encounter. Once in the zone, B cells become fully activated as a result of $CD4^{+}$ T cell interaction (23). This reaction requires the interaction of co-stimulatory B cell-surface receptors with ligands expressed on the surface of T cells and/or APCs (23). The most important co-stimulatory receptors are the Tumor Necrosis Factor (TNF)-receptor family member CD40, expressed by all B cells, and its ligand, CD154 (CD40 Ligand-CD40L), which is expressed by helper T cells (23). Activated B cells can develop directly into either a plasma cell or into GC-precursors and move to primary follicles, an area made of IgM^+IgD^+ B cells and Follicular Dendritic Cells (FDCs) (23). These activated B cells undergo a burst of proliferation that creates the GC, and after proliferation cease, they might differentiate into long-lived plasma cells, if they find survival niches located in the bone marrow, short-lived PCs or memory B cells. In summary, the mechanisms that control B cells during a GC reaction are highly regulated, and uncontrolled changes caused as an unintended side-product of the SHM and CSR mutations, such as translocations and point mutations of oncogenes, are known to lead to pathology such as B cell malignancy.

<u>Microphthalmia Transcription Factor Family (MiT Family)</u>

Transcription factors orchestrate the changes in gene expression that underlie differentiation, proliferation, activation, growth and death of cells. In the B cell lineage, many transcription factors such as, Pax5, E2A, PU.1, Ikaros, Bcl-2, Bcl-6, Blimp-1, Xbp-1, IRF4/8 and Mitf, have essential developmental roles. For example, Pax-5 is necessary for the establishment and maintenance of B cell identity until the plasma cell stage, and it works in concert with E2A and PU.1 to activate expression of B cell specific genes while repressing expression of genes defining other hematopoietic cells. However, in order for B cells to differentiate into plasma cells, Pax5 needs to be repressed. Pax-5 blocks transcription factors required for PC differentiation, factors like X-box binding protein-1 (Xbp-1). Plasma cell development also depends in the expression of Interferon Regulatory factor-4 (IRF-4), which activates B-lymphocyte induced maturation protein-1 (Blimp-1) (24). A factor that inhibits PC differentiation is Mitf. Mitf helps mature B cells to remain in their resting state and in its absence, IRF4 expression occurs more readily and plasma cells form spontaneously more frequently. Thus, Mitf appears to restrain B cell activation by acting as an inhibitor of IRF4, indirectly inhibiting Blimp-1 expression (24).

Mitf is one member of the MiT family. The MiT family includes four genes that are expressed in mammals: MITF, TFE3, TFEB and TFEC, which encode for transcription factors of the basic helix-loop-helix/Leucine zipper (b-HLH-Zip) class (Fig. 2) (25). These proteins can form homodimers or heterodimers with other members of the MiT family but not with other b-HLH or b-HLH-Zip proteins. Homodimers or heterodimers are required for these factors to bind to the DNA via a basic region (Fig. 2).

Fig. 2:

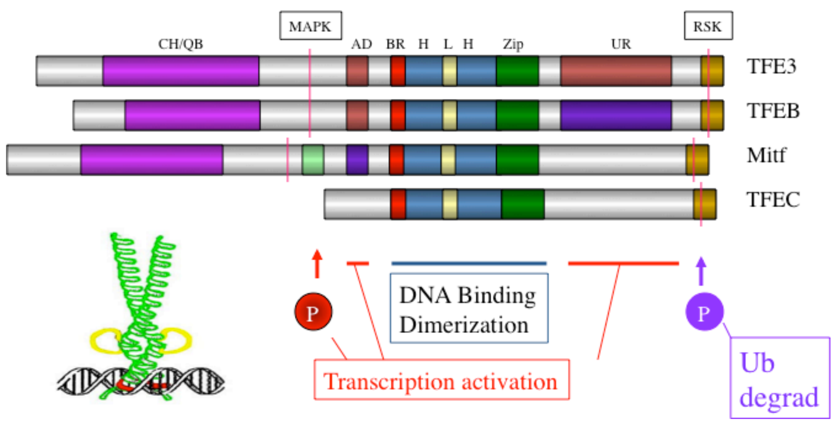


Fig. 2: Structure of the MiT family of transcription factors. All four members share homology sequence among the dimerization domain -Helix-Loop-Helix-Leucine zipper- (middle: blue, yellow and green). The family binds through the DNA binding domain [Basic region (BR) in red]. On the left, there is an example of how this proteins forms dimers and binds to DNA.

Mitf was first isolated as the gene responsible for the *microphtalmia* mouse mutant, being critically important for the development of melanocytes as well as the pigmented retinal epithelium cells (26, 27). Mitf plays an important role in osteoclasts, which are required for bone remodeling, and plays a role in mast cell development (28). Similarly, in humans, Mitf mutations cause deafness and other pigmentation cell disorders, including Waardenburg Syndrome type 2A (WS2A) and Tietz' Syndrome. Other members of the family, TFEB and TFE3, were isolated from B cells through functional screens as genes that encode for E-box DNA binding proteins (27). The homozygous TFEB knockout is embryonic lethal, as it has an essential role in placental vascularization: its absence causes the lack of expression of Vascular Endothelial Growth Factor (VEGF) in labyrinthine trophoblast cells, which form the feto-maternal exchange surface (29).

The importance of TFEB to B cell function is not known, but recent findings of a role for TFEB in autophagy, suggest that this transcription factor could play a role in the maintenance of B-1a cells in the periphery and of PCs (30, 31, 32), both cell types with established dependence on autophagy for homeostasis. TFE3 functions redundantly with Mitf in osteoclast development – both TFE3 and Mitf must be inactivated to reveal an osteoclast defect, which is why only the dominant negative alleles of Mitf that also inactivate TFE3 have osteopetrosis - but TFE3 knockout mice are phenotypically normal with no reported changes to osteoclasts or B cell function (33, 34). However, in T cells, TFEB and TFE3 together are necessary for CD40L expression, having a redundant role in this capacity (35). Thus, the lack of an obvious phenotype of TFE3 deficiency may be due to functional overlap with TFEB or another MiT family member. The last family member, TFEC, is expressed in monocytes but is not expressed (and has no role defined so far) in lymphocytes, and TFEC knockout mice appear to be phenotypically normal. Similarly, it is possible that the reason for the lack of phenotype in the TFEC$^{-/-}$ animals is that the expression of another MiT family member is acting in a redundant manner (36). Together, the MiT family of transcription factors has diverse and critical roles in the immune system of mammals, but the exact role of these proteins have over B cells still unclear.

V. SPECIFIC AIMS:

A. Aim I: To determine the role of the MiT family of transcription factors in intrinsic B cell development.

Mitf is thought to work by regulating B cell activation and thus restrain potentially autoreactive B cells (24). However, in those studies, the nature of the Mitf mutation as a dominant negative protein (Mitf$^{mi/mi}$) that could also inhibit TFE3 and TFEB, and the experimental design, using transplantation of Mitf$^{mi/mi}$ bone marrow, left open the questions of the extent that these effects were B cell intrinsic and whether other members of the MiT family were involved. To answer these questions, I analyzed B cell development and function in a panel of genetically modified mice in which MiT family members – either individually or collectively - were inactivated in different ways (see Table I). In the TDN-B model, all MiT family members are inhibited only in B cells. Relatedly, in the TDN-B/T model, all MiT members are inhibited in B and T cells. Impaired T cell help due to reduced CD40L expression in T cells of the TDN-B/T mice will help determine if any changes I observe in TDN-B mice are dependent on T cell help. Complementary models of genetic MiT deficiency are mice that carry null alleles of Mitf, TFE3, and/or TFEB. Mitf$^{vga/vga}$ mice are Mitf recessive null due to a deletion in the promoter region of Mitf (24, 26), whereas the TFE3 mutation is a conventional germline knockout (33). Because of the embryonic lethality of the genetic absence of TFEB during development, the role of this MiT member in lymphocytes required creation of a conditional allele of TFEB that could be deleted exclusively in B cells using the B cell specific *Cre* transgene CD19-Cre to generate TFEB-deficient B cells (38). Using these animals, the status of B cells and their antibody products were evaluated. B cells in different lymphoid organs - spleen, peripheral and mucosal lymph nodes – were enumerated and characterized by flow cytometry, and splenic architecture evaluated by immunohistochemistry and immunofluorescence. Antibody titers in sera were measured by ELISA.

<u>B. Aim II: To examine whether the deficiency of the MiT family of transcription factors in B cells promotes the development of autoimmunity.</u>

To further characterize the role of the MiT family of proteins in immune dysregulation and autoimmunity, we crossed our B cell specific TDN-B mice and our T and B cell specific TDN-B/T mice into the autoimmune disease prone B6.*lpr* background. B6.*lpr* mice have a defect in Fas dependent negative selection of both B and T cells and exhibit elevated spontaneous auto-Ab production (39, 40). The severity of disease caused by the Fas mutation *lpr* is strain specific: models like the MRL.*lpr* show accelerated autoimmune disease by 6 months of age, whereas B6.*lpr* mice exhibit manifestations of only mild autoimmune pathology, at 9 month-1 year of age. B6 mice harboring *mi/mi* B cells do not display autoimmune pathology. They synthesize auto-Abs, but the spectrum of these auto-Abs is not pathogenic (24, 39).

My prediction was that B cell specific MiT-inactivation in the B6.*lpr* background would cause an amplification of spontaneous B cell activation and possibly cause increased and earlier production of pathogenic auto-Abs such as anti-dsDNA, as well as renal disease. If correct this would suggest that the MiT and *Fas* pathways both regulate aspects of B cell negative selection and activation, possibly synergizing to prevent the emergence of pathogenic B cells. An analysis of B cell subsets would help discern which B cells are most affected by the combination of genetic changes and producing the pathogenic antibodies.

<u>VI. AIM I: To determine the role of the MiT family of transcription factors in intrinsic B cell development.</u>

<u>A. Background and Summary of Aim I</u>

<u>Background:</u>

<u>MiT Family in B cell development, homeostasis and activation</u>

My thesis aims to reveal the mechanisms of MiT family involvement in B cell activation and regulation. As mentioned above, Mitf is thought to play a role in helping B cells maintain their resting state. Once B2 cells are stimulated by activating signals via the BCR receptor and CD40 engagement from T cells, B cells proliferate and become GC B cells. Upon activation, Mitf expression is turned off, causing the up-regulation of IRF-4, which further activates Blimp-1 and Xbp-1 through the inhibition of Bcl-6, allowing GC B cells to differentiate into plasma cells (41).

The previous reports that demonstrated the importance of Mitf to B cell activation studied a Mitf mutant called *mi/mi,* which are Mitf deficient due to a mutation in the Mitf basic region of the gene. However, the mutation creates a trans-dominant negative protein that does not bind DNA but can still bind to and inactivate other MiT family members (identical mechanism to our TDN model). The Mitf *mi/mi* mouse presents with lymphopenia, including B cell deficiency, considered a secondary effect of severe osteopetrosis caused by the combined loss of both Mitf and TFE3 function in osteoclasts given that TFE3 was inactivated by the dominant mutant Mitf protein. Thus, it was not possible to discern whether or not the lymphocyte abnormalities observed were due to a B cell intrinsic phenomenon. In a later study, the Peng group (24) revealed through microarray data that Mitf levels in resting naïve B cells are higher than after activation, when they are reduced dramatically. Given the osteopetrosis in *mi/mi* mice, to better elucidate the role of Mitf in B cells and the hematopoietic system, these investigators adoptively transferred *mi/mi* HSCs to syngeneic wild-type irradiated hosts as a means to provide a normal bone marrow environment to study the maturation of *mi/mi* HSCs. They observed that cells from the $Mitf^{mi/mi}$ mice could indeed repopulate the bone marrow of normal mice. However, they also observed that many more B cells were spontaneously differentiating into plasma cells as well as producing benign autoantibodies in naïve mice. This phenotype was recapitulated in cell culture by *de novo* enforced or repressed expression of Mitf in resting B cells, which either suppressed or promoted respectively, the differentiation into plasma cells and secretion of Ig, in response to antigen stimulation. Evidence showed that Mitf acted to restrain B cell activation by repressing of IRF4, itself a transcription factor, which is needed for plasma cell development; in Mitf absence, basal IRF4 levels were elevated (25).

It is important to note that the *mi/mi* model was a transplantation model and the mutation was expressed in all HSCs, so the question as to whether or not the observed effects in vivo were B cell intrinsic or extrinsic remained open, given that other hematopoietic cells transferred could have had their function altered and therefore indirectly affect the B cell. Thus, the major biological question that still remains and that will be addressed by my experiments is how MiT proteins control intrinsic B cell responses to antigen and other environmental cues to secrete antibody, and in what ways it may differ among different B cell subsets. Specifically, my first Aim addresses how the MiT family of transcription factors, which comprises Mitf, TFE3, TFEB, and TFEC, regulates these decisions.

To accomplish this, one model I used was a transgenic mouse model in which an artificial trans-dominant protein, TDN, which forms inactive dimers with all MiT family proteins (akin to the Mitf *mi* protein), was expressed exclusively in B cells (TDN-B). My lab successfully used a similar strategy to reveal the importance of TFE3 and TFEB in T cell function using transgenic mice that expressed the TDN protein exclusively in T cells (TDN-T) (Fig. 3) (35). As it will be shown, this allowed me to evaluate MiT defective intrinsic B cell development *in situ* rather than relying on adoptive transfer (Aim

I). I employed other MiT deficient models including TDN-B/T mice, which express the TDN protein in both B and T cells, Mitf$^{vga/vga}$, with a recessive mutation of Mitf, TFE3-deficient mice, and a TFEB conditional knockout model, which enable me to study the individual and combined contributions of these proteins in B cells (See Table I). I examined and compared B cell development, homeostasis, and activation in these models of MiT family deficiencies.

Fig. 3

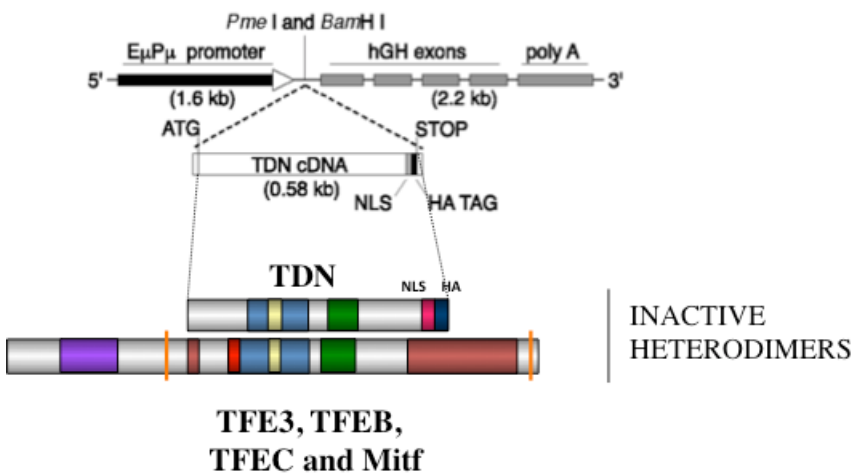


Fig. 3: Schematic of a transgene encoding a Trans-Dominant negative (TDN) protein used to inhibit the MiT family. Top, the transgene used to express the TDN in B cells contains the EμPμ promoter (a human Ig HC enhancer and promoter) the TDN cDNA and sequences (hGH and poly A) needed for RNA processing and stability. Below, the TDN is aligned with a representative MiT member to illustrate the lack of a DNA binding basic region (red) and the presence of the dimerization domains (blue and yellow) that will allow it to form inactive heterodimers. In addition, there is a nuclear localization signal (NLS, a function normally performed by the BR), and a hemaglutinin epitope for immunodetection (HA)

Summary:

To determine the role of the MiT family proteins in B cells, I analyzed mice deficient for all MiT members or deficient for one or more MiT members. MiT deficiency was either global or B cell specific (see Table I). Analysis of the B cell compartments of the mice shown in Table I helped us identify the role of the single or combined mutations and their effects on B cell activation.

Approach: Secondary lymphoid organs of mice were harvested; these included: spleen, peripheral and mesenteric lymph nodes, and Peyer's patches. Leukocytes from the peritoneal cavity that include resident lymphocytes were obtained by lavage. Spleens were weighed, single cell suspensions were made from all organs, cell recoveries counted, and flow cytometry was used to enumerate and characterize the different B cell subsets in each. Histological studies were performed to document splenic architecture and immunoassays (ELISA) were used to analyze the spectrum of antibodies in the serum made from these mice.

Table I. MiT-deficient and MiT member deficient mouse models.

Mouse Models	MiT Inhibition	Cell type in which MiT activity is affected
TDN-B	All MiT members	B cells
TDN-B/T	All MiT members	B and T cells
Mitf ^{vga}	Mitf recessive	All cells
TFE3 $^{-/-}$	TFE3	All cells
TFEB $^{+/-}$ TFE3 $^{-/-}$	TFEB/TFE3	All cells
TFEB $^{flox/-}$ CD19Cre	TFEB	B cells only

B. Results:

TDN-B mice exhibit splenomegaly and increased numbers of spontaneous splenic germinal centers.

Animals used in this Aim, unless otherwise indicated, were ~ 6 months of age. Both males and females were used in all experiments, unless otherwise indicated. TFE3$^{-/-}$ mice used in the next experiments were all males. Control animals were cosanguineous non-Transgenic (non-Tg) C57BL/6 (B6) mice. The contribution of T cell dependent changes was evaluated by comparison of TDN-B mice (B cells only) to TDN-B/T mice. As stated above, TDN-B/T mice express the TDN protein in B cells as well as in T cells, therefore I expected the GC reaction, the hallmark of TD responses, to be impaired because of lower CD40L expression on TDN-B/T T cells (35, 42).

