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Regulation of Autoimmune Germinal Center Reaction by Tfh Cells and Application of Tfr-like Cells for the Treatment of Autoimmune B Cell Responses

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REGULATION OF AUTOIMMUNE GERMINAL CENTER REACTION BY Tfh CELLS AND APPLICATION OF Tfr-LIKE CELLS FOR THE TREATMENT OF AUTOIMMUNE B CELL RESPONSES

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A

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By

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DEDICATION

To my family for their love, encouragement and support to achieve my goals.

To myself, Young Uk, Cheers!!

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REGULATION OF AUTOIMMUNE GERMINAL CENTER REACTION BY Tfh CELLS AND APPLICATION OF Tfr-LIKE CELLS FOR THE TREATMENT OF AUTOIMMUNE B CELL RESPONSES

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Excessive follicular helper T (Tfh) cell responses to self-antigens are associated with antibody-mediated autoimmune diseases in humans including systemic lupus erythematosus (SLE). Numeral and functional aberrations of T regulatory T (Treg) cells are common in patients with autoimmune diseases. Although different types of immunosuppressive agents have been used clinically to treat antibody-mediated autoimmune diseases, they generally have side effects due to the lack of target-specificity. To minimalize the adverse effects, there is a need to develop target-specific therapeutics which specifically control auto-reactive B cell responses and auto-reactive Tfh cell responses. Recent studies unveiled that $Foxp3^+$ Treg cells expressing CXCR5 can migrate into the germinal center (GC) zone where they specifically suppress GC reactions *in vivo*, presumably by directly suppressing B cells and/or Tfh cells. These $CXCR5^+$ $Foxp3^+$ Treg cells are termed as follicular regulatory T (Tfr) cells. Due to their ability to specifically suppress Tfh cell and GC B cell responses, use of Tfr cells may be a promising target-specific therapy for the treatment of autoantibody-mediated autoimmune diseases. To evaluate the role of Tfr cells in autoantibody-mediated autoimmune

diseases, we employed a BXD2 mouse model of spontaneous autoimmune lupus. Immune balance between Tfh and Tfr cell responses is crucial for the prevention of self-destructive antibody generation. However, the contribution of Tfh cells and Tfr cells to auto-reactive B cell responses in the BXD2 strain had not been evaluated. Therefore, we examined Tfh, Tfr and other relevant immune cellular responses in this autoimmune strain. We found no differences in both the frequency of Th17 cells and the levels of IL-17 in the circulation between wild-type and BXD2 mice. By contrast, the frequency of Tfh cells was significantly increased, and the numbers of Tfh cells were positively correlated with the levels of autoantibodies. In addition, we observed that IL-21-producing Tfh cells, but not IL-17-producing Th17 cells, efficiently promoted the production of IgG from BXD2 B cells *in vitro*. These results supported the role of Tfh cells in the development of auto-reactive B cell responses. In addition, the frequency of Tfr cells was reduced in BXD2 mice. Therefore, imbalance between Tfh cells and Tfr cells in BXD2 mice likely caused the self-destructive antibody generation, thereby providing additional support that Tfr cell-based immunotherapy may ameliorate antibody-mediated autoimmunity. Unfortunately, sufficient numbers of Tfr cells that will be required for immunotherapy will be difficult to obtain since they are only found in low frequency *in vivo*. To address this problem, we employed retroviral transduction of CXCR5 onto $Foxp3^+$ Treg cells, which are more abundant and less difficult to purify than Tfr cells. We termed these engineered CXCR5 overexpressing $Foxp3^+$ Treg cells as eTfr cells. We demonstrated that transduction of CXCR5 in the eTfr cells did not affect the expression of other genes important for Treg or Tfh cell function. Furthermore, eTFr cells migrated in response to CXCL13 and had T cell suppressive capacity *in vitro*, demonstrating that eTfr cells maintained critical Tfr cell-like properties *in vitro* and were potentially a cell

source for Tfr cell-based immunotherapy. To test the therapeutic potential of the eTfr cells, we performed *in vivo* adoptive co-transfer experiments using TCRβ-deficient mice. Unfortunately, the results from these *in vivo* investigations were inconclusive, indicating the further refinement of the model system will be required to determine the viability of the eTfr therapeutic approach for the autoantibody-mediated autoimmune disease. In summary, we demonstrated that **imbalance between Tfh cell and Tfr cells and IL-21, produced by Tfh cells, lead to auto-reactive GC B cell responses in BXD2 mice**, suggesting that similar imbalances may have relevance in human autoantibody-mediated autoimmune diseases. Moreover, eTfr cells can migrate in response to CXCL13 and suppress T cell responses *in vitro*, supporting the **possibility that eTfr cells may provide a novel immunotherapeutic approach for the treatment of antibody-mediated autoimmune disorders**.

Table of Contents

List of Illustrations

List of Tables

CHAPTER 1

General Introduction

1. Autoimmune diseases

Autoimmune diseases contain more than 100 different types of disorders characterized with various pathogenesis [1]. Many of the pathogenic mechanisms causing autoimmunity are not clearly defined, in general, however, failure of apoptosis of self-reactive lymphocytes (T and B cell), defects in number and function of regulatory T (Treg) cells, inadequate function of inhibitory molecules, and abnormal activation of antigen presenting cells (APCs) are known to cause autoimmune reactions [2]. Moreover, genetic susceptibility, epigenetic, hormones, and environmental factors are also associated with development of autoimmune diseases [3]. There are two major types of autoimmune diseases; T cell-mediated and autoantibody-producing B cell-mediated autoimmune diseases. Among them, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), and Sjögren's syndrome are characterized by the existence of auto-reactive T cells and/or the development of auto-reactive B cell producing specific autoantibodies [4]. General features of each antibody-mediated autoimmune disease and its pathogenic mechanism are described in Table 1.

Table 1. List of autoantibody-mediated autoimmune diseases and their clinical symptoms and mechanisms

Disease	Major Clinical Manifestations	Disease mechanism
Systemic lupus erythematosus	Inflammation of tissues, glomerulonephritis, vasculitis, rash	Autoantibodies and auto-reactive T cells against DNA and nucleoprotein antigens
Rheumatoid arthritis	Inflammation of the joints, joint stiffness and pain, loss of range of motion	Autoantibodies and auto-reactive T and B cells against joint-associated antigens
Multiple sclerosis	Formation of sclerotic plaque in brain, leading to muscle weakness, ataxia, and other symptoms	Autoantibodies and auto-reactive T cells against brain antigens
Sjögren's syndrome	Inflammation in salivary and lacrimal glands, dry eyes and mouth	Autoantibodies and auto-reactive T cells against apoptotic cells

Adapted with permission from Gharagozloo M, Majewski S, and Foldvari M, *Therapeutic applications of nanomedicine in autoimmune diseases: From immunosuppression to tolerance induction.* Nanomedicine, 2015. **11**(4): p. 1003-1018.

1.1. Systemic Lupus Erythematosus

SLE is one of the representative autoimmune disorders caused by excessive autoantibodies to self-antigens. Production of multiple polyclonal autoantibodies, which causes irreversible tissue damages, is a common clinical manifestation of SLE [5]. Dysregulation of adaptive immunity and overproduction of autoantibodies leads to chronic inflammation in multiple organs, such as skin, joints, kidney, serous membranes, central nervous system and blood [5]. SLE predominantly affects women, particularly during their

pregnancy, and has strong genetic and environmental components [6]. Symptoms of SLE may vary with the individual, common symptoms of SLE include fever, fatigue, anorexia, myalgia, and weight loss [7]. Currently, there is no cure for SLE and therapeutics used only relieve the symptoms [2].

1.2. Current clinical therapeutics in autoimmune diseases

Currently, several non-biological agents (Chemical based) and biologics (recombinant proteins and antibodies) are being used for the treatment of autoimmune diseases (Table 2). In the past, treatment of autoimmune diseases has primarily focused on the induction of systemic immunosuppression, interfering with the activation and migration of immune cell, or cell-targeted immune suppression [2]. For instance, non-biological reagents such as cyclosporine and cyclophosphamide suppress a broad range of immune reactions including pathologic autoimmune responses. On the other hand, biological reagents regulate immune responses by blocking pro-inflammatory cytokines or targeting pathogenic immune cell types (see table 2). There is currently no cure for SLE, and to control symptoms non-biological reagents such as Prednisone and Methotrexate are prescribed, which suppress a broad range of immune reactions [2]. However, these therapeutics suppress systemic immune responses broadly and thus can lead to several side effects such as renal, gastrointestinal, neurologic, hematologic, immunologic toxicities or increase the risk of developing certain types of cancer [2, 8]. Generation of auto-reactive autoantibody is mediated by certain types of T cells including Tfh cells [9]. However, these drugs have no target specificity for the inhibition of Tfh cells and auto-reactive B cells. The development of

alternative therapies with specificity is, therefore, urgently needed for the treatment of autoantibody-mediated autoimmune diseases in humans.

Table 2. Current clinical immunosuppressive therapies in some autoimmune diseases.

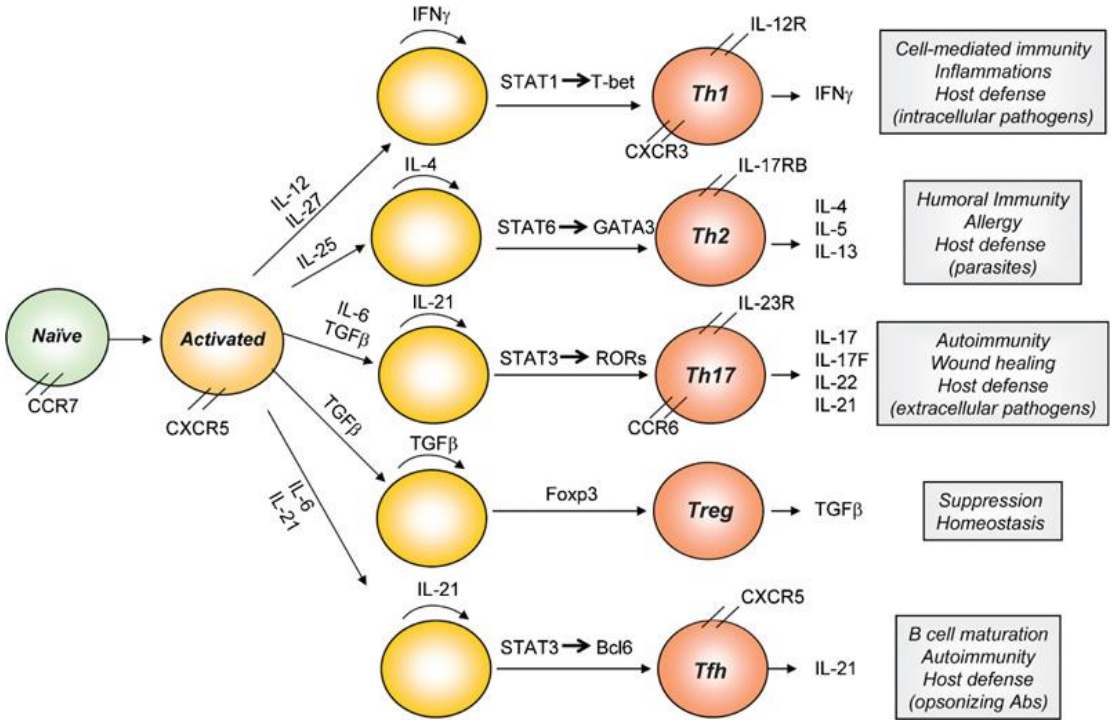
	Therapeutic group	Agent	Mechanism of action	Indications
Non-biological	Glucocorticoid	Prednisone, methylprednisolone	Inhibit many processes involved in immune response and inflammation	MS, RA, SLE
	Calcineurin inhibitor	Cyclosporin	Block T cell activation and IL-2 production	RA
	Anti-metabolite	Azathioprine, Leflunomide, Mycophenolate mofetil Methotrexate	Interfere with the synthesis of culeic acids and cell proliferation	RA, SLE
	Anti-proliferative	Sirolimus	Inhibit T cell proliferation	SLE, RA
Biological	Anti-cytokines			
	IL-1	Anakinra	IL-1 receptor antagonist	RA
	IL-6	Tocilizumab	Inhibit IL-6 signaling	RA
	TNFα	Infliximab, Adalimumab, Golimumab, Cerolizumab, Etanecept	Block TNFα activities	RA
	Anti-cell surface ligands			
	CTLA4	Abatacept	Block T cell co-receptors	RA
	CD20	Rituximab, Crelizumab, Ofatuaumab	Target and deplete B cells	RA
	α4 integrin	Natalizumab	Blocking cell adhesion and migration	MS
	Immuno-modulating proteins			
	IFNβ	Betaferon	Suppress T cell activity	MS
	Glatiramer acetate	Copaxon	Suppress T cell activity	MS
	Intravenous immunoglobulin	IVIg	Suppress or neutralize autoantibodies, activated complement components, and inflammatory cytokines	MS

Adapted with permission from Gharagozloo M, Majewski S, and Foldvari M, *Therapeutic applications of nanomedicine in autoimmune diseases: From immunosuppression to tolerance induction.* Nanomedicine, 2015. **11**(4): p. 1003-1018.

1.3. Role of T helper cell subsets in autoimmune diseases

T helper cells are key cellular regulators of the immune response. Upon antigen-specific stimulation through the T cell receptor, naïve $CD4^+$ T cells can be differentiated into different subsets of T helper (Th) cells depending upon different stimulatory conditions such as costimulatory signals as well as cytokines produced by antigen-presenting cells (Figure 1) [10]. In particular, the types of cytokine stimulation, termed 'signal 3', are known to be essential for activated T cells to acquire distinct transcription factors that govern each helper T cell lineage. Among diverse Th cell lineages, follicular helper T (Tfh) cells play a crucial role in controlling B cell maturation within germinal center (GC) during normal immune responses [9, 11]. GC is a histologically distinct structure that develops within B cell zones of secondary lymphoid tissues, where B cell affinity maturation, somatic hypermutation and selection, class switch recombination (CSR), plasma cell (PC) differentiation, and memory B cell differentiation occurs [9]. While Tfh cells are necessary for humoral immune responses to infectious pathogens and cancerous cells, excessive Tfh responses likely facilitate autoimmune B cell responses by promoting production of autoantibodies [12, 13]. The existence of autoantibodies is a hallmark of a number of autoimmune diseases. Escaping B cell tolerance against self-antigens results in the development of autoantibodies [13]. Increased Tfh responses are strongly associated with systemic autoimmunities, such as SLE, and spontaneous generation of GCs and expansion of Tfh cells are primary features of SLE [13-15].

Figure 1. The differentiation of CD4$^+$ T cell subsets. Effector Th cell differentiation. Upon encountering antigen presented by professional APCs, naive CD4$^+$ T cells differentiate into effector subsets (Th1, Th2, Th17, Treg and Tfh) that are characterized by their cytokine production, expression of transcription factors and chemokine receptors, and immune regulatory functions. (APC, antigen-presenting cell; Bcl6, B-cell lymphoma 6; CCR, CC chemokine receptor; CXCR, CXC receptor; Foxp3, Forkhead box p3; IFN, interferon; ROR, retinoic acid-related orphan receptor; Tfh, T follicular helper; TGF, transforming growth factor; Th, T helper; Treg, regulatory T)



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1.4. Role of regulatory T cells in autoimmune diseases

The disruption of immune tolerance to self-antigens by T cells and B cells is consistently observed in autoimmune conditions. However, the underlying mechanisms of this phenomenon by which self-reactive immune cells escape from immune tolerance and regulatory mechanisms remain incompletely understood [16]. Understanding the cellular and molecular mechanisms of how auto-reactive immune cells are controlled will allow us to develop alternative therapeutic approaches for the treatment of autoimmune diseases.

Deletion or mutation of Foxp3 in mice or humans causes multi-organ autoimmunity, inflammatory disease, and allergy [17]. The Foxp3 expressing Treg cell population is the key component of peripheral tolerance. Numeral and functional abnormalities of Treg cells are common in patients with SLE and RA [18]. In a lupus-prone animal model of autoimmune disease, transfer of *ex vivo* expanded Treg cells showed relevant improvements in renal disease severities and survivals [19]. An increase of Tfh cells is positively correlated with autoantibody titers and disease symptoms and severity in many autoantibody-mediated autoimmune diseases [20-22]. Recent studies have described that different types of Treg subsets regulate distinct T helper cell responses [23]. In 2011, three independent groups reported follicular regulatory T (Tfr) cells as a specialized subset of Treg cells suppressing germinal center reactions *in vivo* (Table 3) [24-26]. Moreover, the number of circulating $CD4^{+}Foxp3^{+}CXCR5^{+}$ T cells from SLE patients is critically fewer than in healthy controls [27]. Therefore, a more complete understanding of the Tfh-specific regulation at a mechanistic level would greatly assist the development of target-specific therapeutics for the treatment of antibody-mediated autoimmune diseases.

Table 3. Diverse subsets of Foxp3$^+$ regulatory T cells

Subsets	Transcription factor	Chemokine receptor	Function	Ref.
Th1 Treg	Foxp3 T-bet	CXCR3$^+$	T-bet$^+$ Foxp3$^+$ Treg cells express CXCR3 and colocalize with Th1 cells	[28-31]
Th2 Treg	Foxp3 IRF4 GATA3		IRF4-mediated CTLA4 expression on Foxp3$^+$ Treg cells has a disproportionate effect on Th2 cells	[28, 32]
Th17 Treg	Foxp3 STAT3		STAT3-mediated IL-10 expression by Foxp3$^+$ Treg cells has a disproportionate effect on Th17 cells	[28, 31, 33]
Tfr	Foxp3 Bcl-6	CXCR5$^+$	Bcl-6 induced expression of CXCR5 on Foxp3$^+$ Treg cells enables Tfr cells to migrate into B cell follicles	[24, 25, 28]

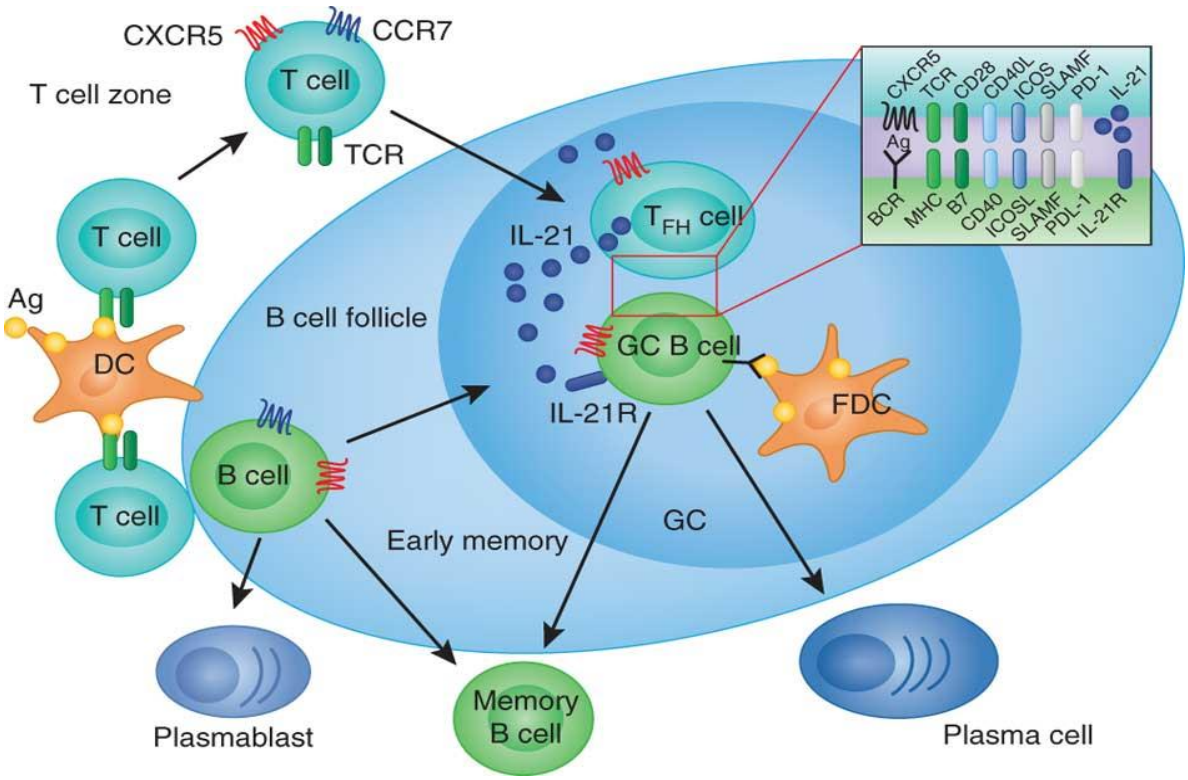
Adapted with permission from Chang JH and Chung Y, *Regulatory T cells in B cell follicles.* Immune Netw, 2014. **14**(5): p. 227-36.