A striking observation was that naïve TDN-B mice have significantly larger spleens compared to naïve non-Tg and TDN-B/T mice (Fig. 4). In addition, the total number of splenocytes was increased significantly in TDN-B compared to control and TDN-B/T animals (Fig. 4 and Appendix A).

To further characterize this observation, histology of the spleens from non-Tg and TDN-B littermates was performed. Hematoxylin and Eosin (H&E) stain of spleen sections revealed an increase in size and in number of follicles and GCs of the spleens of TDN-B mice when compared to non-Tg littermates. As shown in Fig. 4., TDN-B mice displayed elevated numbers of GCs by histology as compared to non-Tg mice (Fig. 4).

Fig. 4

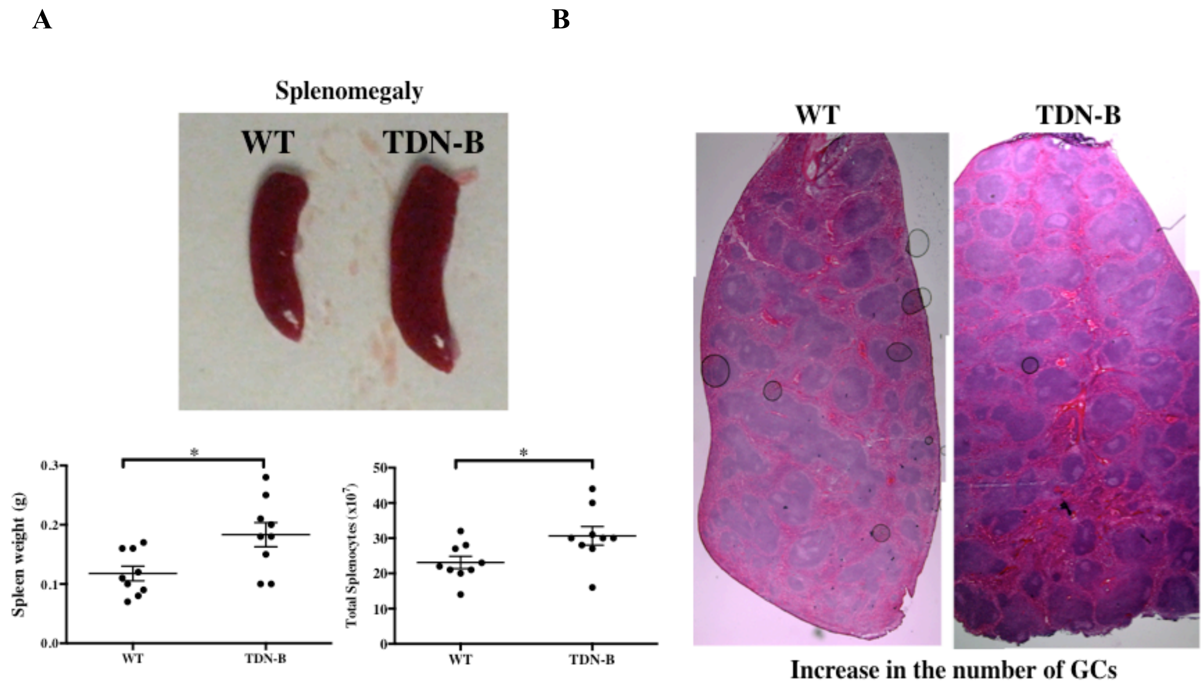


Fig. 4: Splenomegaly caused by MiT inactivation in B cells. A. Photographs of representative spleens from 6-month-old non-Tg (WT) and TDN-B mice are shown only as an illustrative example of a typical relative size difference between each group; the absolute length of an average non-Tg spleen is approximately 1.5 cm (not shown). For a more precise metric of mass, the weights of spleens and number of total splenocytes from non-Tg and TDN-B mice are shown below the photo. Error bars are SEM with n=9. * $p<0.05$ B. Representative spleen sections stained with H&E to visualize GCs of non-Tg and TDN-B mice. Magnification 50X.

I further analyzed the spleens of both groups by immunohistochemistry. Spleen sections of non-Tg and TDN-B animals were stained as described in the methods section. I observed that the $B220^+$ B cells (green) in TDN-B animals were not only in the follicles, as in the non-Tg, but also obscuring the $CD3^+$ T cells (red) in the PALS, as would happen after antigenic stimulation (Fig. 5) (42). It is important to note that, in the TDN-B animals, the CD3 staining is dispersed; in other words, some T cells appear to be out of the follicle (Fig. 5), however, flow cytometry data show that $CD3^+$ proportions are unaffected in TDN-B mice (data not shown). This phenotype is consistent with a hyper-responsive B cell phenotype of the type observed in the *mi/mi* transplantation model; however, whether or not the expansion of B cells in these TDN-B mice is due to hyperactivation still needs to be formally established.

Fig. 5

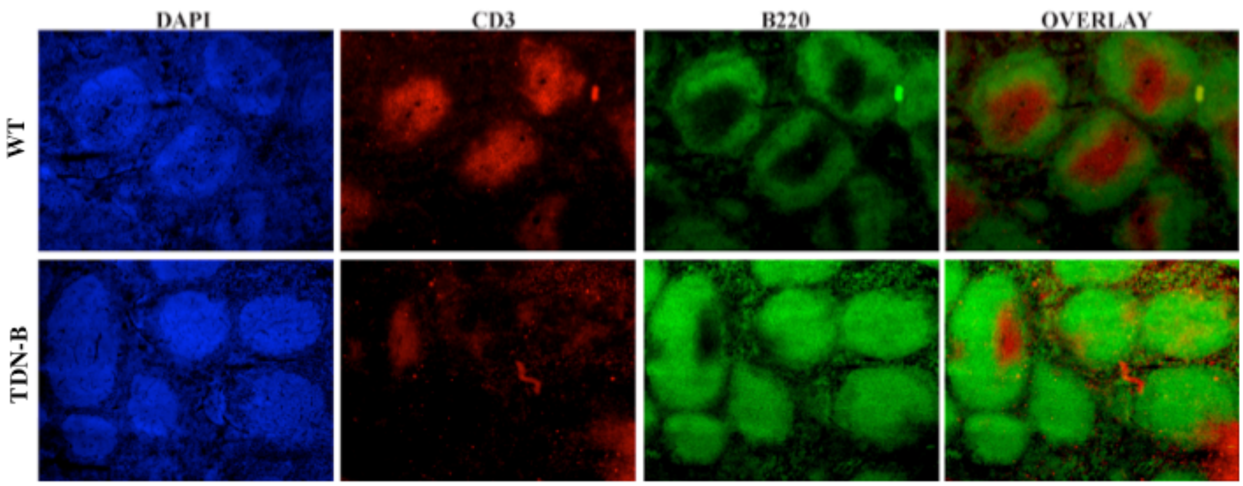


Fig. 5: TDN-B spleens show follicular dysplasia. Photographs of representative spleens from 6-month-old non-Tg (WT) and TDN-B mice are shown. Immunofluorescence dyes and antibodies used were: DAPI (blue; all nucleated cells), CD3 (red; T cells) and B220 (green; B cells). Data is representative of n=5. Magnification 100x.

<u>MiT inactivation in B cells leads to spontaneous PC production and increase in immunoglobulin secretion.</u>

Increased numbers of GCs and splenomegaly in the absence of an intentional immune stimulation are evidence of dysregulation. A prediction is that there should be other indications of perturbations to B cell activation. I observed than in unimmunized TDN-B and Mitf$^{vga/vga}$ mice there was an elevated proportion of plasma cells when compared to non-Tg and TDN-B/T mice (Fig. 6). This PC elevation was also observed in the Mitf$^{mi/mi}$ transplantation model reported by Peng et al (24) I deduced that because of CD40L impairment in T cells in TDN-B/T mice, PCs were not elevated. The elevated PC numbers are indicative of abnormally heightened B cell responsiveness and are in line with the increase in GCs in TDN-B spleens.

Fig. 6

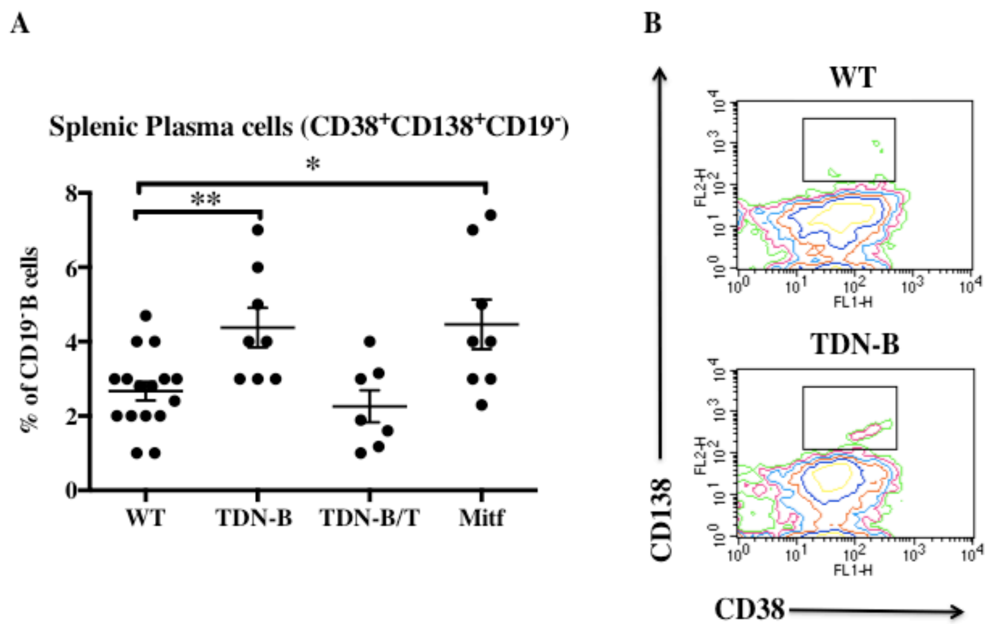


Fig. 6: Mitf inactivation leads to spontaneous generation of plasma cells. A. Scatter plot of percentages of $CD19^-$ cells that are identified as PCs based on being $CD38^+$ and $CD138^+$ ($CD38^+CD138^+CD19^-$), from Non-Tg (WT), TDN-B, TDN-B/T and Mitf $^{vga/vga}$ mouse groups. Each circle represents a mouse. Error bars are SEM with n=7-16 animals per group. *p<0.05 and ** p <0.01. B. Representative staining profiles of plasma cells from one pair of non-Tg and TDN-B mice from A are shown.

The observation that unimmunized TDN-B mice exhibit evidence of B cell dysregulation - spontaneous hyperactivation and elevated PCs - predicts elevated antibody levels. First, I determined the titers of total IgM and total IgG in TDN-B and their non-Tg counterparts. The amount of total IgM in the serum of naïve TDN-B transgenic mice was not statistically different when compared to their non-Tg littermates (Fig. 7, left). However, there was a significant increase in total IgG levels in naïve TDN-B transgenic mice when compared to their non-Tg littermates (Fig. 7, right). This observation correlates with the fact that in naïve TDN-B mice there is spontaneous formation of germinal centers in the spleen and an increase in the percentages of splenic plasma cells, possibly due to hyper-responsiveness of FO B cells and their conversion to IgG^+ B cells.

Fig. 7:

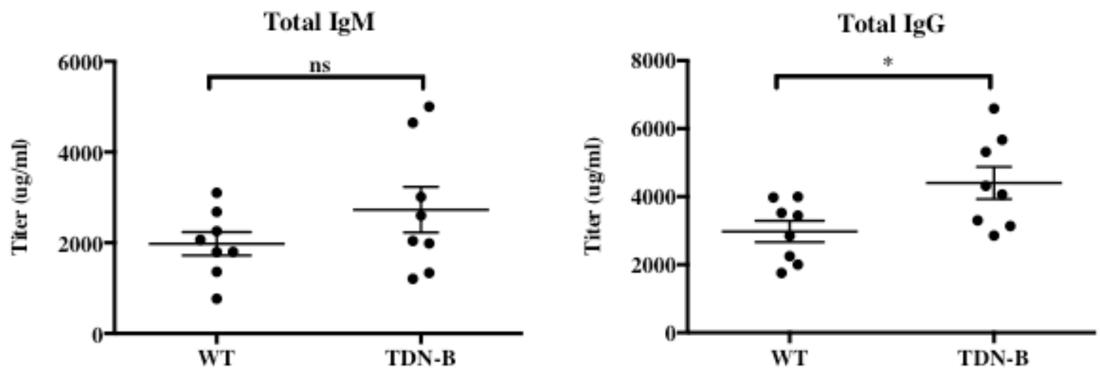


Fig. 7: Increase in antibody titers in unimmunized TDN-B mice. Immunoglobulin (IgM and IgG) assays of sera from naïve TDN-B mice and non-Tg littermates. Each circle represents one mouse. Error bars are SEM with n=8 per group,*p<0.05 and ns, not significant.

Next, I determined which IgG isotypes were responsible for the increase in the total IgG titers in TDN-B mice. IgG isotypes were analyzed from the sera of the animals used for the total IgG assay. I observed that TDN-B transgenic mice did not have a significant increase in the titers of IgG1 and IgG2a when compared to non-Tg littermates (Fig. 8, top panel). However, they do have a significant increase in the titers of IgG2b isotype, while the titers of IgG3 are significantly decreased (Fig. 8, bottom panel).

Fig 8

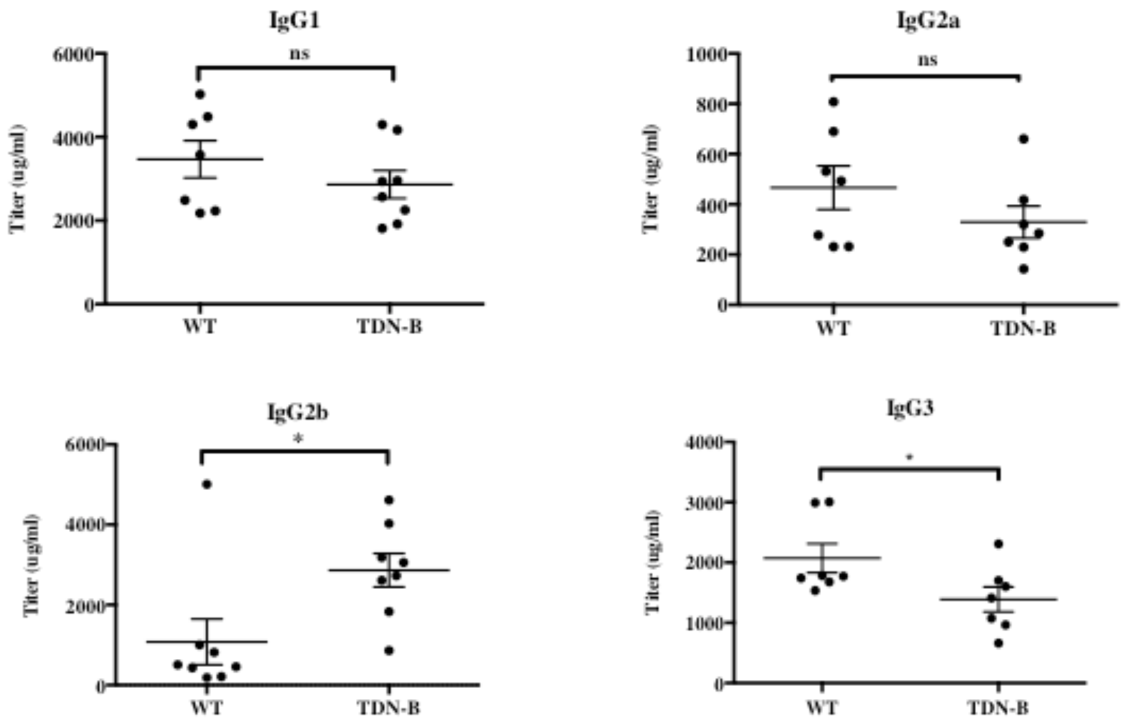


Fig. 8: Naïve TDN-B transgenic mice show differences in the secretion of various IgG immunoglobulin isotypes. Analysis of immunoglobulin IgG isotypes from sera of naïve TDN-B transgenic mice and non-transgenic littermates (WT). Each circle represents a mouse. Error bars are SEM with n=7-8 animals per group; *p<0.05 and ns, not significant.

In summary, TDN-B mice exhibit evidence of B cell dysregulation that leads to a spontaneous increase in total IgG, due to an increase in titers of IgG2b.

<u>**B-1a and MZ B cells are differentially affected by the inactivation of the MiT family.**</u>

On the other hand, analysis of TDN-B mice revealed a significant decrease in serum titers of IgG3 when compared to their non-Tg littermates. Given that T cell independent antigens, either polysaccharides or glycolipids from encapsulated bacteria, stimulate MZB cells in the marginal zone to produce IgM and IgG3 antibodies, next I analyzed the MZ B cell subset. I observed that splenic MZ B cells ($CD21^{high}CD23^{low}CD19^{+}$) were significantly decreased in the TDN-B and TDN-B/T as compared to non-Tg littermates (Fig. 9).