2. Follicular helper T cells

Depending on the types and levels of costimulation and environmental cytokines, naïve $CD4^+$ T cells can be differentiated into different subsets of Th cells. Among them, follicular helper T (Tfh) cells are a specialized Th cell subset that induces the differentiation of B cells into PCs and memory B cells, and are essential for GC formation, affinity maturation as well as CSR of immunoglobulins (Ig) [11]. In the 1960s, the concept of Tfh cells was first suggested based on the fact that Th cells are required for the development of antibody responses [34, 35]. Numerous studies supported the helper role of $CD4^+$ T cells for the development of GC, a distinct structure in secondary lymphoid organs where B cell maturation and high-affinity B cell selection occur [36]. However, which Th subset was engaged in helping B cells has remained unclear for decades. The C-X-C chemokine receptor type 5 (CXCR5), mainly expressed in B cells was discovered [37] and shown to be critical for the migration of B cells into follicles in lymphoid organs [38]. The expression of CXCR5 was also found in a subpopulation of activated Th cells in the secondary lymphoid organs from immunized mice, and its expression was essential for the migration of Th cells into follicles [39]. CXCR5-expressing $CD4^+$ T cells in human tonsils enhanced immunoglobulin production from B cells *in vitro* compared to CXCR5-negative $CD4^+$ T cells obtained from the same tonsil [40, 41], and they were mainly localized in GCs [42]. This CXCR5 expressing $CD4^+$ T cell subset was also found in mouse lymph nodes [43]. These cells help B cells by secreting cytokine IL-21 [44] as well as by providing activating signal CD40 ligand (CD40L) [45, 46]. The transcriptional repressor B-cell lymphoma 6 protein (Bcl-6) was discovered to be essential for the generation of Tfh cells *in vivo* in animal models [47-49].

Tfh cells express CXCR5, ICOS, programmed cell death protein 1 (PD-1), basic leucine zipper transcription factor ATF-like (BATF), signal transducer and activator of transcription 3 (STAT3), c-Maf and interferon regulatory factor 4 (IRF4), Achaete-scute complex homolog 2 (Ascl2), as well as the cytokines IL-21, IL-4 and IL-10 [11]. Other surface molecules, such as CD40L, B and T lymphocyte attenuator (BTLA), CD84, and SAP, are also expressed in Tfh cells. The simplified schematic germinal center reaction is described in Figure 2.

Figure 2. The overview of germinal center reaction. Activated antigen-specific T cells in the T cell zone upregulate ICOS, PD-1, and CXCR5 and migrate to the B cell follicles. After interacting with their cognate B cells, these T cells differentiated into Tfh cells. When follicular B cells encounter antigen, they move to the border of the T cell zone and can further differentiate into extrafollicular plasmablasts, generate early memory B cells or re-enter to the follicle and form a GC. In the GC, interactions of TCR:MHC, CD28:B7, CD40:CD40L, ICOS: ICOSL; SLAM:SAP, PD-1:PDL-1, and IL-21:IL-21R between Tfh and GC B cells are important for GC formation. Cytokines from Tfh cells, particularly IL-4 and IL-21, influence the generation of affinity-matured memory B cells and long-lived plasma cells.



Adapted with permission from Nutt SL and Tarlinton DM, *Germinal center B and follicular helper T cells: siblings, cousins or just good friends?* Nat. Immunol, 2011. **12**(6): p. 472-7.

2.1. Tfh cell differentiation

Tfh cell differentiation is a multi-step and multi-signal process [11], which is initiated by dendritic cell (DC) priming of a naïve $CD4^+$ T cell [50]. Concomitant upregulation of CXCR5 by Bcl-6 [47-49] and Ascl2 [51], and downregulation of CCR7 and PSGL-1 expression [52, 53] migrate Tfh precursor cells to the border of the B cell follicle and interact with antigen-presenting B cells at the border of the B cell follicle and the T cell zone [39, 53].

IL-6, ICOS, IL-2 and T cell receptor (TCR) signal strength are known as key regulatory factors during early Tfh cell differentiation [11]. Upon TCR signal and non-TCR signal combination, naïve T cells can be differentiated into different effector cell types. High TCR affinity possessing $CD4^+$ T cells preferentially differentiate into Tfh cells in a pigeon cytochrome C model [54], but not in a Friend virus infection [55]. IL-6 induces transient Bcl-6 expression by newly activated $CD4^+$ T cells [48]. The Bcl-6 expression is required for early CXCR5 expression in Tfh cells in multiple models [56-58]. Since IL-6 is also necessary for Th17 cell differentiation, other stimulatory signals may be involved in Tfh cell differentiation. ICOS is important for Tfh cell differentiation [59] and migration [60]. Several studies also described the importance of ICOS in Tfh cells. Roquin inhibits ICOS and deficiency of Roquin1 and Roquin2 results in spontaneous GC development and Tfh cells [61, 62]. Phosphatidylinositol-3-OH kinase (PI3K) pathway is the only known ICOS downstream signal pathway [63, 64] and the miR-19~72 complex is necessary for Tfh cell differentiation and migration by suppressing the expression of a phosphatase PHLPP2, and partially PTEN, which are inhibitors of ICOS signaling [65, 66]. Antigen-specific B cells in the follicle provide ICOSL [59], and the ICOS-ICOSL interaction induces directional migration of $CD4^+$

T cells to the B cell follicle [60]. IL-2 plays a suppressive role in Tfh cell differentiation and can act early during T cell priming by inducing STAT5-mediated Blimp-1 [67, 68].

Other factors are also involved in Tfh cell differentiation. Foxp1 is a critical negative regulator of Tfh cell differentiation [69]. By regulating IL-21 and ICOS, Foxp1 suppresses Tfh cell differentiation. Naïve $CD4^+$ T cells from Foxp1-deficient mice preferentially differentiate into Tfh cells and show enhanced GC and antibody responses. A transcription factor Foxo1 binds to the Bcl-6 promoter region [70], and is also a target of E3 ubiquitin ligase Itch [71]. By ubiquitinating and degrading Foxo1, Itch regulates Bcl-6 expression. $CD4^+$ T cells from Itch-deficient mice show reduced expression of Bcl-6 and low frequencies of Tfh cells [71]. This result is in conflict with a previous study, which showed a positive correlation between Bcl-6 gene expression and binding of Foxo1 in the Bcl-6 promoter [70].

2.1.1. Germinal center resident Tfh cell differentiation

After Tfh precursor cells interact with GC B cells at the T:B border, migration into and retention in the follicles are necessary for GC-resident Tfh (GC Tfh) cells. Most of the GC-resident Tfh cells are $CXCR5^{hi}$ $PD1^{hi}$ $Bcl6^{hi}$ Maf^{hi} SAP^{hi} $PSGL1^{lo}$ $CD200^+$ $BTLA^{hi}$ $CCR7^{lo}$ and secrete C-X-C motif chemokine 13 (CXCL13), IL-21 and IL-4 [9, 11]. Adhesion molecules on GC Tfh cells have key roles in regulating their interaction with GC B cells and localization. The signaling lymphocyte activation molecule (SLAM) family receptors SLAMF6 (also known as Ly108), CD84, and SLAM are self-ligands differentially expressed in GC Tfh cells and/or GC B cells [72]. These SLAM family receptors can recruit SAP, a SH2-domain adaptor protein that binds to the cytoplasmic tails of SLAM family receptors and is specifically upregulated in GC Tfh cells [73]. SAP expression is critical for GC Tfh

cell development, GC development, and the generation of the majority of memory B cells and memory plasma cells [73-76]. In the absence of SAP, Tfh cells cannot be recruited to or retained in a nascent GC to maintain the GC reaction [74]. Moreover, SAP prevents binding of phosphatase SHP-1 to immunotyrosine switch motifs (ITSM) in SLAMF6 and provides positive signals within the Tfh cells [77]. These positive signals sustain T:B cell adhesion and helper functions.

2.2. The functions of Tfh cells

The most critical role of Tfh cells is their contribution to B cells for GC development and function. In the light zone (LZ) in GCs, GC B cells bind to the antigen and present antigen peptide:MHC complexes to Tfh cells, while activated Tfh cells provide help signals to GC B cells, essential for their survival and proliferation [9, 36]. GC B cells receive survival signals from Tfh cells then migrate to the dark zone (DZ), where GC B cells undergo proliferation and somatic hypermutation [9, 36].