Fig. 9

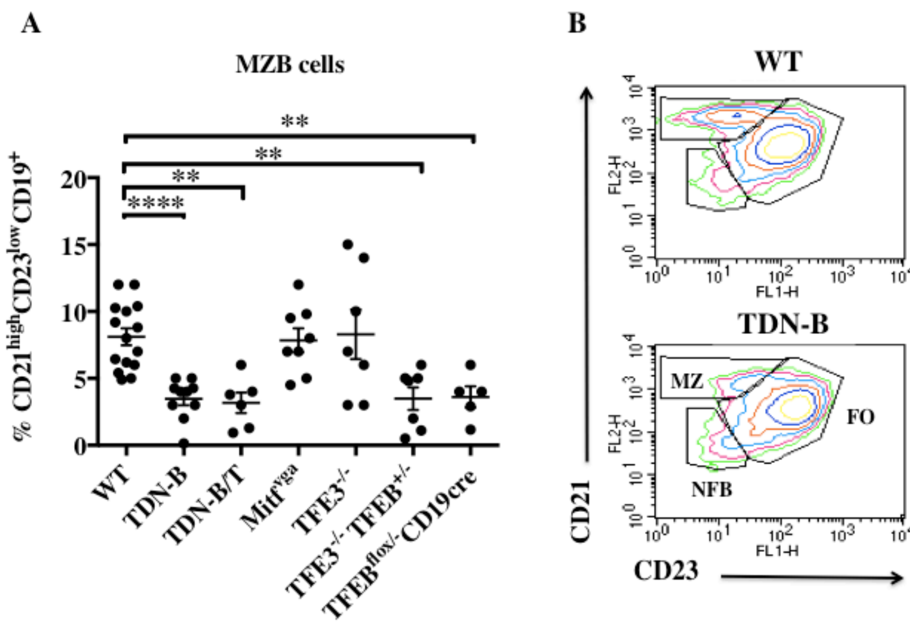


Fig. 9: Inactivation of TFE3 and TFEB affects splenic Marginal Zone (MZ) B cells. A. Percent of total $CD19^{+}$ splenocytes identified as MZ B cells ($CD21^{high}CD23^{low}CD19^{+}$) from Non-Tg (WT), TDN-B, TDN-B/T, Mitf $^{vga/vga}$, $TFE3^{-/-}$, $TFE3^{-/-}TFEB^{+/-}$ and $TFEB^{flox/-}$ CD19cre mice groups are shown. Each circle represents a mouse. Error bars are SEM with n=5-15 animals per group ** $p<0.01$ and **** $p<0.0001$. Representative flow cytometric analysis of splenocytes from Non-Tg (WT) and TDN-B mice are shown.

Interestingly, the decrease in MZB cells was also observed in both younger (< 2 month old) TDN-B and TDN-B/T mice as compared to non-Tg mice (Appendix B), indicating not only that the phenotype is not dependent on T cells, but that it possibly may arise from a defect at an earlier stage of B cell development.

In order to pinpoint which MiT family member is responsible for the observed MZB cell phenotype, we next analyzed individual MiT member deficient mouse models. I observed that there was no difference in proportions of MZ B cells in Mitf$^{vga/vga}$ and TFE3$^{-/-}$ mice compared to non-Tg mice. However, TFEB$^{+/-}$TFE3$^{-/-}$ and TFEB$^{flox/-}$ CD19cre mice showed a significant decrease in the proportions of MZ B cells when compared to non-Tg mice, indicating that TFE3 and TFEB are involved the MZ B defect.

Fig. 10

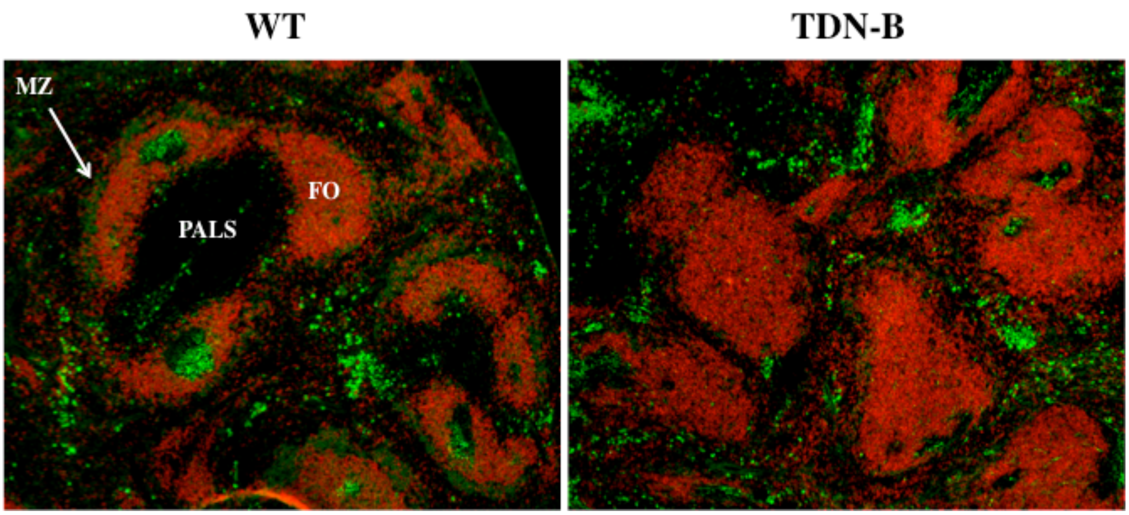


Fig. 10: Marginal Zone of TDN-B mice shows decrease in MZ B cells. Representative spleen sections from 6-month-old non-Tg (left) mice and TDN-B (right) were stained with IgD (red) and IgM (green). MZ B cells appear as faint green around the red follicle, while FO B cells are bright red and present inside the follicles. Bright green staining could represent PCs. This represents an n=5. Original magnification= 200x.

To evaluate at the anatomical distribution of MZ B cells, we used immunofluorescence. Histological sections from both TDN-B and non-Tg littermates were stained with IgD and IgM antibodies to identify the FO and the MZ B cells. TDN-B mice show a decrease of MZ B (IgM^{+}IgD^{low}) cells that in non-Tg controls appear as a faint green halo around the red follicle, which parallels the flow cytometry data. Those few that remained in TDN-B spleens occupied the same regions as in non-Tg. In addition, FOB (IgM^{+}IgD^{high}) cells in the TDN-B mice, were obscuring the PALS area as seen in the immunofluorescence pattern seen with B220^{+} staining (Fig. 10). We believe the bright green positive cells might be PCs (or immune complexes) circulating in the spleen (Fig. 10)

Given that inactivating some members of the MiT family, like TFE3 and TFEB, causes a decrease in MZ B cells, which are known to be independent of T cell activation, I next determined if other T-cell independent B cell subsets were also affected by inactivating the MiT family. I therefore analyzed B-1a cells from the peritoneal cavity (PerC) of the animals. I observed that PerC B-1a cells (B220^{low}CD5^{+}CD19^{+}) were significantly decreased in the TDN-B and TDN-B/T compared to non-Tg littermates (Fig. 11)

Fig. 11

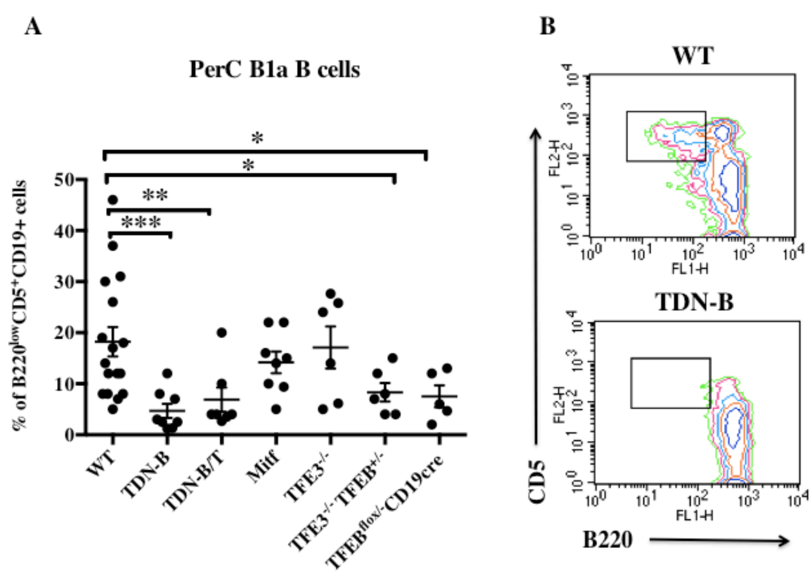


Fig. 11: Inactivation of TFE3 and TFEB affects maintenance of peritoneal cavity (PerC) B-1a cells. A. Percent of total CD19^{+} PerC cells identified as B-1a cells (B220^{low}CD5^{+}CD19^{+}) from Non-Tg (WT), TDN-B, TDN-B/T, Mitf $^{vga/vga}$, TFE3$^{-/-}$, TFE3$^{-/-}$TFEB$^{+/-}$ and TFEB$^{flox/-}$ CD19cre mouse groups are graphed. Each circle represents a mouse. Error bars are SEM for n=5-16 animals per group. * p <0.01, ** p <0.01 and *** p <0.001. **B.** Representative flow cytometric analysis of a Non-Tg (WT) and TDN-B.

I also analyzed other MiT deficient mouse models, in which one or more MiT members were inactivated, in order to determine which MiT member is responsible for the B-1a phenotype. I observed that there was no difference in the proportions of PerC B-1a cells in Mitf$^{vga/vga}$ and TFE3$^{-/-}$ mice. However, TFEB$^{+/-}$TFE3$^{-/-}$ and TFEB$^{flox/-}$ CD19cre mice showed a significant decrease in the proportion of PerC B-1a cells, indicating that TFE3 and TFEB are involved the PerC B-1a cell phenotype.

Because B-1a cells develop during embryogenesis and are the first B cell subset to arise, I analyzed the PerC B-1a subset in younger cohorts of TDN-B, TDN-B/T and non-Tg (WT) littermates to ask whether, like the MZB cell phenotype, it starts early in post-natal development. I observed that PerC B-1a cells (B220^{low}CD5^{+}CD19^{+}) are present in younger (< 2 month old) TDN-B, TDN-B/T at levels comparable to the non-Tg littermates (Appendix E). I concluded that as these animals aged from 2 to 6 months, these mature TDN-B mice experienced a loss of B220^{low}CD5^{+}CD19^{+} PerC B-1a cells.

Upon B-1a cell activation in the PerC, cells can migrate to Gut-Associated-Lymphoid Tissues (GALT), including the Peyer's patches (PP), mesenteric lymph nodes, and the lamina propria. Therefore, I analyzed the PP of 6-month-old TDN-N and non-Tg littermates. Although there was no significance difference in the number of B-1a cells in the PP of TDN-B compared to non-Tg mice, the B-1a cells are starting to be lost from this cavity (Appendix D). In conclusion, the B-1a cells are found in the GALT but are not maintained in the PerC throughout development in mice with inactivation of both TFE3 and TFEB.

<u>C. Materials and Methods:</u>

Mouse models for MiT family inactivation and deficiency. TDN protein expression in the B cell or B plus T cell lineages was directed by immunoglobulin heavy-chain enhancer and promoter sequences [Fig. 4: (35)]. The TDN protein contains an HA-tag used to immunologically identify the protein, and transgenic line-specific expression patterns were confirmed by western blot (data not shown) and by flow cytometry (using anti-HA FITC; data not shown). TFE3-deficient mice have been described (26) and were crossed with mice with a conventional germline knockout allele of TFEB to generate compound heterozygotes (TFEB$^{+/-}$TFE3^{+}). The Mitf recessive (*vga/vga*) mice are described in ref (24). The conditional allele of TFEB will be described in completion elsewhere, but briefly, it was created by insertion of lox P sites flanking the exons encoding the basic region and helix-loop-helix regions; expression of *Cre* deletes these and creates a null allele. A cross with EIIa- *Cre* mice, which express *Cre* in a mosaic fashion early in embryogenesis, allowed for the selective deletion of the TFEB in some gamete progenitors without compromising the whole embryo, and progeny mice carrying such deleted alleles were used in the intercrosses with TFE3 knockout mice. CD19cre was used to delete this allele exclusively in B cells (38). Deletion was confirmed by PCR.

Animals. Mice were maintained in specific pathogen-free (spf) conditions (5 mice per cage and 12 hour light/dark cycle) with sterilized food and water. Mice were euthanized by CO_2 asphyxiation according to DLAR and IACUC standards, and under an IACUC approved protocol.

Serum and Cell Suspensions. Serum was prepared from blood taken immediately after CO_2 asphyxiation by cardiac puncture. To isolate B cells and their specific subsets such as B-1 and B2 cells from multiple organs and sites, the PerC cells were first harvested by a lavage with ice-cold isolation buffer (3% FBS in 1x PBS). Next, the spleen was isolated and weighed, followed by lymph nodes and Peyer's patches. To make splenic single cell suspensions, the spleen was gently ground using a pestle and the suspension is passed through a 100μm cell strainer (Fisher). Bone marrow cells were isolated from femurs and tibias of both legs by washing them out with ice-cold 1X PBS using a syringe. RBCs are removed from all cell suspensions by RBC lysis buffer (Miltenyi Biotech). Recovered bone marrow, spleen and peritoneal cells were then stained with Trypan Blue 0.4% (Gibco, Grand Island, NY) and counted using a hematocytometer.

Antibodies. The following antibodies were used: for B2 cells: anti-mouse IgM-PE, IgD- FITC, B220-FITC, CD21-PE, CD23-FITC and CD19-PerCP-Cy5.5 (BD Biosciences). B-1 cells: anti-mouse B220-FITC, CD5-PE, CD11b-FITC, CD19-PerCP-Cy5.5 (BD Biosciences) and CD9-FITC (eBiosciences). T cells: anti-mouse CD4-FITC and PerCP-Cy5.5, CD3-PE and CD8-PE and PerCP-Cy5.5 (BD Biosciences). GC B cells: CXCR5-PE, CD38-FITC, B220-FITC, GL-7-FITC, CD95-PE, PD-1-FITC (BD Biosciences, eBiosciences). For PCs: anti-mouse CD38-FITC, CD138-PE and CD19- PerCP-Cy5.5 (BD Biosciences). The following activation markers for B cells were used: CD80-PE, CD86-PE, MHCII-FITC, GL7-FITC (BD Biosciences), and CD69 (Southern Biotechnology). For intracellular staining of HA protein, rat monoclonal anti-HA Fluorescein High Affinity antibody was used (Roche, Mannheim, Germany). For western blots, rat monoclonal anti-HA Peroxidase High Affinity antibody (Roche, Mannheim, Germany) and mouse monoclonal GAPDH (Millipore, Temecula, CA) were used.

Flow cytometry. A maximum of 5 x 10^5 cells were used per stain, with the exception of intracellular staining (at least $1x10^6$ cells). Cells were incubated on ice and in the dark with an antibody cocktail (FACS buffer + 100-fold dilution of the antibodies of interest). After the incubation period, samples were washed twice with FACS Buffer (0.2% BSA, 0.1% Sodium Azide and 2% NGS in 1x PBS) and centrifuged. After the last centrifugation, cells were suspended in 1x PBS (or fix with 2% paraformaldehyde if not processed the same day) followed by FACScan analysis using the Cell Quest Pro software (Version 6.0, BD Biosciences, Mountain View, CA). 10,000 events were collected for routine analyses except for PCs (50,000 cell counts). After collection lymphocytes are gated based on their characteristic scatter properties to exclude large, granular non-lymphoid cells, cell aggregates and dead cells from further analyses. Representative flow cytometry data in the figures are contour plots: the z axis for these contour plots is cell number, in which contours define 5% increment changes in population "altitude"; colors are used only to facilitate visualization of contours.

Enzyme Linked-ImmunoSorbent Assays (ELISA). 96-well plates were incubated with each respective coating antibody, anti-IgM and anti-IgG at 10ug/mL, followed by RT incubation for one hour. Plates were washed with 1x TBS-Tween20 (TBS-T) 5 times. Blocking buffer was then added (1% BSA in 1X TBS) to each well to block any non-specific binding sites on the surface, and incubated for one hour at RT. Sera were diluted in blocking buffer at 1:500, further diluted, IgM (1:10,000) and IgG (1:50,000), added to each well on a 96-well plate, and incubated at RT for 1 hour. Plates were then washed 5 times with TBST. HRP antibody against either IgM or IgG was diluted with blocking buffer followed by a series of dilutions, followed by incubation for one hour. Plates were washed again with 1X TBST. TMB was then added to develop positive signal (blue color). The color reaction was stopped with a 1:10 dilution of H_2SO_4 and read immediately in a plate reader (NovoStar, BMG Labtech). For the IgG isotypes, the same protocol was followed with a variation in the concentration of the isotypes. IgG1 (1:10,000), IgG2a (1:40,000), IgG2b (1:40,000), and IgG3 (1:10,000).

Preparation of genomic DNA. High molecular weight genomic DNA was extracted from tail samples of 3-week-old mice. Briefly, tail material was incubated in 500μL of 50 mM of NaOH in a 95ºC heat block for 1 hour. After incubation, each tail was vortexed for 10 seconds, followed by neutralization with 50μL of 1M Tris-HCl (pH 8.0) and re-vortex. After vortexing, tails were subjected to centrifugation for 5 minutes at 14,000 RPM and the DNA containing supernatant isolated.

Polymerase Chain Reaction (PCR). Routine PCR analyses were performed to genotype all experimental mice using oligonucleotides that amplify the TDN transgene or MiT allele-specific amplicons and following standard protocols (24). PCR reactions were carried out in a 50μL volume, using 200ng of template DNA in 1 μL of tail lysate, 0.5 U/μL Go Taq polymerase (Promega, Madison, WI), 0.2 mM dNTPs (Roche, Indianapolis, IN), 0.5μM of Forward and Reverse TDN primers (see description below), 5X Green Go Taq Reaction Buffer (Promega, Madison, WI) and HyClone HyPure Molecular biology Grade Water (Thermo Scientific, Logan, Utah).