Tfh cells control GC size [47, 64, 78], regulate low-affinity B cell entry into the GC, support high affinity B cell occupation of the GC [79], and select high-affinity B cells during affinity maturation [80, 81]. Both cytokines and cell surface molecules secreted and presented by Tfh cells are the help signals to GC B cells. CD40L, together with IL-21 or IL-4 is required for the GC B cells survival and proliferation [9]. CD40L-CD40 engagement is critical for the maintenance of GC B cells [82]. Furthermore, CD40-CD40L interaction is crucial for the migration of T cells into follicles [83]. IL-21 and IL-4 also trigger Ig isotype switching to produce IgG3, IgA and IgG1 in human B cells, or IgG1 in murine B cells [84-86] and IgG1 and IgE [87, 88], respectively. ICOS signal induces the expression of IL-21, IL-4,

c-Maf and CXCR5 [56, 89, 90]. ICOS signaling, mediated by follicular bystander B cells, is a key for motility of Tfh cells into B cell follicles in a Bcl-6 independent manner [60]. High frequency of programmed cell death ligand 1 (PD-1L) in GC B cells leads to a reduction in cell proliferation, activation, ICOS expression and IL-21 secretion [91].

2.3 Tfh cell in humans

The importance of SAP and ICOS in human Tfh cell differentiation and function was reported by analyzing human genetic deficiencies [9]. IL-12-STAT3/STAT4 axis is also associated with Tfh cell development and functions in humans [92, 93]. Recent studies have shown the human Tfh cell differentiation *in vitro* in the presence of transforming growth factor β (TGF-β) and IL-12 or IL-23, but not in murine $CD4^+$ T cells [94]. Although both human and murine Tfh cells share many features, the mechanism of Tfh cell differentiation in human remains unclear.

Human blood memory $CXCR5^+$ $CD4^+$ T cells share phenotypic and functional properties with Tfh cells. Tfh-like cells comprise three subsets: Th1 (Tfh1), Th2 (Tfh2) and Th17 (Tfh17) cells [95]. A recent study from patients with adult SLE showed that while Tfh1 cells were reduced, Tfh2 and/or Tfh17 cells were elevated among blood memory Tfh cells [96]. This imbalance between Tfh1 cells and Tfh2 and/or Tfh17 cells is correlated with disease activity correlated with disease activity, autoantibody titers and/or the frequency of blood plasmablasts [95-97].

2.4. Tfh cells in autoimmune diseases

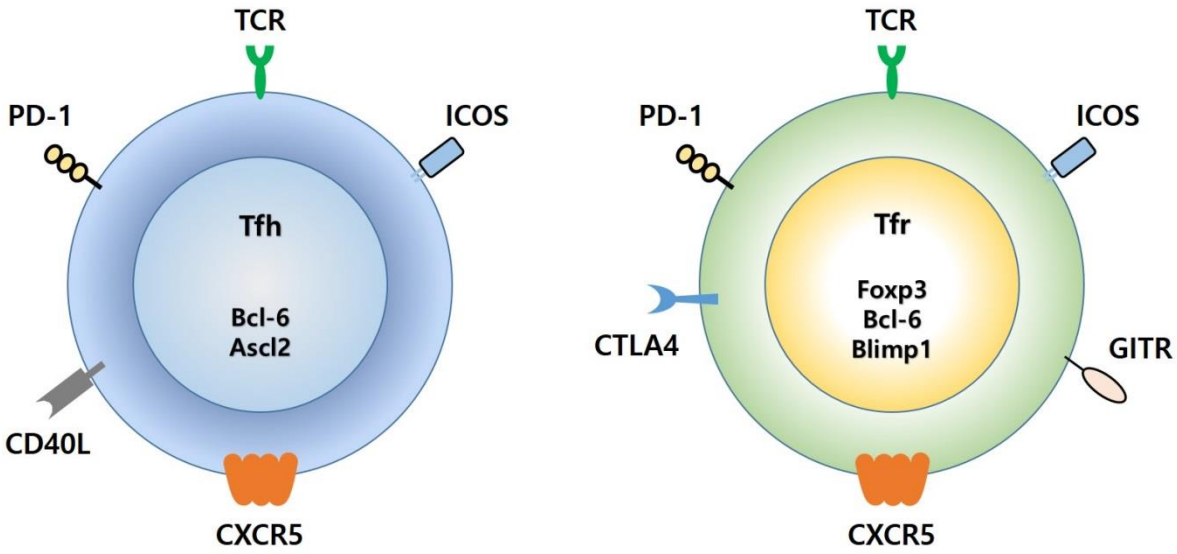
High levels of class-switched autoantibodies and abnormal GC B cells are common symptoms in patients with autoimmune diseases [98, 99]. Recent progress has suggested the role of Tfh cells in the pathogenesis of autoimmune diseases. Increased frequencies of circulating $CXCR5^+$ and $PD\text{-}1^+$ or $ICOS^+$ Tfh cells are found in patients with SLE [20, 22], Sjögren's syndrome [97], and RA [100]. The increase of IL-21 also has been reported in the serum of patients with SLE [13]. Moreover, ectopic GCs formation with accumulation of T and B cells has been found in the kidneys of patients with lupus nephritis [101]. Thus, formation of tertiary lymphoid structures containing Tfh cells seems common in many autoimmune and inflammatory diseases [10, 102]. Furthermore, an increase of Tfh cells is positively correlated with autoantibody titers and symptoms and/or severity of many autoimmune diseases, such as SLE and RA [20-22]. That is, auto-reactive T cells, in this case likely Tfh cells, are the pathogenic T cells mediating the production of autoantibody and subsequent development of antibody-mediated autoimmune disease in humans.

3. Follicular regulatory T cells

Regulatory T (Treg) cells are a subset of $CD4^+$ T cells characterized by the expression of the transcription factor Foxp3 and are central in control immunological self-tolerance and homeostasis [103]. Deletion or mutation of Foxp3 in mice or humans causes multi-organ autoimmunity, inflammatory disease, and allergy [17]. Previous studies reported that Treg cells are found in B cell follicles and GCs and can suppress the B cell responses and auto-reactive B cells [104-106]. More recently, three independent groups identified and characterized a new subset of Treg cells, follicular regulatory T (Tfr) cells [24-26]. Tfr cells are differentiated from thymus-derived $Foxp3^+$ Treg cells and approximately 10~15% of

$CXCR5^+$ $CD4^+$ cells express Foxp3 [24-26]. Similar to Tfh cells, Tfr cells express Bcl-6, CXCR5, PD-1, ICOS, and BTLA (Figure 3). However, they do not express CD40L, IL-4, and IL-21. Tfr cells also express general Treg markers including Foxp3, CD25, CTLA4, KLRG1, GITR and IL-10 [24, 25]. In addition, Tfr cells are distinct from T-bet^+ Treg cells and $CD103^+$ Treg cells as they do not express CXCR5 and CD103 on their surface [24, 26]. Hence, $Bcl6^+CXCR5^+Foxp3^+$ Tfr cell population represents a novel subpopulation of Treg cells with unique expression of transcription factors and surface molecules.

Figure 3. Comparison between Tfh and Tfr cells. Tfh cells express ICOS, PD-1, CD40L and CXCR5 on the surface; Bcl-6 and Ascl2 are known transcription factors. Like Tfh cells, Tfr cells express PD-1, ICOS, and CXCR5, but not CD40L. Instead, they express GITR and CTLA4 on the surface. Foxp3, Bcl-6 and Blimp1 are involved in the differentiation of Tfr cells.



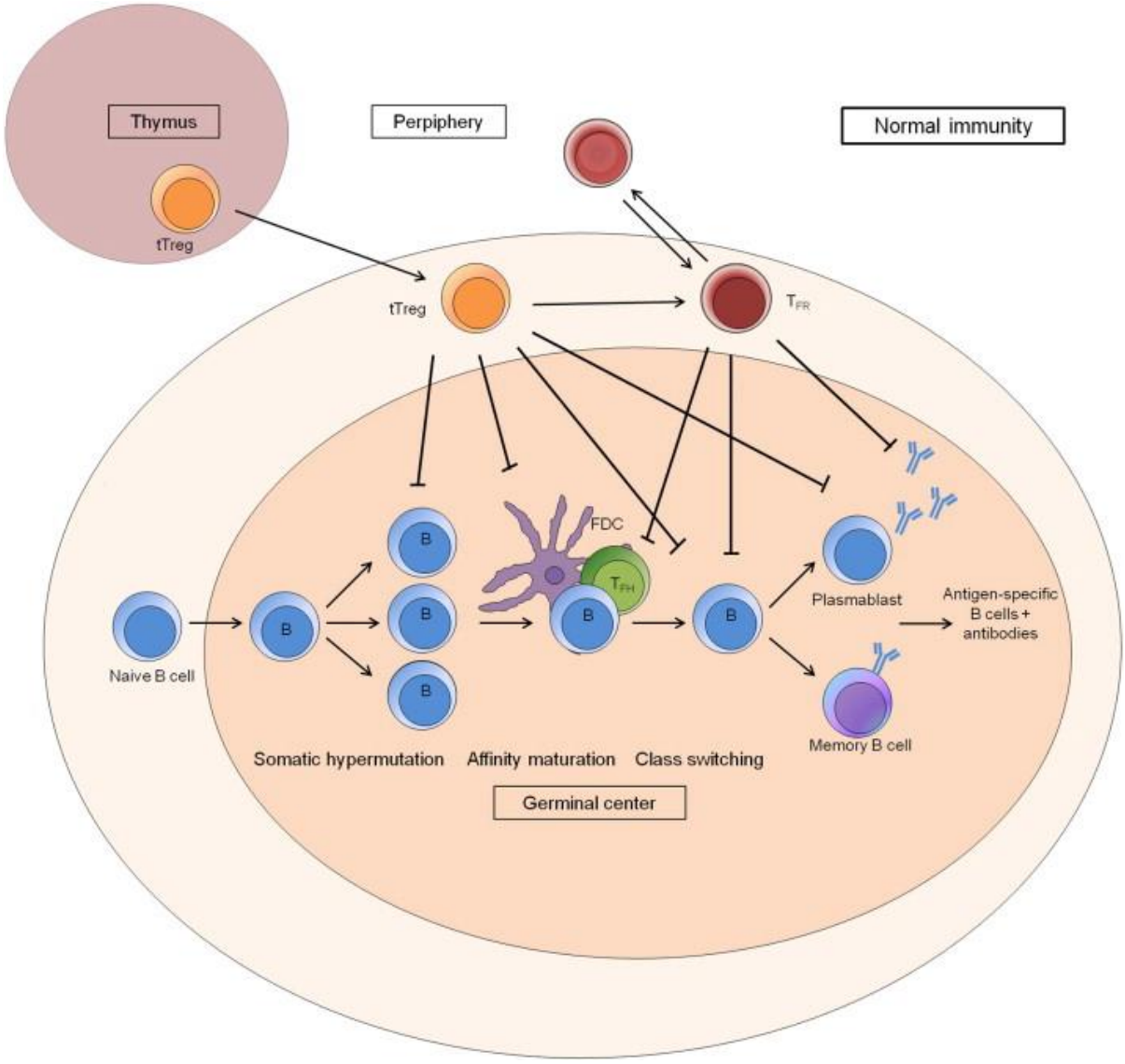
3.1. Tfr cell differentiation and function