Immunohistochemistry. Spleens were preserved in OCT-Tissue-Tek (Sakura, Japan) and frozen. Spleens were cut at 6μm in a cryostat at a cryostat temperature of 20ºC and outside temperature of 18ºC. Sections were stored at -80ºC until staining. Before staining, tissue sections were set at RT for 10 min. Tissue sections were then fixed in ice-cold Acetone (Fisher) for 5-10 min. Slides were then removed from fixation agent and dried for 10 min. Sections were re-hydrated in 2 changes of 1x PBS (or TBS). After re-hydration, tissue sections were blocked for 1hr in a solution containing 1% BSA (Fisher) and 5% of normal serum (Invitrogen) in 1x PBS in a humid chamber. After blocking, primary antibody preparation containing biotinylated-CD3 and B220-FITC in a 1:100 dilution in blocking solution was added and incubated for 1 hour. A secondary antibody, Strepavidin-PE (BD Biosciences) was used to identify CD3, followed by 45 min. incubation. After secondary antibody incubation, tissue sections were washed in 3 changes of 1x PBS. Tissue sections edges were dried and anti-fade Prolong (Molecular Probes, Invitrogen) mounting solution was applied. Sections were allowed to air dry for 24 hrs in a dark chamber and sealed with clear nail polish the next day. Specimens were visualized using a fluorescence microscope (Zeiss, Germany) and pictures taken with Axio 4.7 software. Figures were placed together using Photoshop and Illustrator programs (Adobe).

Statistical Analyses. All data in the thesis is presented as the mean plus or minus standard error (SEM) and was analyzed for statistical significance using Student's t test for parametrically distributed data sets. Non-parametrically distributed data sets were analyzed by Mann Whitney U test and ANOVA using Prism 6-Graph Pad software. The level of significance was set at $p < 0.05$.

D. Appendix of Aim I:

A

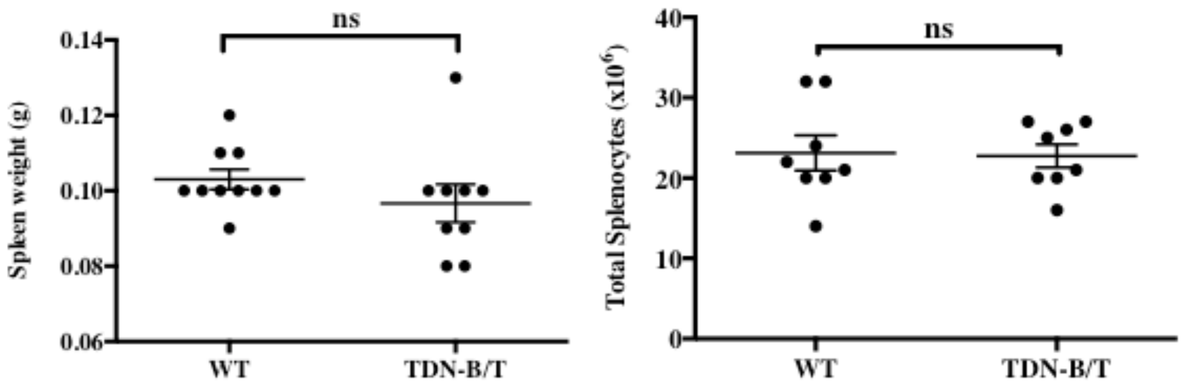


Fig. A: No splenomegaly in TDN-B/T mice group. Weight of spleens and number of total splenocytes from non-Tg (WT) and TDN-B/T mice, Error bars are the SEM with n=8-10, not significant (ns).

B

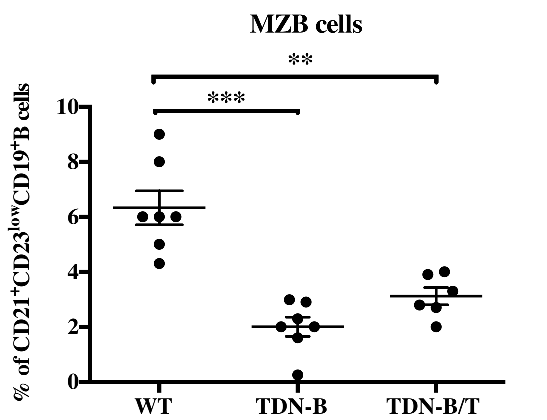


Fig. B: Inactivation of MiT family also affects Marginal Zone B cells in younger animals. Flow cytometric analysis of splenocytes from 1-2 moths old Non-Tg (WT), TDN-B and TDN-B/T mice groups. Numbers of MZB cells ($CD21^{high}CD23^{low}CD19^{+}$) expressed as a percentage of total $CD19^{+}$ splenocytes are shown. Each circle represents a mouse. Error bars are the SEM with n=6-7 animals per group. ** $p < 0.01$ and *** $p < 0.001$.

C

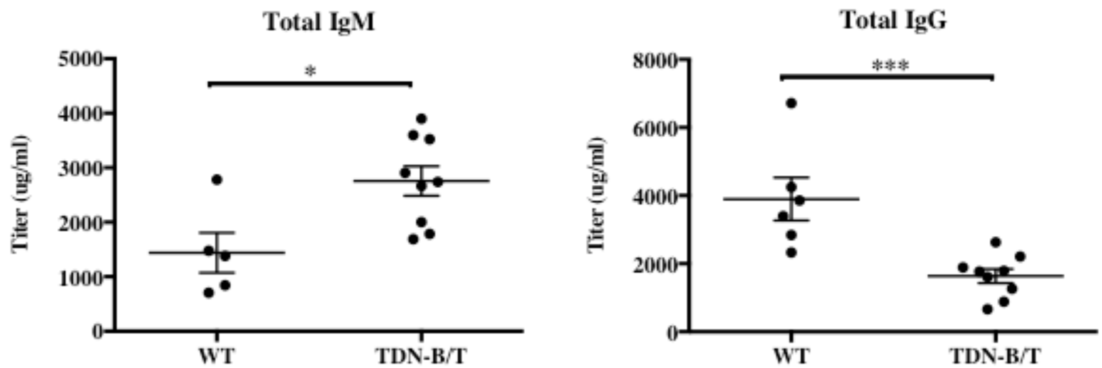


Fig. C: Evidence of hyper-IgM syndrome in unimmunized TDN-B/T mice. Immunoglobulins levels in sera from naïve 6 month-old TDN-B/T mice and non-Tg (WT) littermates. Each circle represents one mouse. Error bars are the SEM with n=5-9 per group. *p<0.05 and ***p<0.001.

D

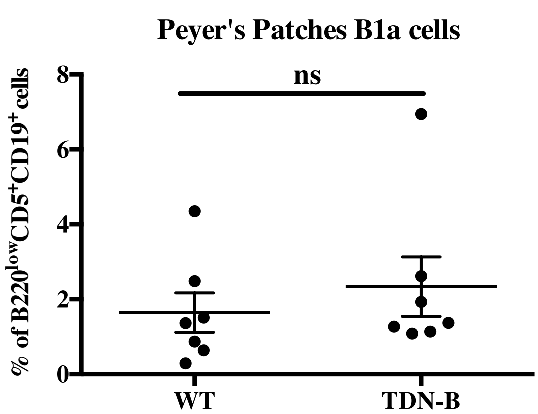


Fig. D. The fraction of B-1a cells present in Peyer's patches is unchanged. Flow cytometric analysis of Peyer's patch cell suspensions from Non-Tg (WT) and TDN-B mice groups. Numbers of B-1a cells ($B220^{low}CD5^{+}CD19^{+}$) expressed as a percentage of the total $CD19^{+}$ cells, are shown. Each circle represents a mouse. Error bars are the SEM with n=7 animals per group. ns, no significance.

E

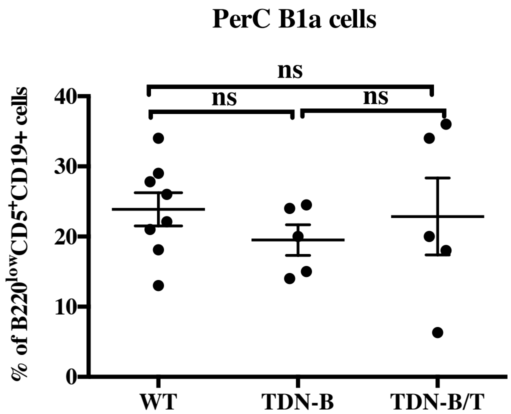


Fig. E: PerC B-1a cells are maintained in younger mice. Percentages of staining profiles of B-1a cells ($B220^{low}CD5^{+}CD19^{+}$) from 1-4 months-old non-Tg (WT), TDN-B and TDN-B/T are shown. Each circle represents a mouse. Error bars are the SEM with n= 5-8 mice per group. ns, no significance.

VII. AIM II: To examine whether the deficiency of the MiT family of transcription factors in B

cells promotes the development of autoimmunity.

A. Background and Summary of Aim II:

Background: Autoimmunity

Systemic Lupus Erythematosus (SLE) is a devastating and life-threatening systemic autoimmune disease in which the mechanisms that enforce tolerance have failed. It is estimated that 1.5 million Americans, and at least five million people worldwide, have a form of lupus (source, Lupus Foundation of America). It has been shown that defects in different immune processes can weaken tolerance mechanisms and lead to lupus.

The adaptive immune system has a prominent role in lupus, with both anti-nuclear autoreactive B and T cells characteristic of this disease. The diversity of antigen binding of BCRs is vast and a there is a dynamic reservoir of B cells some of which some carry incipient autoreactivity. Since VDJ recombination and SHM are random mechanisms, it is unpredictable as to whether the Ig repertoire produced by either mechanism will create an Ig that reacts against self-antigens, and so tolerance mechanisms to keep these in check are ongoing (45). Failure of the mechanisms to eliminate these autoreactive lymphocytes that react strongly to self-antigens causes a breach in tolerance and therefore a role in the generation of disease (46).

However, autoimmune diseases are not only initiated by specific autoreactive lymphocytes, but also cells involved in activating autoreactive lymphocytes and in managing self-molecules. This is because these cells, particularly macrophages and dendritic cells (DCs), recognize invading pathogens as well as self-antigens through a distinct set of receptors. In fact, one of the roles of innate immune cells is the safe clearance of self-debris after cells die by apoptosis. Apoptotic cells are phagocytosed through receptors that recognize opsonized self-antigens. The body's natural IgM antibodies function as opsonins, facilitating the removal of debris with little to no inflammation. Defects in the clearance mechanisms could result in self-antigen overload, consequently, activating FcR and TLRs on innate immune cells, such as DCs and autoreactive T and B cells (47, 48, 49). In addition, the innate cells recognize pathogen associated molecular patterns using a set of germline-encoded receptors such as TLRs, which are conserved in many species. This in contrast to the highly specific and genetically polymorphic antigen receptors expressed by B and T cells (BCR and TCR) that exclusively recognize specific antigens (45). Moreover, both systems crosstalk on B cells; for example, TLRs present on MZ B cells interact with ligands that can induce this cell to secrete IgM, providing with a rapid protection before the delayed adaptive immune response.

B cell tolerance checkpoints

As stated in the Introduction, one of the mechanisms of tolerance to self in humans and mice is the elimination of autoreactive BCR specificities either by editing the receptor or by apoptosis of autoreactive B cells themselves. This is necessary since the quasi-random Ig gene assembly process that underlies the diversity of the naïve B cell Ab/BCR repertoire also naturally generates autoreactive B cells during development, and SHM activated by the GC reaction can generate autoreactive Abs from non-autoreactive ones. It has been estimated that at least half of newly formed immature B cells in the bone marrow are highly polyreactive, producing antibodies against self-antigens, including insulin, DNA and LPS (50). However, these B cells are not found in the periphery of healthy people or mice. This is evidence of central tolerance, operating at discrete steps during B cell development, to ensure prevention of autoimmunity. Cross-linking of autoreactive receptors by self-molecules at different developmental checkpoints is a critical mechanism for tolerance because the strength of these interactions determines the fate of that B cell. For example, if the strength of receptor cross-linking by antigen is strong, the cell is eliminated by apoptosis, whereas intermediate signaling promotes secondary recombination of the IgL genes to replace the one contributing to autoreactivity. This gives the cell a second chance on "life" by editing the autoreactive BCR to eliminate its self-reactivity. Alternatively, if an autoreactive B cell is not eliminated by apoptosis or editing of its receptor, this B cell can become ignorant (anergic), a status of self-antigen unresponsiveness with reduced fitness and half-life. This reservoir of short-lived anergic cells, are thought to be one source of the autoreactive B cells that emerge when the mechanisms that enforce tolerance are breached.

A small percentage of autoreactive cells escape central tolerance in the BM and migrate to the periphery, where they could encounter other self-antigens. Some of these autoreactive cells in the periphery may have particular roles in normal immune defense, but others may have the potential to become pathogenic if activated. In addition, in the periphery, self-antigens not present in the bone marrow become available to these generated and possibly autoreactive B cells, which are deleted upon crosslinking with those self-antigens, and as stated above, autoreactivity to a benign antibody gene can be conferred by SHM. Autoreactive B cells that are not selected out by central tolerance also can be vetted by these subsequent tolerance mechanistic checkpoints collectively known as Peripheral Tolerance.

Those autoreactive B cells that escape might potentially become activated and differentiate into cells that secrete autoantibodies. Normally the process of differentiating from an activated GC B cell to a plasma cell is tightly controlled. Disturbances in this control can occur when chronically activated innate cells such as DCs interact with B cells via TLR and other innate receptors (50). This can have pathogenic consequences including dysregulation of follicular and extra-follicular antibody reactions. These types of reactions are often observed in autoimmune disorders including SLE. When apoptotic pathways such as the Fas-associated protein with death domain (FADD) pathway fail to remove immune cells with autoreactive potential, accumulation of these cells including DCs and autoreactive lymphocytes, is the force that pushes autoimmunity forward (50).

Effective interactions between a T cell and a B cell are required for the secretion of high affinity antibodies, and autoreactive T cells also exist and can provide help to autoreactive B cells. In addition to abnormal T cell help, heightened activation of mitogenic/survival pathways from abnormally activated innate cells or through innate receptors expressed on B cells themselves can protect and even activate autoreactive B cell clones. B cells that arise from dysregulated follicles, such as those in the TDN-B.B6 mice, are suspected to be autoreactive because they arose without exogenous antigenic stimulation and could secrete high levels of autoantibodies, which are associated with many autoimmune diseases (51).

In summary, under normal circumstances, humans and mice have evolved mechanisms to eliminate autoreactive B cells, first by removing immature autoreactive B cells in the BM and then, by eliminating the ones that escape into the periphery. If autoreactive B cells escape these mechanisms of tolerance, these cells could potentially become activated by self-molecules and secrete autoantibodies involved in the pathogenesis of autoimmune diseases.

Autoimmune diseases: SLE in humans and mice

SLE is a well-known and devastating autoimmune disease whose features overlap with RA and Sjörgens syndrome. What these diseases have in common is a failure of B cell tolerance resulting in inflammation that is instigated by autoantibodies and results in organ damage. Although the primary target organs are different, there is some overlap. In humans, SLE is a systemic autoimmune disease that mostly affects females, and is characterized by the loss of tolerance to nuclear self-antigens such as DNA and RNA-containing complexes like chromatin and its components, and ribonucleoproteins (RNPs). These pathogenic autoantibodies that interact with them lead to multi-organ failure (50). Human lupus characteristics vary from patient to patient and these could include, inflammatory lesions in the skin, joints, kidneys, the central nervous system, and the vasculature (51). In mice, individual strains show manifestations of lupus-like disease, such as the presence of different anti-nuclear autoantibodies and immune complex deposition in the kidneys leading to renal failure (52, 53).

The disease in SLE patients arises in early years (20-30 yrs. old), with characteristic flares (aggravation of symptoms) and remissions that often affect multiple organs. Elevated serum interferon-alpha (IFN-α), a critical cytokine for anti-viral immunity, is another characteristic feature of lupus flares, and is thought to propel a misguided anti-nuclear autoimmune response. Renal failure, a major cause of death in SLE patients, is the result of immunoglobulin complex deposition, direct complex formation or anti-nuclear antibody deposition, leading to activation of complement. Complement activation causes the mesangial cells to proliferate and endothelial cells to undergo apoptosis. Debris from apoptotic cells alert inflammatory cells, such as neutrophils and lymphocytes, to infiltrate the glomeruli leading to inflammation and destruction. All these events, together, cause structural changes in the glomeruli preventing the kidney from filtering properly and resulting in eventual organ failure (53).

Over the years, many immune suppressive medications have been used to treat or control the symptoms of SLE. Today, we have a limited armamentarium of immunosuppressive therapies to treat them. However, the great improvement in the survival of SLE patients is largely due to the management of life threatening conditions such as renal failure rather than targeted therapies directed at the disease itself (54).

What are the underlying pathophysiological mechanisms of SLE? There are a number of single genetic deficiencies, such as those that affect components of the classical complement activation pathway or intracellular nucleases, that are strongly associated with SLE but which account for only a subset of patients (54). Recently, genome-wide association studies (GWAS) that use millions of common single nucleotide polymorphisms as genomic markers to type increasingly large patient and control populations, have definitively linked a number of genetic variants to disease susceptibility. These genetic variations, together with environmental factors such as virus infection, for which the anti-nucleotide TLR7 and TLR9 receptors, and IFN-α responses are important, are believed to combine to trigger the breach of immune tolerance, inducing SLE in humans.

Mouse models are important tools to study SLE and, although not identical to humans, they provide powerful insights to understand the genetics of this devastating disease. The serological findings in the classic lupus MRL.Fas$^{lpr/lpr}$ model (commonly known as MRL.*lpr*) relates more closely to those observed in lupus patients than most mouse models, and those antibodies generated in this line, such as anti-dsDNA, are considered to be pathogenic and involved in the induction of severe lupus nephritis. Lpr is a null mutation of the pro-apoptotic Fas gene (the *lpr* allele). This mutation impairs the deletion of autoreactive lymphocytes and is one of many murine models that has provided greater understanding of the cellular and genetic basis of lupus disease (56). However, despite severity of lupus-like disease in this model, they do not generate anti-Sm antibodies that are a characteristic of most human lupus. This is an example of the fact that the manifestations of the disease differ from one lupus prone strain to another, as much as they do in individual patients. In addition to MRL.lpr, the other most commonly studied strains for spontaneous lupus include the F1 hybrid between New Zealand Black (NZB) and New Zealand White (NZW) strains (NZB/W F1) and the BXSB/Yaa strains, whereas the exogenously induced models include the pristane-induced model and the chronic graft-versus-host-disease (cGVHD) model. All of these models have some similarities to human lupus, displaying a subset of parameters seen in human disease, namely, anti-nuclear autoantibody production, lymphoid activation and hyperplasia, and often, immune complex mediated nephritis (55, 56, 57). In some of these mouse models, both females and males develop disease, whereas in certain specific models, the symptoms are only seen in females.

The severe phenotype seen in the MRL.lpr model – death of lupus nephritis and vascular events at 9 months of age on average - is due to the manifestations of a defect in Fas and the MRL background strain in both lymphoid and non-lymphoid cells such as DCs. These DCs, as mentioned previously, also play a role in systemic autoimmunity because abnormal DCs drive activation of autoreactive lymphocytes. In contrast – and as an example of the importance of strain differences in non-lymphoid cells in autoimmunity - the B6.*lpr* exhibits a much milder disease with later onset than the MRL.*lpr* model and a much longer lifespan ($\geq$ 1 year). This late onset correlates with anti-nuclear antibody titers and glomerulonephritis, but there is no report of a DC defect in the B6.*lpr* model (55, 56). The B6.*lpr* model was chosen so that I would be able to detect an acceleration of disease,.

Similarities and differences on Fas mutation in humans and mice

Defects in pathways used to cull autoreactive lymphocytes arise from mutations in genes critical for that process, such as the Fas receptor (Fas), a cell surface receptor involved in apoptosis. If there is a failure in the Fas pathway either due to a mutation in the Fas or Fas ligand (FasL), immune cells may escape immune tolerance mechanisms (58, 59, 60). Deletion of Fas, as in the $Fas^{lpr/lpr}$ model, leads to the accumulation of potentially autoreactive lymphocytes. Fas, also termed CD95, are members of the tumor necrosis factor (TNF) and nerve growth factor (NGF) family of receptors expressed on a variety of immune and non-immune cells and are associated with the induction of apoptosis, as well as inflammatory signaling (60). Fas mediates apoptotic signaling through FasL (CD95L) expressed on the surface of other cells, making this an important interaction in aspects of the immune response, such as immune surveillance, removing transformed cells and cells infected by viruses.

In humans, Fas mutation leads to a disease called Autoimmune Lymphoproliferative Syndrome (ALPS). ALPS is defined as an impairment of lymphocyte apoptosis that results in chronic non-malignant lymphadenopathy, splenomegaly, and the expansion of rare lymphocyte populations such as double negative ($CD4^-CD8^-$) T cells expressing α/β TCR, and in some cases, development of lymphomas (61).

In these patients, autoimmune symptoms occur at some point later in disease once they have manifested elements describe above. The clinical manifestation of ALPS varies from patient to patient, depending on the type of mutation, but lymphoproliferation is the most dramatic and consistent finding with enlargement ranging from slight to massive. Autoimmunity in ALPS patients is largely against blood cells, with a very small percentage of patients (1-2%) developing renal pathology (62).

This is in contrast to mice, in which Fas mutations are characterized by lymphoid hyperplasia and immune-complex formation that involves the kidney as an end target organ, and mimic SLE to a varying extent depending on the background strain. The timing of onset and severity are strongly influenced by background strain, with C57BL/6 Fas$^{lpr/lpr}$ mice (commonly referred as B6.*lpr*) exhibiting milder disease than other strains.

Although the original mouse model with Fas deficiency was developed as a model for SLE in humans, there are no reports of mutations in *TNFRSF6* in patients with SLE. The similarities between humans with ALPS and the Fas$^{lpr/lpr}$ mice come from a single report (NIH-1992) in which two children had massive lymphoid hyperplasia as well as autoimmune disease. Nevertheless, the Fas$^{lpr/lpr}$ models have been informative models to study lupus like disease in mice.

Autoantibodies and TLRs

Many autoimmune diseases differ in the spectrum of autoantibody specificities. For example, SLE is associated with anti-DNA and anti-ribonucleo proteins (RNP) antibodies commonly known as antinuclear antibodies (ANAs), whereas Sjörgen syndrome, an autoimmune disease with salivary pathology, is associated with antibodies against SSA (Ro) and SSB (La) antigens, which are RNP antibodies. Ro/SSA antigens may recognize one or both of two cellular proteins with molecular weights of approximately 52 and 60 kD, and these autoantigens are referred to as “Ro-52” and “Ro-60,” respectively. Ro-60 binds small, non-coding RNAs termed "Y RNAs" and Ro-52, is an interferon-inducible protein. La/SSB antigens interact with a 47-kD protein, which shuttles between the nucleus and cytoplasm but which is predominantly found in the nucleus, and binds cellular transfer RNAs (tRNAs). Although these autoimmune diseases are different in their targets and pathologies, the key autoantigens are part of a complex with either DNA or RNA.

It is well known that nucleic acid components are of importance in the immune activation, but how cells do recognize nucleic antigens? As discussed earlier in this section, similar to innate cells such as dendritic cells and macrophages, most B cells can also respond to nucleic acid antigens through their TLRs (63), which are crucial in microbial detection and this play a critical role in the innate as well as the adaptive immune response (63, 64). TLRs are a large family of receptors each of which has different ligands and activation mechanisms (64) but only TLR3, TLR7, TLR8 and TLR9 are located in intracellular endosomes and recognize nucleic acids. In autoimmune disease models, only TLR7 and TLR9 are involved in pathologies in mice. TLR7 recognizes ssRNA, whereas TLR9 is the only TLR that recognizes dsDNA (64). In patients with SLE, the rate of lymphocyte apoptosis is very high, as measured by ELISA. Due to a defect in macrophages in these patients, phagocytosis of cellular debris is less efficient allowing material from these apoptotic cells, such as nucleosomes, to serve as a source of intracellular self-antigens. These self-antigens, mostly nucleic ligands such as ssRNA and dsDNA, are endocytosed by autoreactive B cells and directed to endolysosomal compartments synergizing with TLR7 and TLR9 (64). This pathway places these receptors as key players in the production of autoantibodies against DNA- or RNA-associated autoantigens. Therefore, agents that target these dysregulated pathways could serve as an alternative therapy to treat this devastating disease.

MiT family in SLE

SLE is a very complex disease that involves the loss in tolerance of both innate and adaptive immune system pathways, which fail to be controlled by regulatory mechanisms. It is believe that multiple triggers, such as genetic predisposition, environment, tissue injury and infections, are involved in the initiation of disease. The combination of all these factors leads to systemic inflammation and inflammatory responses that could increase and accelerate disease.

In this Aim, we test the hypothesis that the Mitf family is important in enforcing B cell tolerance. The absence of Mitf leads to spontaneous plasma cell differentiation, and presence of benign autoantibodies in the Mitf$^{mi/mi}$ bone marrow chimeras, but no disease (24). However, in a different genetic setting or under conditions of immune provocation, formation of spontaneous plasma cells could lead to autoantibody production potentially causing a form of autoimmunity. Given that the TDN-B mice have a defect in several B cell subsets, I asked whether murine lupus was affected by the absence of the MiT function in B cells. I found that lupus like disease is accelerated and more severe when MiT is inactivated in B cells.

Summary

I analyzed naïve TDN-B and non-transgenic littermates for the presence of autoantibodies, and then crossed the B cell specific TDN-B and TDN-B/T transgenes into a mouse line that is prone to a mild form of lupus-like autoimmunity, namely the B6.*lpr* strain, to evaluate disease manifestations between B6.*lpr* mice that contained or lacked the TDN transgene in B cells. We chose a strain with a milder form of disease because our hypothesis is that autoimmunity will be worsened if I inactivate the Mitf family in B cells. It is also important to note that the TDN-B transgene has been backcrossed several generations into B6 mice (>5), so background strain should not be a confounding factor.

Approach: As shown in Aim I, our preliminary studies demonstrate that in unimmunized TDN-B mice, there is an increase in plasma cell numbers that correlates with the formation of more GCs in the spleens. We hypothesize that because these reactions are hallmarks of B cell activation, these mice would also be spontaneously secreting more Igs in the absence of specific stimuli.

B cell specific TDN-B mice and B and T cell specific, TDN-B/T mice, were crossed with the B6.*lpr* mouse strain, which is susceptible to autoimmune disease. The three cohorts (B6, TDN-B.$Fas^{lpr/lpr}$ and $Fas^{lpr/lpr}$) were evaluated by flow cytometry to enumerate and characterize B cell phenotypes. We also evaluated characteristics for disease, such as; lymphoid abnormalities, immunoassays to analyze the spectrum of antibodies and autoantibodies made, and presence of protein in urine and histology to assess renal dysfunction.

B. Results of Aim II:

Naïve TDN-B mice have an increase in total antibody and some autoantibody production than non-transgenic mice.

Since it is possible that B cells of the TDN-B mice might be hyper-responding and possibly activated against a particular antigen, we tested for the presence of autoantibodies in these animals. To observe if unimmunized TDN-B mice have an increase in the amount of antibodies and autoantibodies being secreted, I performed a series of ELISAs to identify their presence in sera. In lupus, anti-DNA autoantibodies are involved in the pathogenesis of the disease, and are key mediators of autoimmune complex mediated glomerulonephritis in mice and humans (56). Therefore, given the B cell defects observed in the TDN-B and since in the Mitf$^{mi/mi}$ transplantation model, the dominant negative mutation in Mitf, the production of auto-Abs including anti-ssDNA and RF was observed (24), the presence of these auto-Abs was analyzed as well as in our TDN-B model. Since I observed that naïve TDN-B mice spontaneously secreted more IgG antibodies, I investigated whether these were auto-Abs as observed in the Mitf$^{mi/mi}$ bone marrow chimeras. Using ELISAs as a tool, I tested for the presence of ssDNA, dsDNA and chromatin autoantibodies.

Fig. 12:

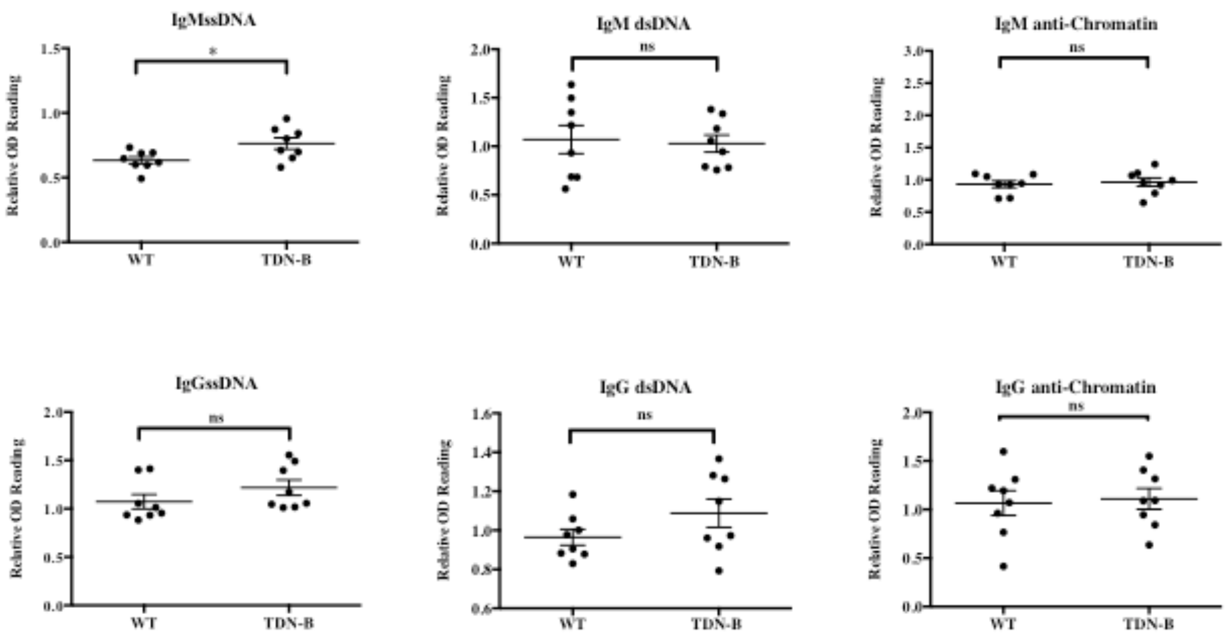


Fig. 12. Naïve TDN-B transgenic mice secreted IgM ssDNA autoantibodies. Autoantibody titers in the sera from naïve TDN-B transgenic mice and non-transgenic (WT) mice littermates. ELISA activity is expressed relative to a titrated standard serum. Each circle represents a mouse. Error bars are SEM of n=8 per group. *p<0.05, and ns, not significant.

There was no significant increase in the amount of IgG ssDNA, IgM and IgG dsDNA, or IgM and IgG chromatin antibodies in TDN-B mice when compared to their non-transgenic littermates (Fig. 12, top and bottom panel). However, there was a significant increase in the amount of IgM ssDNA autoantibodies in naïve TDN-B mice when compared to non-Tg (WT) animals (Fig. 12, first in top panel). This observation is similar to that which was observed in the Mitf$^{mi/mi}$ transplantation model (24). This is evidence that Mitf inhibition in B cells causes a break in tolerance in the immune system.

TDN-B.Fas$^{lpr/lpr}$ mice spontaneously develop accelerated lymphoproliferative disease.

Our first striking observation was that 5-month-old TDN-B.Fas$^{lpr/lpr}$ animals developed visible lymphadenopathy of lymph nodes, in the axillary area as well as in the salivary region, and visible splenomegaly, all disease manifestations not observed in non-Tg or control Fas$^{lpr/lpr}$ mice. This is months sooner than is expected for disease to manifest in the Fas$^{lpr/lpr}$ strain. Some TDN-B.Fas$^{lpr/lpr}$ animals that died soon after were identified as sick, probably due to massive progression of systemic autoimmune disease that lead to renal failure. Therefore, I analyzed the remaining animals at 6 months of age. At that point, all other animals that were positive for the TDN protein in the *lpr* background, as well as their controls, were analyzed.

Since there is a defect the Fas apoptotic pathway, B and T cells in the *lpr* mice are not eliminated, and these cells accumulate in the lymphoid organs, which enlarge. I observed that at 6 months of age TDN-B.Fas$^{lpr/lpr}$ mice showed greater splenomegaly, which in some cases was severe, compared to Fas$^{lpr/lpr}$ animals (Fig. 13). Cells were also enumerated from the spleens of Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ mice, and their total recovery correlated with the weight and size (Fig. 13). Another characteristic of lupus-like disease in mice is the sporadic appearance of lymphadenopathy. I observed that TDN-B.Fas$^{lpr/lpr}$ and Fas$^{lpr/lpr}$ animals showed diffuse and localized lymphadenopathy, respectively (see Table II). This was observed in axillary and brachial lymph nodes, as well as mesenteric, pancreatic, inguinal, and paraortic nodal areas of the TDN-B.Fas$^{lpr/lpr}$ mice.

Fig. 13

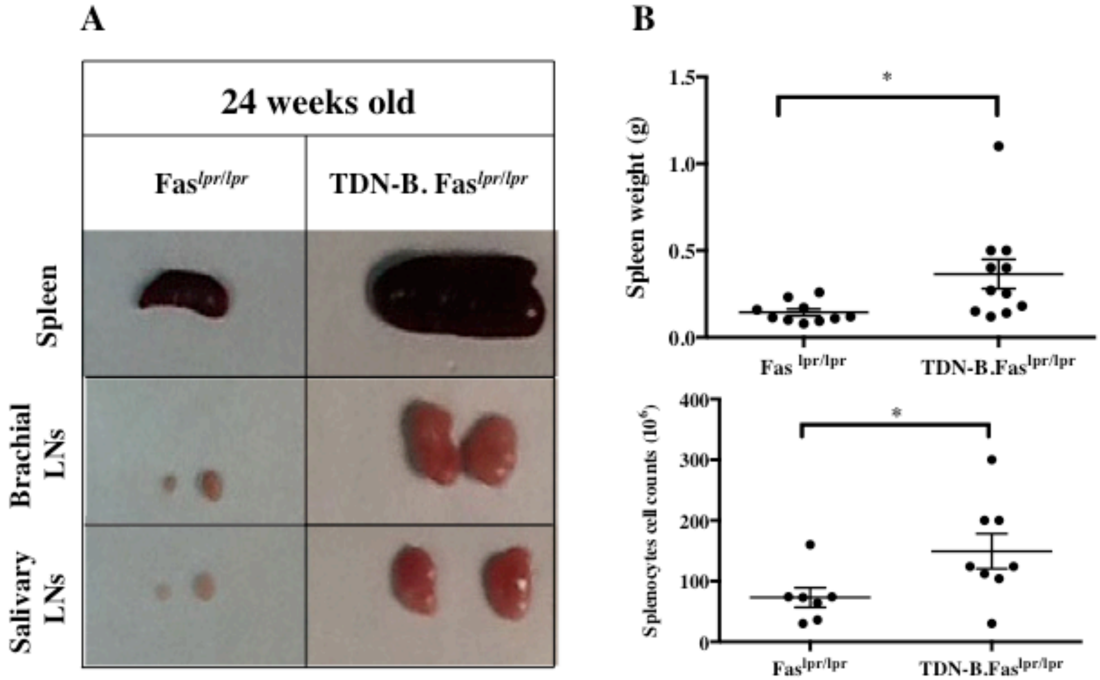


Fig. 13:. Spontaneous development of lymphoproliferation in TDN-B.Fas$^{lpr/lpr}$ mice. A. Spleens and lymph nodes from 24-week-old Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ mice for illustrative purposes only. The TDN-B.Fas$^{lpr/lpr}$ spleen shown is the most severe case of splenomegaly observed in this group (see outlier in B) and is extreme degree of enlargement is not representative of this set; however, the lymph nodes from the same animal are representative of lymphadenopathy that typified this group. B. Top, spleen weights from mice of both groups; bottom, total numbers of splenocytes, as enumerated by cell counting. The outliers in both graphs are a representation of severe splenomegaly, as shown in the picture on the left. Cells derived from lymph nodes were also enumerated (data not shown). Bars are SEM with n=5-12 mice per group. *$p<0.05$.