Differentiation of Tfr cells depends on Bcl-6, CD28, ICOS, SAP and B cells [25, 107]. TNF receptor-associated factor 3 (TRAF3), Id2, Id3, and nuclear factor of activated T cells 2 (NFAT2) are also necessary for Tfr cell differentiation [107-110]. A considerable decrease of Tfr cells in the CD28 and ICOS deficient mice indicates the essential role of costimulatory signals for Tfr cell differentiation [25, 107]. Treg-specific deletion of TRAF3 mice demonstrated higher GC responses and high-affinity antibodies [108]. Without changing the overall frequency of Treg cells, deletion of TRAF3 in Treg cells attenuated antigen-stimulated Tfr cell production. Since TRAF3 mediates ICOS expression, loss of TRAF3 in Treg led to reduced expression of ICOS, which is essential for Tfr cell generation. A helix-loop-helix family Id protein interacts with E protein and suppresses the DNA-binding activity of E protein [109]. Activation signaling through TCR in Treg cells decreased the expression of Id2 and Id3, inducing Tfr cell specific program of gene expression, including CXCR5 and IL-10. Recently, NFAT2 has been identified as a critical factor for the up-regulation of CXCR5 in thymus-derived Treg cells [110]. NFAT2 deletion in $CD4^+$ T cells or Treg cells led to impaired numbers of Tfr cells and enhanced GC responses, respectively.

Blimp-1 is expressed by a certain Treg population residing in mucosal sites, which produces IL-10 in a Blimp-1 dependent manner [111]. *Prdm1*, the gene encoding Blimp-1 and *Il10* gene transcription levels were increased in Tfr cells [25]. Blimp-1-deficient mice demonstrated a significant increase in the frequency of Tfr cells [25]. An increase of Tfr cells was reported in PD-1-deficient mice, without affecting Tfr cell migration into GCs, and these Tfr cells possess greater suppressive ability [107]. Therefore, both Blimp-1 and PD-1 have

important roles in Tfr cell homeostasis. CTLA4 is a critical factor for controlling the GC reactions in both Treg and Tfr cells [112, 113]. The loss of CTLA4 on Tfr cells failed to suppress Tfh cells or antigen-specific antibody responses [113]. Treg cells from CXCR5, Bcl-6, and SAP-deficient mice show less efficiency in suppressing T cell-dependent antibody production *in vivo* [24, 25]. Therefore, Tfr cells play a fundamental role in regulating GC reactions. However, it is unclear how Tfr cells regulate GC reactions. One study proposed that Tfr cells might directly suppress B cells [24], while another study showed that Tfr cells suppress the differentiation of Tfh cells [25]. The current concept, how Treg and Tfr cells are involved in GC reaction is depicted in Figure 4. There are several unsolved questions remaining regarding, including 1) what is the primary mechanistic role of Tfr cells *in vivo,* and 2) do Tfr cells regulate the immune response in an antigen-specific manner [11].

Figure 4. Regulatory T cells regulate B cells in the germinal center reaction in normal immunity. Follicular regulatory T (Tfr) cell regulates GC responses by suppressing Tfh cell or GC B cell.



Adapted with permission from Dhaeze T, Stinissen P, Liston A, and Hellings N, *Humoral autoimmunity: A failure of regulatory T cells?* Autoimmun Rev, 2015.

3.2. Tfr cells in autoimmune diseases

The production of autoantibody is the primary feature of autoimmune disease. Most of self-antigen recognizing immature B lymphocytes were deleted or changed their specificity in the bone marrow (BM) [114]. Despite the mechanism in BM, self-reactive B cells can escape the negative selection in BM. However, because self-reactive T cells are generally deleted or anergized in the thymus and in the periphery, self-reactive B cells cannot get T cell help in normal condition. However, in autoimmune patients, autoreactive T cells are abnormally activated and lead to the activation of autoreactive B cell responses in an antigen-specific manner. Therefore, B cell tolerance is important for preventing emergence of auto-reactive B cells. However, malfunction of B cell tolerance of self-antigens leads to the development of autoantibodies. Various studies support that excessive Tfh responses result in self-reactive B cell production [115]. Many Tfr cell studies have supported the regulatory role of Tfr cells in Tfh cell or GC B cell reactions [116]. Therefore, a defect in Tfr cell might drive autoimmune diseases by elevating Tfh cell activity and generation of auto-reactive B cells. Lupus-like disease model using chromatin isolated from syngeneic-activated lymphocytes in NFAT2 mice exhibited an increase of Tfh and GC B cells, but decreased the frequency of Tfr cells [110]. These studies highlight the importance of Tfr cells in preventing loss of tolerance. BXD2 mice, one of the autoimmune disease mouse models, display a higher number of Tfh cells and autoantibody producing B cells, and development of spontaneous GCs [117, 118]. IL-21 promotes Tfh cells, but inhibits Tfr cells in BXD2 mice. IL-21 deficient BXD2 mice have increased the frequency of Tfr cells compared to wild-type BXD2 mice and transfer of Tfr cells from IL-21-deficient BXD2 mice into BXD2 mice

ameliorates the autoimmune phenotype of BXD2 mice [119]. There are several studies of Tfr cells in mice [116]. However, although the existence of human Tfr cells expressing BCL6 and FOXP3 has been reported [120], little is known if human Tfr cells display similar functions as murine Tfr cells. The Tfr cell population within GC in human tonsils is much less abundant than in mice [115]. However, the impact or significance of this decrease is currently uncertain and still it is unveiled in this field.

4. The role of CXCR5 in GC responses.

Certain chemokines regulate chemoattraction during immunologic events, and cell migration is a critical factor in the development of lymphoid tissues [121]. CXCR5 is a member of the seven transmembrane G protein-linked receptors involved in mediating cell migration [121]. CXCR5 is a reliable marker for Tfh cells and the expression of CXCR5 helps Tfh cells migrate into the B cell follicles in response to CXCL13 [39-41]. CXCR5-deficient T cells have increased Blimp-1 expression and exhibit hampered migration into the B cell follicle resulting in decreased Tfh cell frequency [47, 52, 54, 122-124]. Moreover, constitutive expression of CXCR5 increases the follicular presence of T cells [60]. Retroviral constitutive expression of Ascl2 in $CD4^+$ T cells induces CXCR5 expression *in vitro* and $CD4^+$ T cell-specific deletion of Ascl2 decrease Tfh cells in both viral infection and immunization models [51]. Roles of CXCR5 in Tfr cells are also similar to those of Tfh cells. Transferred Treg cells from CXCR5-deficient mice were inefficient in controlling GC reactions and migration into the GC area [24]. NFAT2 regulates CXCR5 expression in Treg cells and NFAT2 deficient Tfr cells fail to ameliorate chromatin induced lupus-like disease

[110]. Thus, expression of CXCR5 is one of the critical events for $CD4^+$ T cells to differentiate into Tfh or Tfr cells and migrate into B cell follicles.

5. Animal models of antibody-mediated autoimmune lupus

To understand the pathophysiology of autoantibody-mediated autoimmune diseases, researchers have developed many mouse models of autoimmune lupus, which are summarized in Table 4.

Table 4. Mouse models of autoimmune lupus

Name	Target gene	Autoantibody	Arthritis	Tfh cell	Ref.
Sanroque	*Rc3h1*	Yes	-	Increased	[5, 31, 32]
NZB/W F1	-	Yes	-	Increased	[33, 34]
BXSB-*Yaa*	-	Yes	-	Increased	[35-37]
MRL/*lpr*	*Fas*	Yes	Yes	Increased (extrafollicular)	[38, 39]
BXD2	-	Yes	Yes	Increased	[22, 40-50]

5.1. *Sanroque* mouse animal model of autoimmune lupus

Sanroque mice have a single amino acid mutation in the RNA-binding protein Roquin-1, which disturbs inducible costimulator (ICOS) repressor expression and develops lupus-like symptoms [125]. This single mutation in *sanroque* mice causes an impaired ability to suppress the expression of ICOS and interferon gamma (IFN-γ) and promotes Tfh cell generation [125, 126]. Adoptive transfer of Tfh cells from *sanroque* mice into wild-type mice

promotes spontaneous GC formation and production of autoantibodies, whereas SLAM-associated protein (SAP) deficiency or Bcl-6 deletion in the *sanroque* mice prevents this lupus-like pathology [12].

5.2. NZB/W F1 mouse animal model of autoimmune lupus

The NZB/W F1 mice exhibit spontaneous lupus-like disease [127]. Like other mouse models, NZB/W F1 mice also exhibit increased numbers of Tfh cells and enhanced GC B cell responses. The lupus-like phenotype of NZB/W F1 mice is dependent on the ICOS/ICOS ligand (ICOSL) pathway since anti-ICOSL treatment ameliorates the disease severity by decreasing Tfh cells and GC B cell responses [128].

5.3. BXSB-*Yaa* mouse animal model of autoimmune lupus

BXSB-*Yaa* mice, which display duplication of the *Tlr7* gene, also exhibit SLE-like phenotypes, such as spontaneous GC formation and Tfh responses [129, 130]. Unlike *sanroque* mice, SLE-like autoimmune phenotypes in BXSB-*Yaa* mice are dependent on IL-21 signals [130, 131]. Deletion of the IL-21 receptor in BXSB-*Yaa* mice significantly decreases multiple parameters of SLE [130].

5.4. MRL/*lpr* mouse animal model of autoimmune lupus

The production of autoantibodies in MRL/*lpr* lupus mice, which are characterized by deficiency of the proapoptotic molecule Fas, is from B cells in the extrafollicular foci, and this process is mediated by extrafollicular helper T cells [53, 132]. Bcl-6 and ICOS are essential for the development of extrafollicular Th cells and IL-21, and CD40L are important for their function [53].

5.5. BXD2 mouse animal model of autoimmune lupus

As described above, to study the pathophysiology of antibody-mediated autoimmunity, several animal models of experimental autoimmune lupus and arthritis have been developed. MRL/*lpr* mice spontaneously develop autoimmune lupus and arthritis [133]. However, the deficiency of the *Fas* gene is not common in patients with lupus. NZB/W F1 mice are another autoimmune lupus model which also spontaneously develops autoimmune lupus phenotypes [127]. However, this strain does not develop arthritis symptoms, which are common in patients with lupus. In this regard, BXD2 mice represent an alternative animal model to study the complex features of antibody-mediated autoimmune diseases that more closely mimic the pathophysiology of autoantibody-mediated diseases in humans.

BXD2 is a recombinant inbred strain established by intercrossing the F2 generation of C57BL/6 and DBA/2J strains for more than 20 generations [134, 135]. BXD2 mice excessively produce rheumatoid factor and autoantibodies and spontaneously erosive arthritis as well as autoimmune lupus symptoms including glomerulonephritis [118, 136-138]. Genetic linkage analysis revealed that BXD2 mice have several autoimmune loci such as *Lbw*, *Sle*, *Sles*, *Lmb* and *Asm2* as a result of complicated interaction of multiple genes from the original parental B6 and DBA/2 mice [118]. In addition, increased expression of CD28 in $CD4^+$ T cells of BXD2 mice can induce the expansion of $CD86^+$ GC B cells and the expression of activation-induced cytidine deaminase (AID) in B cells [138]. These multiple genetic and immunologic characters together seem to enhance spontaneous autoimmune phenotypes in BXD2 mice, thereby providing a novel animal tool to study the mechanism of naturally occurring autoimmune GC responses.

A series of studies done by Mountz and colleagues have demonstrated the role of IL-17 in developing spontaneous GC formation and autoantibody-mediated autoimmune phenotypes in the BXD2 mice [117, 139, 140]. In their works, IL-17 was shown to induce the expression of regulator of G-protein signaling (RGS) proteins on B cells and these RGS proteins stabilized the interaction of the germinal center B cells with nearby T cells [117, 139]. Without affecting the total number of Tfh cells and their function *in vitro*, IL-17 was indicated to play a critical role in the localization of Tfh cells in the GC light zone in BXD2 mice [140]. A recent study also showed that an increased level of IL-21 is associated with the development of autoimmune diseases [141]. In BXD2 mice, IL-21 plays a critical role in developing spontaneous GC formation and autoimmune pathophysiology [140]. The high level of IL-21 in BXD2 mice upregulates Tfh cell differentiation but suppresses Tfr cell development and functions [119].

6. Overall hypothesis and specific aims

Abnormalities of Tfh cells are strongly correlated with many autoimmune diseases. Currently, many immunosuppressive therapies have been developed and used to treat autoimmune diseases. Nevertheless, these reagents have significant disadvantages including systemic immune suppression and only partial target-specificity. Therefore, the main goal of the present dissertation is to develop a new therapy for auto-reactive B cell responses by regulating Tfh cell-mediated GC responses. Expression of CXCR5 on $Foxp3^+$ regulatory T cells enables cells to migrate into GC zone and these Tfr cells specifically regulate GC reactions *in vivo*. In addition, loss of Tfr cells leads to autoimmune diseases. Although Tfr cells are a good candidate for the treatment of auto-reactive B cell responses, their low

frequency and unknown stability are possible drawbacks in the development of Tfr cell-based immunotherapy. We selected BXD2 mice as our model system to examine the therapeutic potential of Tfr cells for the treatment of autoimmune diseases.

Our overall hypothesis is that the development of auto-reactive B cell responses is mediated by Tfh cells, and that, adoptive transfer of Tfr cells will ameliorate the development of autoantibodies in autoimmune prone-hosts by inhibiting autoimmune B cell responses. For this purpose, in Chapter 2, we have investigated the role of Tfh cells in the auto-reactive B cell responses in BXD2 mice. We also hypothesized that retroviral transduction of CXCR5 onto $Foxp3^{+}$ Treg cells will enable these cells to control GC reactions. In Chapter 3, we have tested this hypothesis by determining whether adoptively transferred CXCR5 overexpressing Treg cells will inhibit GC responses.

CHAPTER 2

Regulation of Autoimmune Germinal Center Reactions in Lupus-Prone BXD2 Mice by Follicular Helper T cells

This chapter is based on and reproduced from the following journal article. As per PLoS policy, no permission is required to reprint:: Kim YU, Lim H, Jung HE, Wetsel RA, and Chung Y (2015) Regulation of autoimmune germinal center reactions in lupus-prone BXD2 mice by follicular helper T cells. PLoS ONE 10(3): e0120294.

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1 Rationale and Hypothesis

Tfh cell responses are essential for the generation of effective humoral immune responses against infectious agents [9]. By contrast, excessive Tfh cell responses to self-antigens are shown to be associated with antibody-mediated autoimmune diseases, such as SLE, RA, Sjögren syndrome, and juvenile dermatomyositis [12, 13]. Among multiple animal models of experimental autoimmune lupus and arthritis, BXD2 mice provide many advantages since they spontaneously develop autoimmune lupus, arthritis and subsequent glomerulonephritis without Fas deficiency [142]. Therefore, BXD2 mice offer a novel and robust animal model to study comprehensively the pathophysiology of antibody-mediated autoimmunity. A series of studies by one laboratory have shown a critical contribution of IL-17 and Th17 cells in mediating autoimmune B cell responses in these mice [117, 140]. Moreover, Th17 cells were found to reside in spontaneous GCs and IL-17 enhanced the formation of GCs in BXD2 mice. In addition, IL-17 did not affect the total number of Tfh cells or their function *in vitro*, whereas IL-17RA-deficient Tfh cells in BXD2 mice were shown to be impaired in their ability to localize to the GC LZ. Although Th17-related phenotypes of BXD2 mice were characterized in these initial studies, the contribution of Tfh cells to auto-reactive B cell responses in this autoimmune strain was not fully elucidated.

We hypothesized here that the role of Tfh cells in BXD2 mice is critical for autoantibody-mediated autoimmunity. We found that both the frequency of Th17 cells and the levels of IL-17 in the circulation of BXD2 mice were comparable to those of wild-type. On the other hand, the frequency of Tfh cells was increased in BXD2 mice while the

frequency of Tfr cells was reduced. Also, the frequency of Tfh cells was positively correlated with the GC reaction factors. Lastly, we found that IL-21 secreting Tfh cells are greatly responsible for regulating autoimmune GC reactions in BXD2 mice. Our findings indicate that Tfh cells rather than Th17 cells are the major contributors to the autoimmune GC reactions in BXD2 mice.

2 Results

2.1. Spontaneous germinal center reactions in BXD2 mice

As a first step to determine the contribution of each Th cell response to autoimmune lupus in BXD2 mice, we comparatively analyzed the production of autoantibodies and the generation of spontaneous germinal center reactions between BXD2 mice and C57BL/6 (wild-type, WT) control mice at the age of 6 months or older. Consistent with previous reports [117, 118, 137, 140], we observed that the levels of autoantibodies to double-stranded DNA and histone in the sera of BXD2 mice were significantly higher than those in control mice (Figure 5A). Similarly, we also observed spontaneous generation of GCs in the BXD2 mice (Figure 5B and 5C), which was associated with increased frequency and number of $GL7^{+}Fas^{+}$ germinal center B cells in the spleens (Figure 6A). Increased autoantibodies and spontaneous GCs were observed as early as 3 months of age in the BXD2 mice (data not shown). However, the frequency of $GL7^{+}Fas^{+}$ germinal center B cells remained comparable between the two groups in other secondary lymphoid organs, such as peripheral lymph nodes and Peyer's patches (Figure 6B).