On the other hand, only a few Fas$^{lpr/lpr}$ animals showed enlarged axillary lymph nodes, with no visible enlargement of the other lymph nodes. We described this as focal lymphadenopathy (Table II). None of the non-Tg mice showed evidence of splenomegaly or lymphadenopathy (data not shown). In a similar way, TDN-B/T.Fas$^{lpr/lpr}$ mice did not show signs of sickness at 24 weeks, nor splenomegaly or lymphadenopathy (data not shown). Thus, the inhibition of the MiT family of proteins in B cells only resulted in the accelerated spontaneous development of a lymphoproliferative disorder that affects both males and females, whereas its inhibition in both T and B cells prevented this acceleration.

Fas expression is important for the elimination of GC B cells when they are rendered with a non-functional or autoreactive BCR after SHM, as well as for the elimination of autoreactive T cells in the thymus. To further characterize the lymphoproliferative disease exhibited in TDN-B.Fas$^{lpr/lpr}$ mice, I performed a detailed flow cytometric analysis to determine if these *lpr* mice show any abnormalities that could relate to a dysregulated immune response.

Table II. Lymphadenopathy of Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ mice.

Lymphadenopathy	Fas$^{lpr/lpr}$	TDN-B.Fas$^{lpr/lpr}$
Brachial LNs	2 /10	12/12
Salivary LNs	9/10	12 /12
Mesenteric LNs	1/10	9/12
Inguinal LNs	0 /10	8 /12
	localized	diffuse

Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ both exhibited marked aberrancies within the B cell compartments, which we not only characterized as an increase in the numbers of B cells, but also as an expansion of GC B cells and an accumulation of plasma cells (Fig. 14 and 15). GC B cells were defined as GL7$^+$C38$^+$CD19$^+$ cells (Fig. 14). TDN-B.Fas$^{lpr/lpr}$ mice showed an increase in the percentages of GC B cells compared to Fas$^{lpr/lpr}$ mice and non-Tg mice (Fig. 14). In contrast, TDN-B/T Fas$^{lpr/lpr}$ mice showed a similar percentage of GC B cells as non-Tg, in contrast to Fas$^{lpr/lpr}$ mice (Appendix E).

Fig. 14:

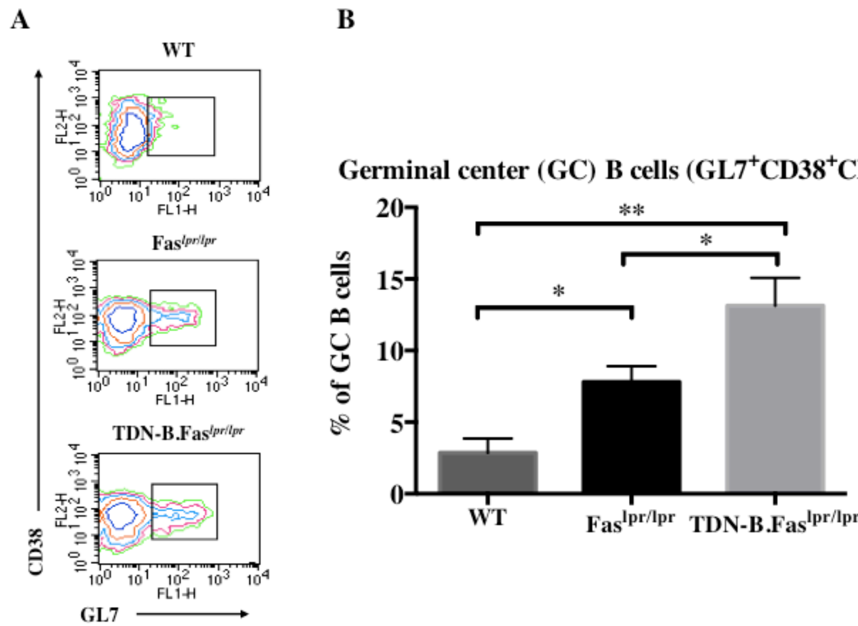


Fig. 14: MiT inhibition contributes to an increase in splenic GC B cells in TDN-B.Fas$^{lpr/lpr}$ mice. (A) Representative staining profiles and (B) percentages of CD19$^+$ splenocytes identified as GC B cells (CD19$^+$GL7$^+$CD38$^+$) based on A, are shown, from non-Tg B6 (WT), Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ mice. Bars are SEM of n=5-12 mice per group. *p<0.05 and **p<0.01.

Along with the increase in GC B cells, Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ mice had increased PCs numbers (CD38^{low}CD138$^+$CD19$^-$), compared to non-Tg mice (Fig.15). However, TDN-B.Fas$^{lpr/lpr}$ mice had an even greater percentage of plasma cells than Fas$^{lpr/lpr}$ mice (Fig.15). In contrast, TDN-B/T.Fas$^{lpr/lpr}$ mice have a lower percentage (~6%) of splenic plasma cells than Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ mice (Appendix F).

Fig. 15

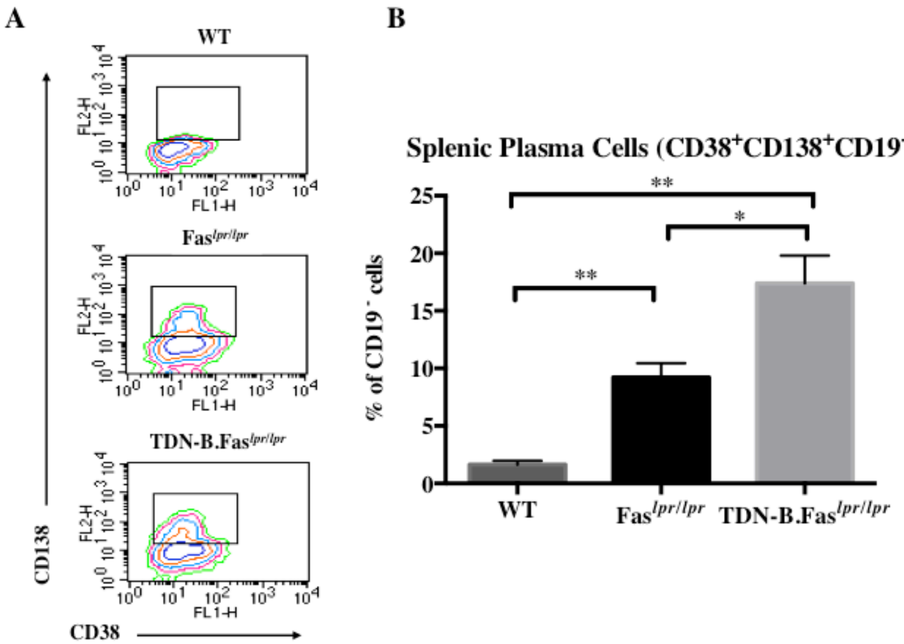
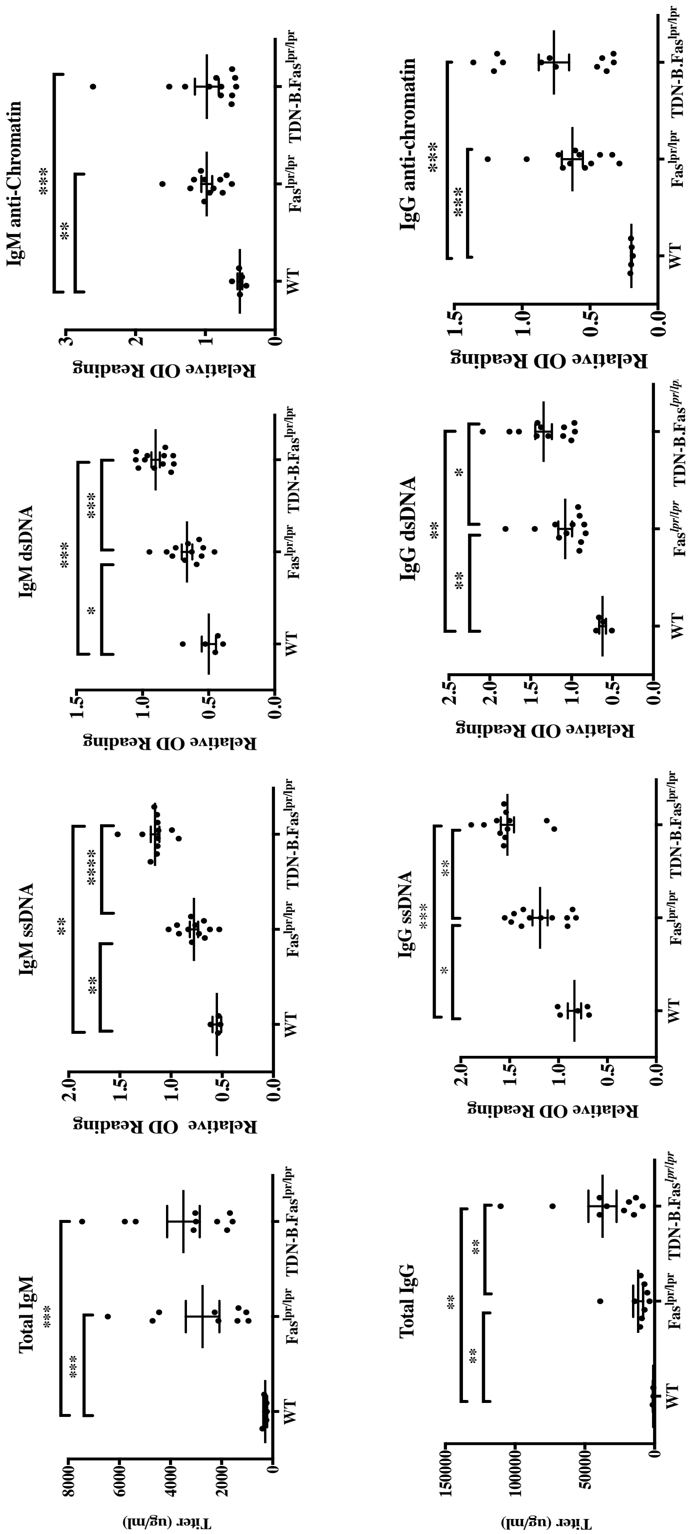


Fig. 15: MiT inhibition contributes to an increase in splenic PC cells in TDN-B.Fas$^{lpr/lpr}$ mice. (A) Representative staining profiles, and (B) percentages of CD19$^-$ splenocytes identified as PCs (CD38^{low} CD138$^+$CD19$^-$) are shown, from non-Tg B6 (WT), Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ mice. Bars are SEM of n=5-12 mice per group. *$p<0.05$ and **$p<0.01$.

TDN-B.Fas$^{lpr/lpr}$ mice have increased secretion of autoantibodies and kidney pathology.

Lpr lymphadenopathy is coincident with the presence of autoantibodies. Next, we performed an extensive serological analysis of the different groups of mice at 6 months of age and showed that both Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ mice developed IgM and IgG autoantibodies against ssDNA. There was an even greater amount of IgM and IgG ssDNA autoantibodies in TDN-B.Fas$^{lpr/lpr}$ than in the Fas$^{lpr/lpr}$ group of mice, whereas in TDN-B/T.Fas$^{lpr/lpr}$ mice, ssDNA autoantibodies titers were similar to the Fas$^{lpr/lpr}$ group (Fig. 16, Appendix G).

Fig. 16



Total IgM
Titer (ug/ml)
IgM ssDNA
IgM dsDNA
IgM anti-Chromatin
Relative OD Reading
Total IgG
IgG ssDNA
IgG dsDNA
IgG anti-chromatin
WT
Fas$^{lpr/lpr}$
TDN-B.Fas$^{lpr/lpr}$

Fig. 16: Production of autoantibodies in TDN-B.Fas$^{lpr/lpr}$. Spontaneous production of IgM (top) and IgG (bottom) autoantibodies in non-Tg B6, TDN-B.Fas$^{lpr/lpr}$ and Fas$^{lpr/lpr}$ mice. Antibody levels in sera from each strain of mice as measured by ELISA. Error bars are SEM of n=5-12 mice per group. *p<0.05, **p<0.01, and ***p<0.001 and ns, not significant. Circles represent individual mice.

dsDNA autoantibody titers were also elevated in both Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$mice (Fig. 16). ssDNA and dsDNA autoantibodies were even higher in TDN-B.Fas$^{lpr/lpr}$ mice than Fas$^{lpr/lpr}$ mice alone. Little to no autoantibodies against ssDNA or dsDNA were detected in WT mice (Fig. 16).

Given that TDN-B.Fas$^{lpr/lpr}$ mice exhibit an increase in DNA antibodies, we next determined the presence of antibodies against ribonucleoproteins that are represented in ANAs. We performed an ELISA analysis of the sera from the different groups of mice at 6 months of age showed that both Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ mice developed ANAs. There was an even greater amount of ANAs in TDN-B.Fas$^{lpr/lpr}$ than in the Fas$^{lpr/lpr}$ group of mice (Fig. 17).

Fig. 17

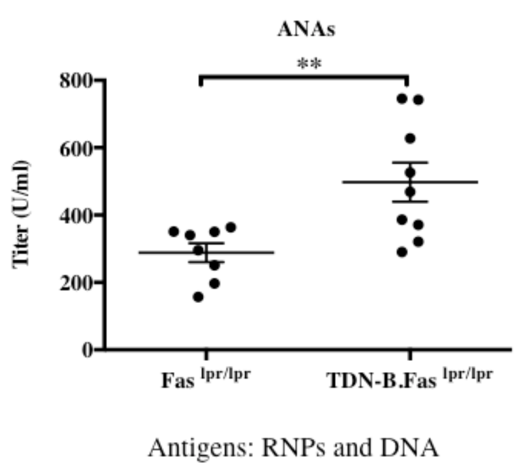


Fig. 17: Production of antinuclear antibodies (ANAs) in TDN-B.Fas$^{lpr/lpr}$. Spontaneous production of ANAs in both TDN-B.Fas$^{lpr/lpr}$ and Fas$^{lpr/lpr}$ mice. Antibody levels in sera from mouse as measured by ELISA. Bars are SEM of n=8-9 mice per group. **p<0.01. Circles represent individual mice.

One of the major morbidities of lupus is impaired renal function caused by glomerulonephritis. An early manifestation of this is proteinuria, due to the deterioration of glomerular function. I therefore measured protein content in the urine, which was scored as follows: grade 0.5 = 'trace' proteinuria; grade 1 = ~ 30 mg/dL; grade 2 = ~ 100 mg/dL; grade 3 = ~ 300 mg/dL; and grade 4 = > 2000 mg/dL. TDN-B.Fas$^{lpr/lpr}$ showed a higher level of protein in the urine than both Fas$^{lpr/pr}$ mice and non-Tg B6 mice, which showed only basal levels of protein in urine (Fig. 18).

Fig. 18

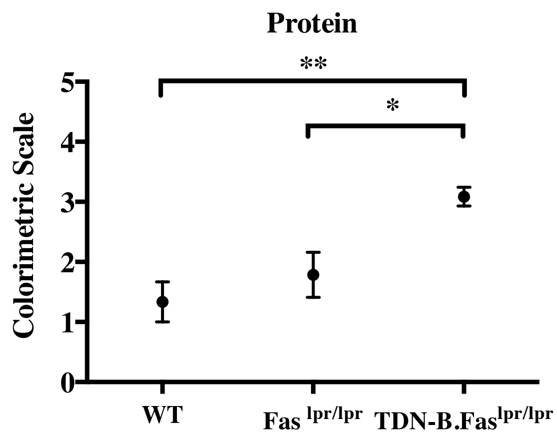


Fig. 18: TDN-B.Fas$^{lpr/lpr}$ mice excrete more protein than Fas$^{lpr/lpr}$ mice. Levels of excreted protein from non-Tg B6 (WT), Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ mice were measured and quantified in a colorimetric scale. Circles represent the mean value for a particular mouse group. Bars are the SEM of n=3 for WT, n=9 mice for both *lpr* groups. *p<0.05 and **p<0.01.

One of the first abnormalities detected in lupus kidneys is the deposition of immune complexes in the basement membrane of the mesangium leading to crescent formation. This prevents the glomeruli from functioning properly. Immune complex deposition in the mesangium and sub-endothelial capillary walls triggers changes in the structure of glomeruli and inflammation (45, 57) creating structures called "crescents."