Figure 5. Spontaneous germinal center responses in BXD2 mice. (A) Auto-reactive autoantibody levels against double-strand DNA and histone in the sera of WT and BXD2 mice at the age of 6 months or older were measured by ELISA. (B) Immunofluorescence imaging of PNA^+ (red) germinal center area of the spleen from WT control or BXD2 mice (×4 magnification). (C) The number of GC per spleen section in the WT and BXD2 mice was enumerated by fluorescence microscopy. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

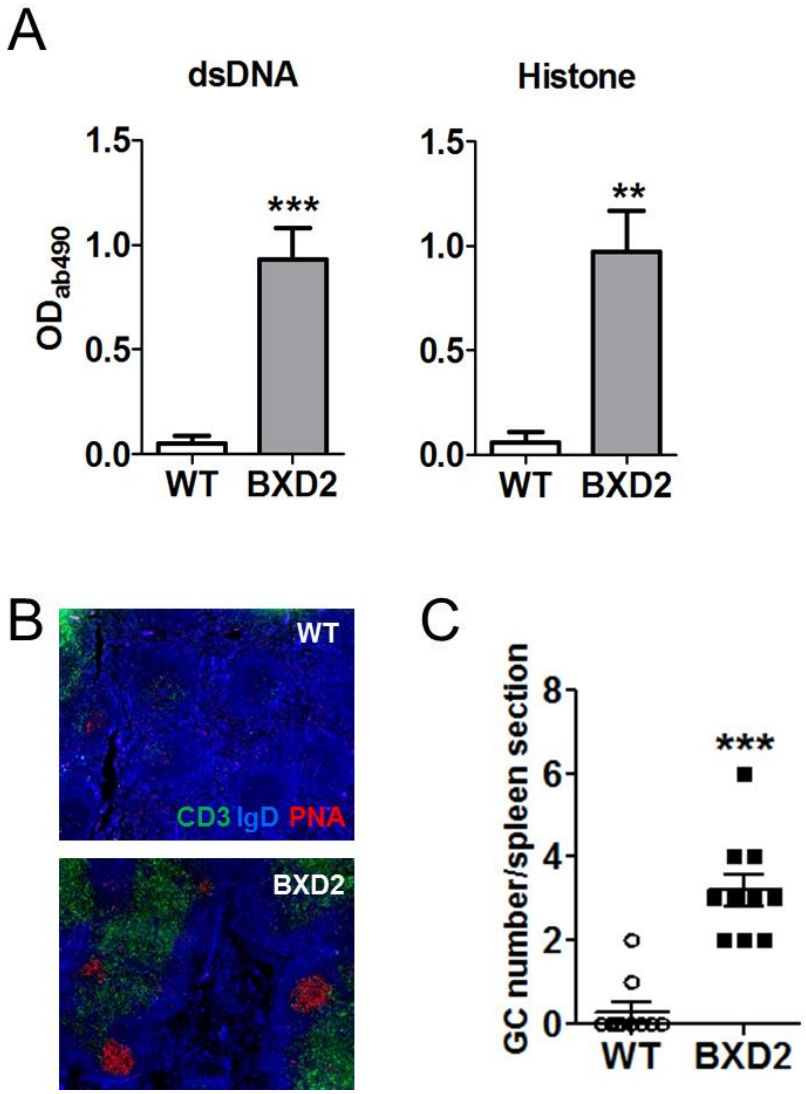
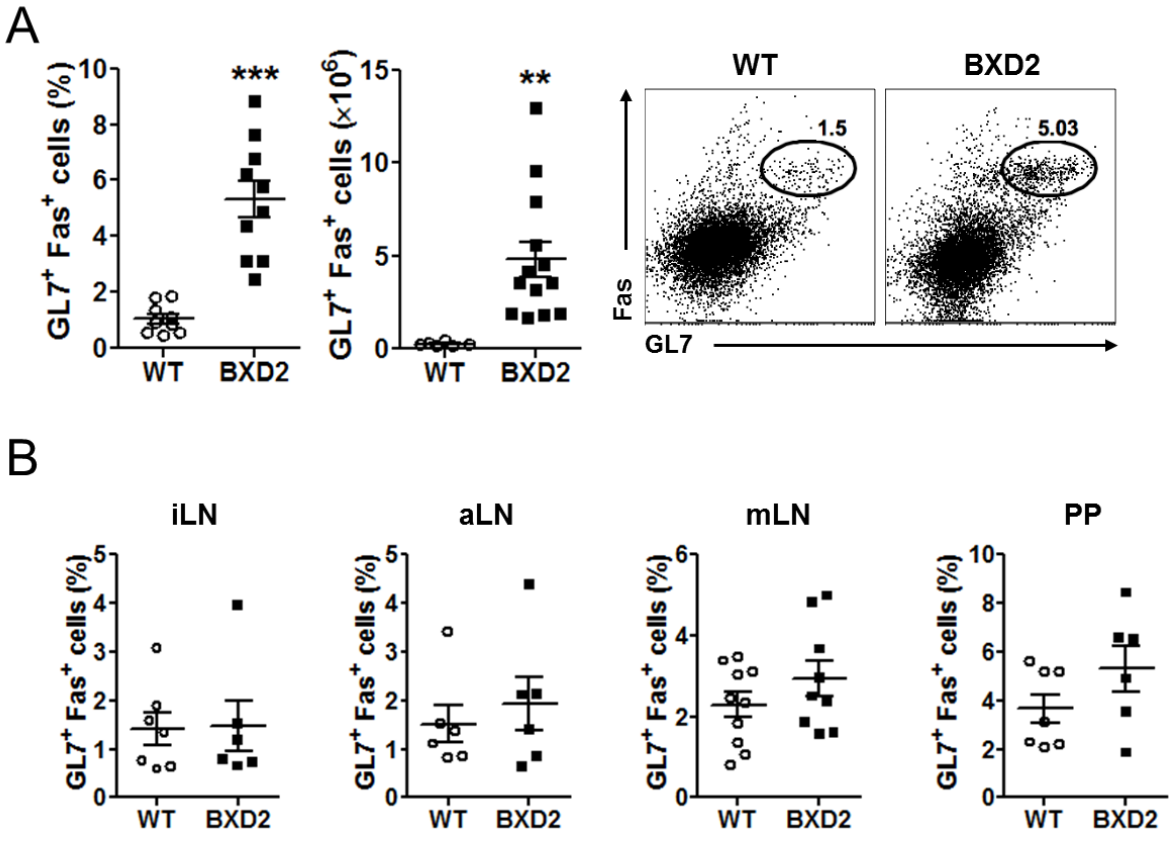


Figure 6. Spontaneous germinal center B cell development in BXD2 mice.

(A) Flow cytometry analysis of the percentage and number of $GL7^{+}Fas^{+}$ germinal center B cells in the WT and BXD2 mice at the age of 6 months or older. (B) Flow cytometry analysis of $GL7^{+}Fas^{+}$ germinal center B cells in the indicated lymphoid organs from WT and BXD2 mice (iLN: inguinal lymph node, aLN: axillary lymph node, mLN: mesenteric lymph node, PP: Peyer's patch). Cells were gated on $B220^{+}$ B cells. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.



2.2. Analysis of helper T cell subsets in BXD2 mice

We next analyzed each helper T cell subset in the spleens of BXD2 mice. The frequency of IFN-γ-producing Th1 cells was slightly but significantly higher in the spleens of BXD2 mice compared to control mice (Figure 7A). In addition, due to increased cellularity in the spleens of BXD2 mice, the absolute number of Th1 cells was remarkably higher in this group. The proportion of Th1 cells were increased in most of the secondary lymphoid organs of BXD2 mice, except Peyer's patches (Figure 7B). However, unlike previous study [117], we observed that the frequency of IL-17-producing Th17 cells in the spleens of BXD2 mice was comparable to that of control mice, although the absolute number of Th17 cells appeared to be increased in the former group (Figure 8A). Similarly, the frequency of IL-17 secreting cells in total splenocytes of BXD2 mice was comparable to that of control mice, ruling out any increased production of IL-17 from non-$CD4^+$ T cell population (Figure 7C). Despite the increased number of Th17 cells, the levels of IL-17 in the circulation of BXD2 mice were comparable to that of control mice (Figure 8A). To further characterize the Th1 and Th17 cell responses, we analyzed the expression of Th1 and Th17-associated genes by using quantitative RT-PCR. As depicted in Figure 7B, we observed that the levels of *Il17a* and *Rorc* mRNA expression in the spleens of BXD2 mice were comparable to those of control mice, while the levels of *Tbx21* (encoding T-bet in mouse) and *Ifng* were increased in the BXD2 mice. Commensal bacteria such as segmented filamentous bacteria (SFB) can modulate Th17 cell responses [143, 144]. To test if any differences of gut microbiota between BXD2 and control mice impact Th17 responses, we co-housed BXD2 mice with control mice for 4 weeks. The frequencies of Th17 cells in both Peyer's patches and

mesenteric lymph nodes were comparable between the two groups (Figure 9). These results together demonstrate that Th17 cell response in BXD2 mice did not significantly differ from control mice in steady state.

Figure 7. Th17 cells are not a major subset of T helper cells in BXD2 mice. (A) Flow cytometry analysis of the percentage and number of IFN-γ or IL-17A positive $CD4^+$ T cells in the spleen from WT and BXD2 mice at the age of 6 months or older. (B) Flow cytometry analysis of IFN-γ^+ Th1 or IL-$17A^+$ Th17 cells in the indicated lymphoid organs from WT and BXD2 mice (iLN: inguinal lymph node, aLN: axillary lymph node, mLN: mesenteric lymph node, PP: Peyer's patch). (C) Frequency of IFN-γ^+ or IL-$17A^+$ cells in whole splenocytes of WT and BXD2 mice. Data are representative of the analysis indicate mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with BXD2-WT mice.