In order to assess if there are any structural changes in the kidneys that could explain the proteinuria, I looked for glomerular deposits of IgM, IgG and complement component, C3. Histological and immunohistochemical staining of kidneys of 6-month old TDN-B.Fas$^{lpr/lpr}$ mice were analyzed and compared to Fas$^{lpr/lpr}$ mice and a control group heterozygous for the Fas *lpr* mutation, Fas$^{+//lpr}$ mice, to assess disease severity. Mesangial proliferation and inflammatory infiltrates were evaluated and a nephritis index score was determined in a blinded fashion by pathologist Dr. Susan Gottesman. Based on that, we concluded that the degree of mesangial cell proliferation and chronic inflammation was increased in TDN-B.Fas$^{lpr/lpr}$ mice when compared to Fas$^{lpr/lpr}$ mice and to Fas$^{Fas/lpr}$ mice (Fig. 19 and data not shown). TDN-B.Fas$^{lpr/lpr}$ mice also showed more hypercellular and larger glomeruli due to inflammation than Fas$^{lpr/lpr}$ and Fas$^{Fas/lpr}$ mice (Fig. 19 and data not shown). Chronic inflammation was observed in interstitial areas in TDN-B.Fas$^{lpr/lpr}$ mice, whereas Fas$^{lpr/lpr}$ mice showed little to no inflammation (data not shown). Indeed 7 out of 10 TDN-B.Fas$^{lpr/lpr}$ mice showed increased proliferation of mesangial cells whereas 1 out of 5 Fas$^{lpr/lpr}$ mice showed mesangial proliferation. As a result of inflammatory cell infiltration and mesangial proliferation, the size of some of the glomeruli of TDN-B.Fas$^{lpr/lpr}$ mice was up to three times the size of the glomeruli of Fas$^{lpr/lpr}$ mice (Fig. 19). PAS staining revealed that the basement membrane in the capillaries of the glomeruli was thickened in TDN-B.Fas$^{lpr/lpr}$ mice (black arrow) whereas Fas$^{lpr/lpr}$ mice at 6 months of age had no apparent change in capillary basement membrane by light microscopy (Fig. 19, lower panel).

Fig. 19

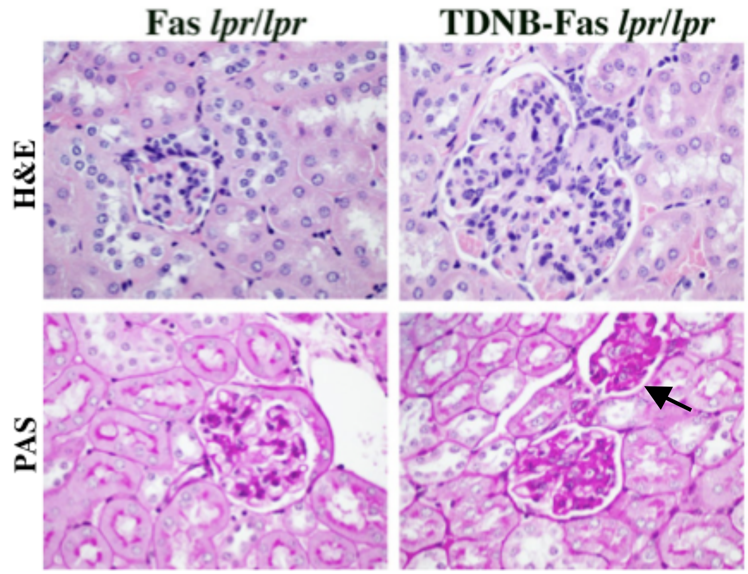


Fig. 19. TDN-B.Fas$^{lpr/lpr}$ mice showed increased kidney pathology compared to Fas$^{lpr/lpr}$ mice. Kidney sections of 6-month-old mice were stained either with hematoxylin and eosin (H&E, top) or periodic acid Schiff (PAS). TDN-B.Fas$^{lpr/lpr}$ mice had chronic inflammation, as evidenced by increased proliferation of mesangial cells, and infiltration by lymphocytes, PCs, and polymorphonuclear cells within the enlarged glomerulus (top panel). PAS staining highlighted in magenta the thickening of the basement membrane in the capillary loops of the glomeruli (black arrow). Magnification is 400X. Representative of 10-12 different mice per group.

Formation and deposition of immune complexes of IgM or IgG antibodies with complement components, such as C3, is part of the hallmark for lupus nephritis in mice and humans. We next asked whether the aggravated systemic inflammation observed in TDN-B.Fas$^{lpr/lpr}$ mice was associated with immune-complex glomerulonephritis. Fas$^{lpr/pr}$, TDN-B.Fas$^{lpr/lpr}$ and TDN-B/T.Fas$^{lpr/lpr}$ group mice showed IgM and IgG deposition (in the mesangium) with TDN-B.Fas$^{lpr/lpr}$ mice showing more deposition, whereas non-Tg animals showed little to no staining for both IgM and IgG (Fig. 20, Appendix I). The IgG deposition was exclusively present in the mesangial area. Significantly, TDN-B.Fas$^{lpr/lpr}$ mice showed more deposition of the complement component, C3d, than the *lpr* animals alone and the TDN-B/T.Fas$^{lpr/lpr}$ Appendix I). Non-Tg animals showed no C3d deposition in the glomeruli (Fig. 20).

Fig. 20

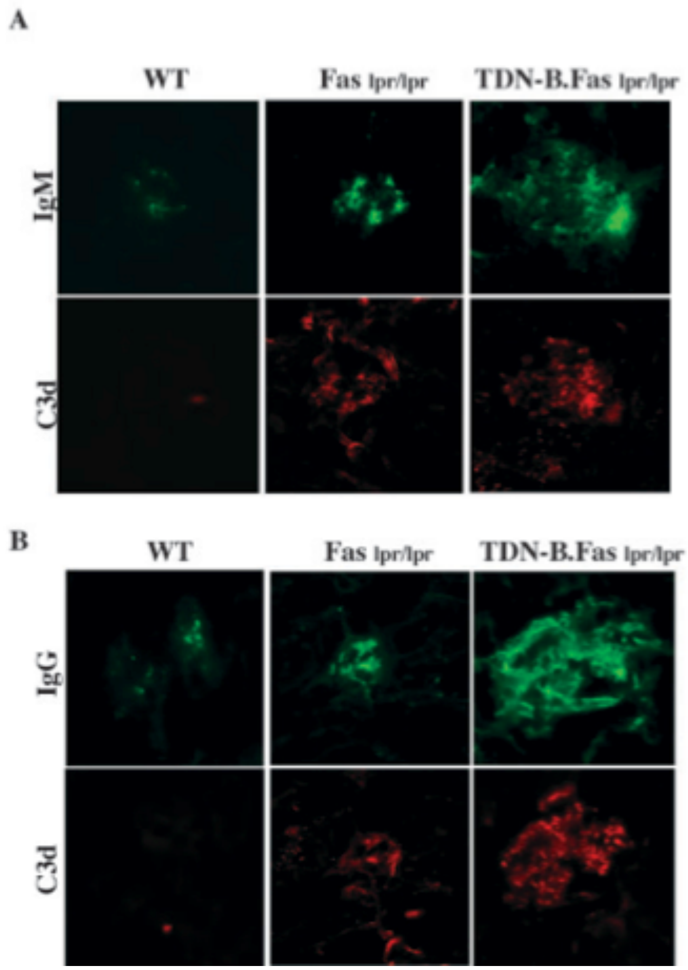


Fig. 20: MiT inhibition induces earlier development of glomerulonephritis in TDN-B.Fas$^{lpr/lpr}$ mice (A, B) Immunofluorescence staining of kidney sections for immune-complex formation of non-Tg B6 (WT), Fas$^{lpr/lpr}$ and TDN-B.Fas$^{lpr/lpr}$ mice. Sections were stained with antibodies against IgM (green), IgG (green) and C3d (red). Magnification 400X. n= 6

In conclusion, autoimmunity manifestations were accelerated in TDN-B.$Fas^{lpr/lpr}$ compared to $Fas^{lpr/lpr}$ mice at 6 months of age. They developed lupus-like disease that included greater lymphoproliferation as documented by splenomegaly and lymphadenopathy, a higher percentage of plasma cells in the spleen, increased anti-nuclear autoantibodies with DNA specificities, ANAs, normally not a characteristic autoantibody type of this model, increased proteinuria, and increased immune-complex deposition in the glomeruli and glomerulonephritis.

C. Materials and Methods:

Mice: TDN-B.B6 mice were backcrossed for > 5 generations into B6 and then $Fas^{lpr/lpr}$ (B6.Lpr) mice, purchased from Jackson Laboratories. All groups of mice ($Fas^{lpr/lpr}$ and TDN-B.$Fas^{lpr/lpr}$) used in the experiments were littermates and were kept in a spf facility. Both genders were used. All animals were provided with food and water under sterile conditions, and maintained in a 12-hr day/night cycle. Animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of SUNY Downstate Medical Center.

Serum autoantibody assays. Serum levels of IgM and IgG anti-ssDNA and anti-dsDNA were analyzed by ELISA assays as described in protocols kindly provided by Dr. Ann Davidson. For dsDNA, poly-L-Lysine plates were coated with 10µg/mL DNA prepared from lyophilized salmon sperm DNA. For ssDNA, untreated plates were coated with 10µg/mL of denatured DNA (denature at 100°C). DNA solutions were allowed to evaporate by incubating plates at 37°C for 48-72 hours (ref). After target antigen binding, plates were blocked with 5% bovine serum albumin (BSA) and 3% fetal bovine serum (FBS) in 1x PBS for 2 hours at room temperature. Sera were incubated at a dilution of 1:100 for one hour at 37°C. Plates were washed 5 times with PBS-Tween 20 (PBS-T) and incubated with either IgM–HRP or IgG –HRP secondary antibodies for one hour at 37°C. Plates were washed again 5 times with PBST and developed with TMB. Reactions were stopped with 1M H_2SO_4, and absorbance was read at 450nm on a BMG Novostar plate reader.

Urine analysis. Urine was collected and the levels of protein were determined semi-quantitatively with Multistix 8 SG (Fisherbrand). Colorometric analysis was made in a gray scale using Digital Color metric program (Macintosh, CA).

Histology. The procedures for freezing tissues, sectioning and immunohistochemical staining were conducted as described elsewhere. Briefly, kidneys were frozen in optical cutting temperature (OCT) compound (Sakura, Japan) and serial 6μm thick frozen sections were cut in a cryostat microtome, thaw-mounted onto poly-L-lysine coated slides (Fisher, USA). Fresh tissues were fixed in buffered 10% Formalin, washed in 70% ethanol, and embedded in paraffin blocks. Paraffin embedded kidneys were cut at 4μm, fixed on slides and stained with hematoxylin and eosin (H&E) or Periodic Acid-Schiff (PAS) stain. The pathology scores of renal tissue of H&E and PAS stained sections were made in a blindly manner by pathologist Dr. Susan Gottesman (SUNY Downstate) and graded on a 1-3 scale.

Immunofluorescence staining. Kidneys were snap frozen in OCT compound and stored at -80°C before being cut at 6μm with a cryostat followed by fixation in ice-cold acetone. Kidney sections were blocked with 10% horse serum and 1%BSA in 1x TBS for 1 hr at room temperature. After blocking, C3d antibody was the first one to be applied at a dilution of 1:400 and incubated for 1 hr in a humid chamber at room temperature. Slides were then washed 3 times for 5 minutes with 1x TBS, followed by incubation with an anti-goat Phycoerythrin (PE) secondary antibody (R&D Systems). Sequential stainings after C3d were done with FITC-conjugated IgM or IgG antibodies (Invitrogen) and 4,6-diamidino-2-phenylindole (DAPI, 0.5ug/mL, Invitrogen) for 1 hour. Slides were then mounted with anti-fade solution (Prolong, Invitrogen). Finally, slides were visualized using a Zeiss microscope and images were taken with Axia Vision 4.7 software.

Statistical Analyses. All data in the thesis is presented as the mean plus or minus standard error (SEM) and was analyzed for statistical significance using Student's t test for parametrically distributed data sets. Non-parametrically distributed data sets were analyzed by Mann Whitney U test and ANOVA using Prism 6-Graph Pad software. The level of significance was set at $p < 0.05$.

D. Appendix of Aim II:

F.

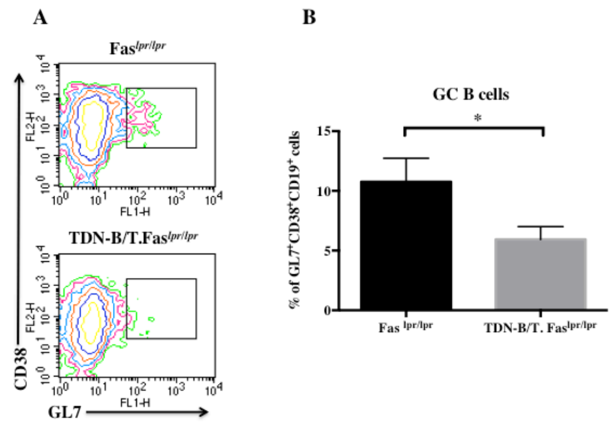


Fig. F: MiT inhibition in both B and T cells decreases splenic GC B cells in TDN-B/T.Fas$^{lpr/lpr}$ mice. Representative staining profiles (A) and percentages of GC B cells (GL7^{+}CD38^{+}) (B) are shown. Flow cytometric analysis of spleen cells of non-Tg B6 (WT), Fas$^{lpr/lpr}$ and TDN-B/T.Fas$^{lpr/lpr}$ mice. Bars are SEM of n=7-8 mice per group. *p <0.05.

G.

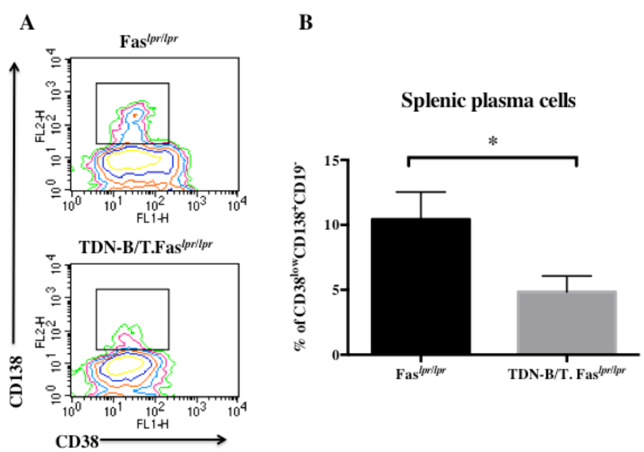


Fig. G: MiT inhibition in both B and T cells decreases splenic PC cells in TDN-B/T.Fas$^{lpr/lpr}$ mice. Representative staining profiles (A) and percentages of PC cells (CD38^{low} CD138^{+}CD19^{-}) (B) are shown. Flow cytometric analysis of spleen cells of Fas$^{lpr/lpr}$ and TDN-B/T.Fas$^{lpr/lpr}$ mice. Bars are SEM of n=5-12 mice per group. *p<0.05.

H.

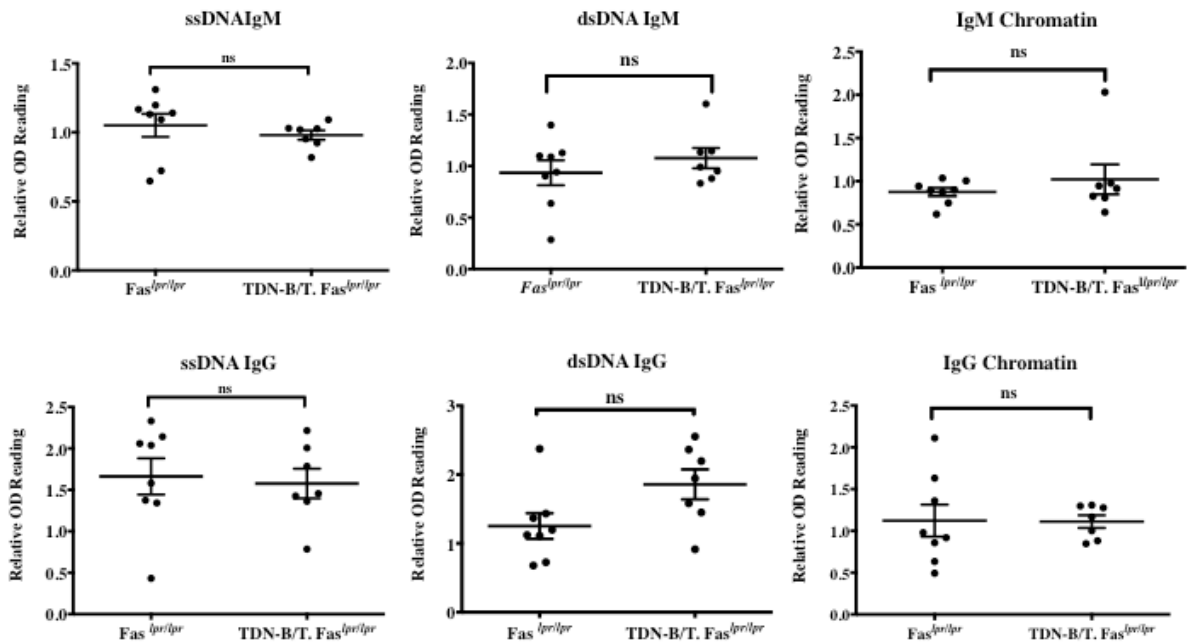


Fig. H: Production of autoantibodies in TDN-B/T.Fas$^{lpr/lpr}$ mice. Spontaneous production of IgM (top) and IgG (bottom) autoantibodies in TDN-B/T.Fas$^{lpr/lpr}$ and Fas$^{lpr/lpr}$ mice. Antibody levels in sera from each strain of mice as measured by ELISA. Bars are SEM of n=7-8 mice per group. ns, not significant. Circles represent individual mice.

I.

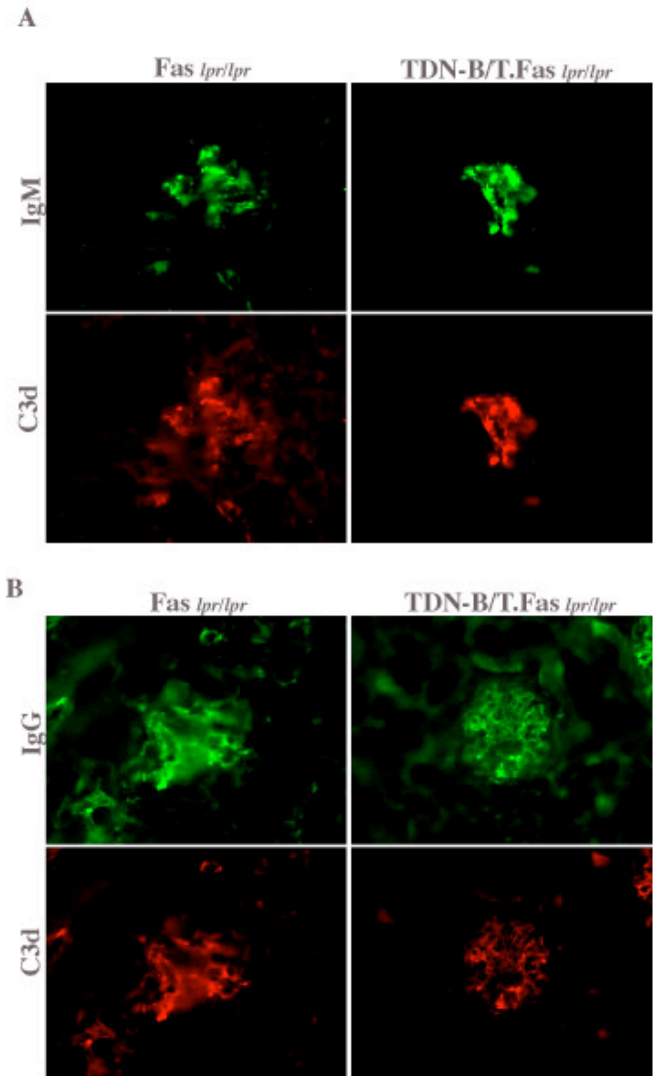


Fig. I. Development of glomerulonephritis in Fas$^{lpr/lpr}$ and TDN-B/T.Fas$^{lpr/lpr}$ mice. (A, B) Immune-complex formation: immunofluorescence staining of kidney sections from Fas$^{lpr/lpr}$ and TDN-B/T.Fas$^{lpr/lpr}$ mice were stained with antibodies against IgM (green), IgG (green) and C3d (red). Magnification 400X, n= 6.

VIII. SUPPLEMENTAL INFORMATION:

Flow cytometry (Contour plots): The essence of using flow cytometry versus other techniques is the provision of detailed quantitative information on every single cell within a larger cell population (e.g. MZ B cells in total splenocytes). One of the most common ways to show flow cytometry data is by two parameters, such as dot plots or contour plots, which shows the distribution of cells on two dimensions (2D). Contour plots are used to delineate the cell density on the *z*-axis at a particular set of 2D coordinates, or the probability that the next cell to be analyzed will display those coordinates (73).

Outliers: an outlier is generally considered to be a data point that is far outside the norm for a variable or population (74). In this case, the correct term to refer to the "outliers" observed in some of the data shown in this document could be referred as "fringe-liers". A fringe-lier could be defined as an event that occurs more often than seldom and reflects part of an authentic biological phenomenon. In the case of some of my data sets, we are analyzing various parameters of the immune system, and although our animals are kept at spf conditions, there exists the possibility that by chance some of these animals might experience infection (e.g. pinworms) that would affect cell lymphocyte numbers and properties in to a degree that deviates strongly from the rest of the population. Another example, which is the case of the appearance of fringe-liers in AIM II, is the spontaneous emergence of autoimmunity. Like in humans, autoimmune manifestations vary from mouse to mouse, e.g. splenomegaly vs. severe splenomegaly in some animals, presence or absence of lymphadenopathy or higher/lower titers of autoantibodies. Therefore, I did not remove the outliers from my data sets because these are an authentic feature of a normal immunological phenomenon that must be incorporated into the analysis. Moreover, their exclusion does not affect the statistical significance of the observations shown.

<u>Brackets used in scatter plots:</u> In this thesis, brackets were utilized to identify the groups being compared to designate a p value. The ends of the brackets indicate only those two groups that are being compared, and do not include those groups underneath the brackets

IX. DISCUSSION:

In this thesis, I addressed the role of Mitf and its family members, TFE3 and TFEB in B cell development and tolerance. Using a combination of different mouse models for MiT deficiency, I have shown that selective MiT inactivation in B cells causes radical disturbances to B cell development and homeostasis, and can break immune tolerance. TFEB and TFE3 were found to be critical for MZ and B1a B cells, whereas Mitf was critical for restraining inappropriate B cell activation, for which T cell help seemed to be crucial. Simultaneous inhibition of these proteins led to T cell help-dependent splenomegaly and accelerated lupus-like autoimmunity in the B6.lpr lupus background. Thus, these three MiT family members have discreet and important roles in different aspects of B cell regulation.

The indicators of dysregulated B cell activation – elevated anti-ssDNA IgM and spontaneous elevated PCs in unimmunized TDN-B mice -- was mostly likely due to the inactivation of Mitf, since both the TDN-B and Mitf$^{vga/vga}$ exhibited this phenotype, similar to the Mitf$^{mi/mi}$ transplantation model, and was not observed in TFE3 and TFEB deficient mice. Moreover, T cells seem to be important for these defects, as they were not observed in TDN-B/T mice. This is in line with the original observations made by the Peng group, which showed evidence that Mitf serves as a repressor of IRF4, a member of interferon receptor family, to prevent spontaneous differentiation into plasma cells (24). Our studies therefore support the conclusion that Mitf is solely responsible for dysregulated B cell activation that results in hyperresponsiveness. However, follow-up studies should examine whether basal IRF4 levels are also elevated in TDN-B and Mitf$^{vga/vga}$ B cells, which would provide stronger evidence for the proposed mechanism for how Mitf regulates PC differentiation.

In contrast, we believe the combined absence of Mitf, TFE3 and TFEB was necessary for splenomegaly and follicular hyperplasia, since these phenotypes were not observed in the Mitf $^{mi/mi}$ bone marrow transplantation model, the Mitf$^{vga/vga}$ mice, or in TFE3 or TFEB-deficient mice. In TDN-B mice, splenomegaly was evident by 6 months of age, but was anticipated by an increase in the number of splenic PCs, increase in GCs and follicular dysplasia in younger TDN-B mice in the absence of stimuli. Evidence that these changes were dependent on T cell help is that older TDN-B/T mice, which have impaired T cell help, do not have any apparent gross changes in spleen size or numbers of germinal centers (GCs). Our working interpretation is that the TDN-B/T animals do not exhibit any abnormal GC formation because there is impaired expression of CD40L on their T cells, impairing an interaction that is required for GC formation by FO B cells (35). This reflects the fact that primed T cells migrate to the T cell-B cell border to encounter B cells in the course of a normal immune reaction. The B cells proliferate and eventually terminally differentiate into PCs (7). In our TDN-B mice, MiT inhibition in B cells lead to a follicular hyperplasia, where FO B cells are located in the PALS area. My explanation that could explain this observation is that TDN-B FO B cells, because of their lower activation threshold, are being activated by ambient antigens that would normally not do so, and attracted by a chemokine gradient causing them to move towards the PALS. B cells and T cells normally move towards each other upon antigen stimulation by the attraction of a chemokine gradient of Chemokine Ligand-13 and a change in expression of their chemokine receptors, CXCR5 and CCR7 (72). Lymphocytes with dysregulated expression of these receptors lead to defects in this repositioning. What is particularly noteworthy in the TDN-B mice is that T cells are repositioning even though B cells are the affected cell type, which suggests that FO T cells are responding to B cell activation without requiring their own cognate antigen stimulation to do so.

TDN-B mice also had a number of additional B cell abnormalities that were not observed in the Mitf$^{mi/mi}$ transplantation model. Namely, we observed changes in non-FO B cell subsets that can function independently of T_H cell activation: MZ B cells and B-1a cells. In younger TDN-B mice we observed a significant decrease of the proportions of MZ B cells, a phenotype that persisted as the mice aged; consequently, a decrease in the titers of IgG3 was also observed. On the other hand, younger TDN-B mice had a normal proportion of PerC B-1a cells, but as the animals aged, the maintenance of these cells was lost. None of the changes observed in these B cell subsets were present in the Mitf$^{vga/vga}$. Why? Concerning the latter point, it is possible is that Mitf is simply not expressed in B-1 or MZ B cells, but that evidently TFEB and TFE3 have the more prominent role in these B cell subsets and possibly earlier during B cell development (18). In addition, my findings indicated that TFE3 knockout mice do not demonstrate any of the B cell abnormalities described for the Mitf$^{mi/mi}$ or the TDN-B mice, suggesting that TFE3 elimination by itself is not sufficient for the phenotype. Rather, analysis of TFEB$^{+/-}$TFE3$^{-/-}$ and TFEB-CD19cre conditional TFEB knockout mice strongly implies that TFE3 and TFEB are together important for MZ B cells and PerC B-1 cells, since we observed a similar phenotype to the one observed in TDN-B mice, in the reduction in the numbers of both B cell subsets. Moreover, I see evidence of functional overlap between TFE3 and TFEB, because whereas TFEB$^{+/-}$ mice also have no phenotype (not shown), the combination of both TFE3 and TFEB haploinsufficiency impacted these B cell subsets. Future experiments should examine double B cell knockouts (TFE3$^{-/-}$ and TFEB-CD19cre) mice to establish that this is truly B cell specific, since the TFEB$^{+/-}$ is germline haploinsufficiency and affects all cells. Finally, TDN-B/T mice also had a decrease in the proportion MZ B cells, and the phenotype persists, as the mice got older. In a similar fashion, there was a marked reduction of B-1a B cells in the peritoneum of older TDN-B/T mice, suggesting that these B cell subsets are not affected by MiT-dependent T cell functions (11, 13, 18).

Given that my findings indicate that TFEB in particular might be the critical MiT member for the development of MZ B and PerC B-1a cells, future studies should examine the molecular basis of this dependency. Some insight as to what might be happening relates to recent findings that show that TFEB is a regulator of autophagy, a catabolic process that allows cells to degrade and recycle constituents and nutrients (31). TFEB regulates the expression of many genes involved in the process of autophagy, such as the *Atg* genes and genes involved in lysosome biogenesis. Published data have shown that ablating the Atg5 gene in B cells – a gene critical for essential steps in autophagy - causes the selective loss of maintenance of PerC B-1 cells, which are known to undergo autophagy homeostatically (30, 32); B2 B cells were not affected. These published observations support the hypothesis that the B-1 phenotype I observed in TDN-B, TDN-B/T, $TFEB^{+/-}TFE3^{-/-}$ and TFEB-CD19cre mice may in part be due to a TFEB-dependent deficiency in autophagy required for B1a B cell homeostasis.

However, the involvement of autophagy in MZ B cells is not known. One possibility is that autophagy might be important in this B cell subset as it is in B1a cells and TFEB might be important in that process. However, there are other possibilities. In the TDN-B and B/T models, the MZB defect is more severe than the B1a in that the MZ phenotype is evident even in younger animals when the MZ compartment is first being established (~1 month of age). MZ B cells are bone marrow derived-B-2 cells that upon early development migrate to secondary lymphoid organs like the spleen in where they transition into a more mature stage. This transition completely depends on the signal that this B cell receives through their BCR, and it involves several pathways including Notch-2. Published observations have shown the Notch-2 pathway is critical for immature B cells to transition into MZ B cells (65). A gene responsible to facilitate transitional B cells into the MZ B cell pool is called *Fos*. Fos (distinct from c-fos) is expressed in MZ B cells, in which its levels increase when these B cells are stimulated with LPS, but deleting Notch-2 in B cells decreases the amount of Fos present in B cells (65). Another pathway that is involved in the MZ B cell development is dependent on PI3K. The PI3K pathway is crucial for B cell development and activation in general (66). It has been shown that deletion of the catalytic subunit of PI3K, p110δ, leads to a severe reduction of MZ B cells, as well as B-1 cells, whereas B2 cells were not affected (66). These results suggest a possible synergism of TFEB with Notch-2 and PI3K pathways in MZ B cells. Given these observations, on one front, future work should address whether TFEB inactivation affects autophagy and related phagocytic processes in MZ and B1a B cells that might be required for their development and homeostasis, such as by evaluating how well these B cells ingest particulates in culture and in vivo when TFE3 and TFEB are deleted when they are still present in sufficient numbers to test. In addition, it would be important to establish the relationship between TFEB and these other genes known to be uniquely important for MZ B cell development compared to other B cell subsets. For example, a possible model to test is that TFEB regulates the genes encoding Fos, PI3K, and/or Notch-2, and when TFEB is absent, the expression of these genes critical for MZ B cells is reduced. This could be tested by examining the effects of knocking down TFEB in these B cells and following the expression of these genes. These findings also would support the idea that B2 cells do not depend on these processes.

A dramatic illustration of the importance of the MiT family in tolerance was in the lupus-prone setting. One logical possibility is that Mitf mutation alone contributed to aspects of B cell dysregulation responsible for accelerating lupus like disease in the mouse model TDN-B.Fas$^{lpr/lpr}$. In this mouse model, we observed that animals at an early age (5-6 months of age) develop accelerated manifestations of lupus-like disease, such as splenomegaly that in some cases was severe, diffuse lymphadenopathy, high titers of autoantibodies with DNA and RNP specificity and glomerulonephritis. The accelerated phenotype could be due to the hyper-responsive FO B cells due to Mitf inactivation that synergized with the *lpr* mutation. This hyper-responsiveness is highly dependent on T cell help since our other autoimmune model, the TDN-B/T.Fas$^{lpr/lpr}$ mice, did not exhibit the severe phenotype observed in TDN-B.Fas$^{lpr/lpr}$; it has been shown that lupus pathogenesis depends in the expression of CD40L (40).

Another hypothesis of why the lupus-like phenotype observed in the TDN-B.Fas$^{lpr/lpr}$ was so robust – and one that does not preclude but rather synergizes with Mitf mutation - could be due to accumulation of debris from apoptotic cells. It is known that natural IgM antibodies serve as regulatory antibodies that aid in the clearance of apoptotic cells or debris (69, 70). Natural IgM antibodies secreted by MZ and B-1a B cells could be decreased in this model due to the low numbers of these B cell subsets. If the number of regulatory natural antibodies is low, there exists the possibility that an accumulation of intracellular debris serves as material to activate autoreactive B cells that are accumulating. Support for this hypothesis would be measuring the different IgG isotypes in the lupus cohort, and/or supplementing TDN-B.Fas$^{lpr/lpr}$ mice with IgG3 from healthy mice to see whether this is protective. Over recent years, autophagy has been implicated in a number of diseases including SLE. Loss of Atg5 dependent effects, which include clearance of dying cells and cell Ag presentation, might contribute to autoimmunity and inflammation, both associated with SLE (68, 70). If this is the case, we could argue that the severity of autoimmune manifestations observed in the TDN-B.Fas$^{lpr/lpr}$ mouse model could be accounted for by the loss of, or inappropriate activation of MZ B and B-1 cells, which require autophagy to process and phagocytized antigenic material (69, 70). Improperly processed self-material might inappropriately activate these B cell populations.

Support for this idea is that the TDN-B. Fas$^{lpr/lpr}$ mice exhibit autoantibody specificities not characteristic of B6. Fas$^{lpr/lpr}$ mice. There is a wide range of autoantibodies identified in patients with SLE, but a different spectrum of autoantibodies is usually captured in mouse models. DNA and RNP autoantibodies have been identified in mice models such as MRL.lpr and NZB/ZW, whereas only autoantibodies with DNA specificity can be identified in the B6.lpr mouse model. However, the spectrum of autoantibodies in TDN-B.Fas$^{lpr/lpr}$ mice inclined more towards those observed in the MRL.lpr model and human lupus patients. This tells us that the specificity of autoantibodies made by these animals is dependent on the inhibition of the MiT family, and may be a reflection of the availability of self-antigens that would normally be cleared with the help of natural IgM.

In summary, my studies demonstrate that the MiT family serves as powerful immune regulators that maintain a balance to prevent immune dysregulation that could lead to autoimmunity. The observations made in this thesis are only the tip of the iceberg, and open new directions for further exploration. Most importantly, it will be critical to determine the molecular mechanisms and signaling pathways that interface with these transcription factors. Although the MiT family of proteins has been implicated in the autoimmune model shown here, disease pathogenesis is still poorly understood. In order to identify if the MiT genes are involved or interact with other genes or pathways involved in autoimmune pathogenesis, a genome-wide mRNA expression analysis of the spleens and kidneys of the TDN-B.Fas$^{lpr/lpr}$ mice throughout the disease course could be performed. This same microarray could be performed in human lupus patients to determine if the MiT genes are also involved in disease pathogenesis. With that information in hand, it might be possible to exploit them for therapeutic purposes to control abnormal B cell responses.

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