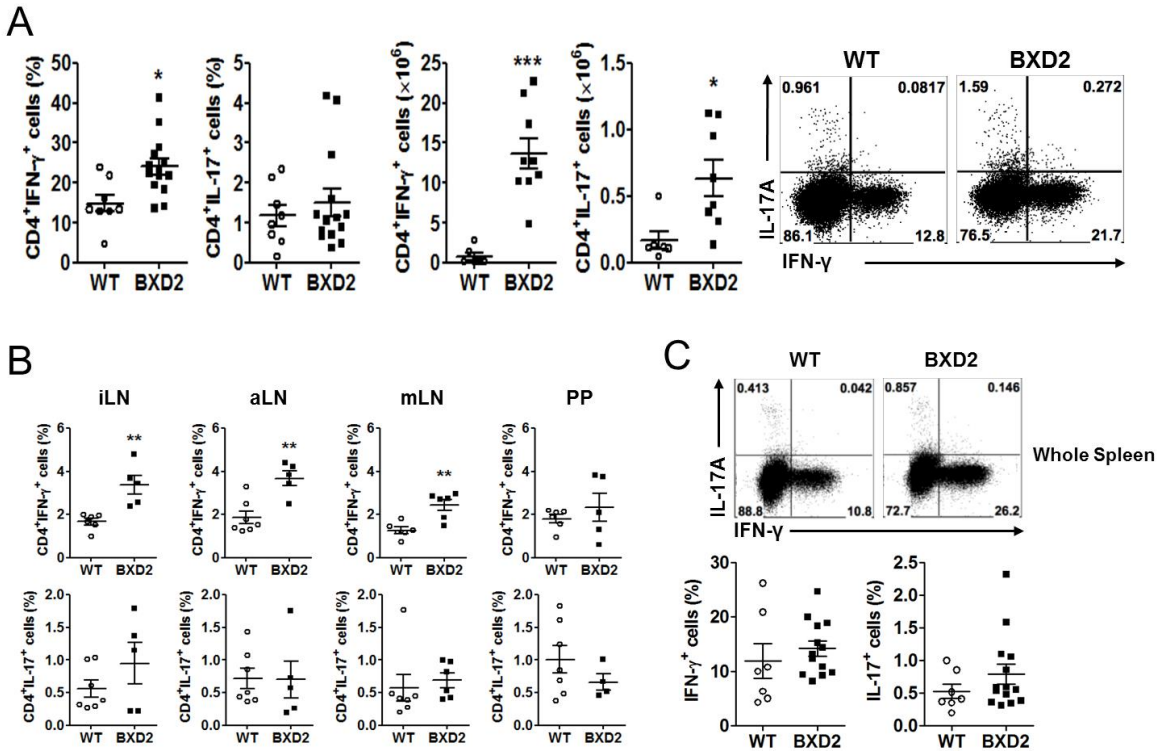


Figure 8. IL-17 in circulation and Th17 related gene expressions are not increased in BXD2 mice. (A) IL-17A levels in the sera of WT and BXD2 mice at the age of 6 months or older were measured by ELISA. (B) Quantitative RT-PCR analysis of Th1 or Th17 cell related genes from WT and BXD2 mice splenocytes at the age of 6 months or older. Data are representative of the analysis indicate mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared with BXD2-WT mice.

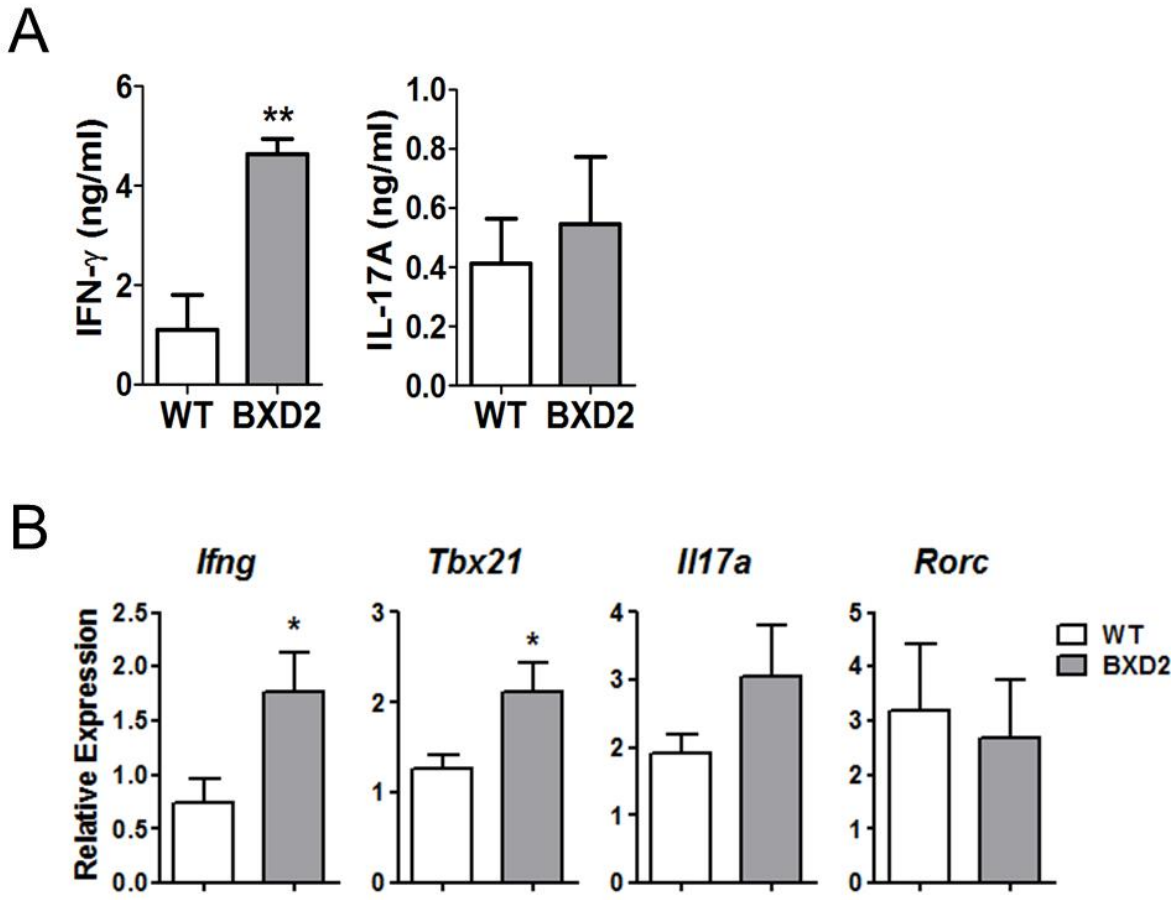
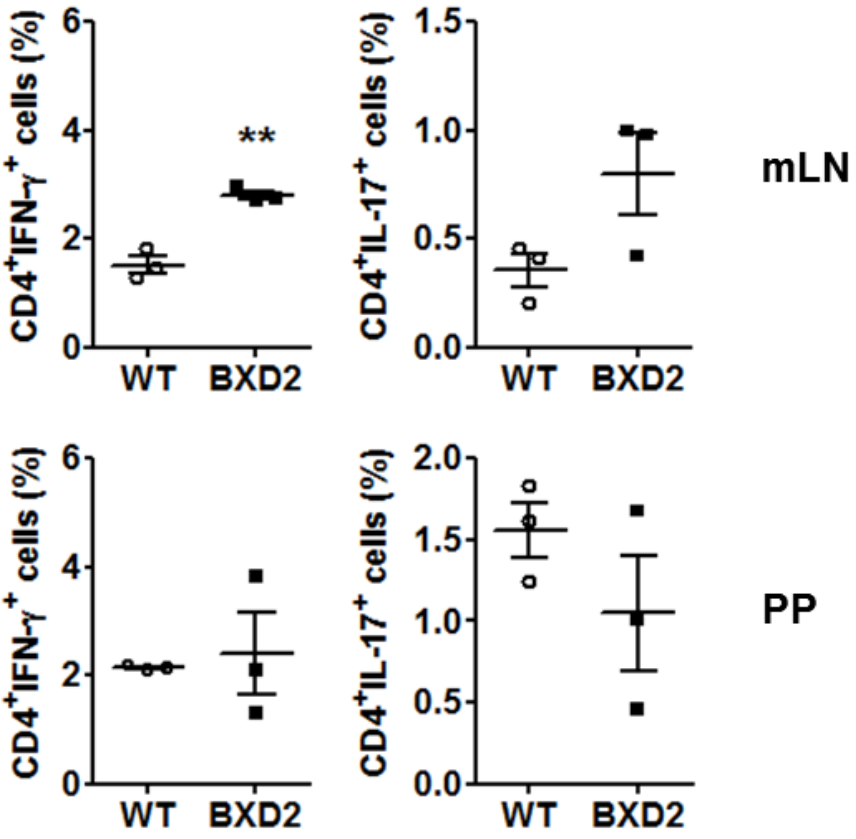


Figure 9. The gap of Th17 cells in BXD2 mice is not due to the difference of gut microenvironment. Percentage of Th17 cells in the mesenteric lymph nodes and Peyer's patches from co-housed WT and BXD2 mice. Data are representative of the analysis indicate mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared with BXD2-WT mice.



2.3. Analysis of Tfh and Tfr cells in BXD2 mice

Since increased Tfh cells are associated with systemic autoimmune diseases [22, 145, 146], we next analyzed Tfh cell responses in the BXD2 mice. As shown in Figure 10A, the frequency and number of $PD1^{+}CXCR5^{+}$ Tfh cells were significantly higher in the spleens of BXD2 mice compared to control mice. Moreover, the levels of Tfh cell signature genes, such as *Il6*, *Il21*, *Bcl6*, *Pdcd1* (encoding PD-1) and *Icos* were all significantly increased in the splenocytes of BXD2 mice compared with those in control mice (Figure 10B). The frequency of Tfh cells in the other secondary lymphoid organs, except axillary lymph node and Peyer's patches, remained comparable between BXD2 and wild-type mice (Figure 10C). Extrafollicular T helper cells are proposed to be involved in the development of antibody-mediated autoimmunity [53]. However, the frequency of $PD\text{-}1^{low}$ extrafollicular T helper cells in BXD2 mice remained comparable to that of control mice (Figure 11A and 11B). Collectively, these results demonstrate that the spleens of BXD2 mice contained significantly increased Tfh cells and, to a lesser extent, Th1 cells, while the frequencies of Th17 cells and $PD\text{-}1^{low}$ extrafollicular T helper cells were similar when compared with control mice.

Figure 10. Tfh cell responses are increased in the BXD2 mice. (A) Flow cytometry analysis of the percentage and number of PD-1^{+}CXCR5^{+} CD4^{+} T cells in the spleens of WT and BXD2 mice at the age of 6 months or older. (B) Quantitative RT-PCR analysis of Tfh cell related genes from WT and BXD2 mouse splenocytes at the age of 6 months or older. (C) Flow cytometry analysis of the percentage and number of PD-1^{+}CXCR5^{+} CD4^{+} T cells in the indicated lymphoid organs from WT and BXD2 mice (iLN: inguinal lymph node, aLN: axillary lymph node, mLN: mesenteric lymph node, PP: Peyer's patch). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

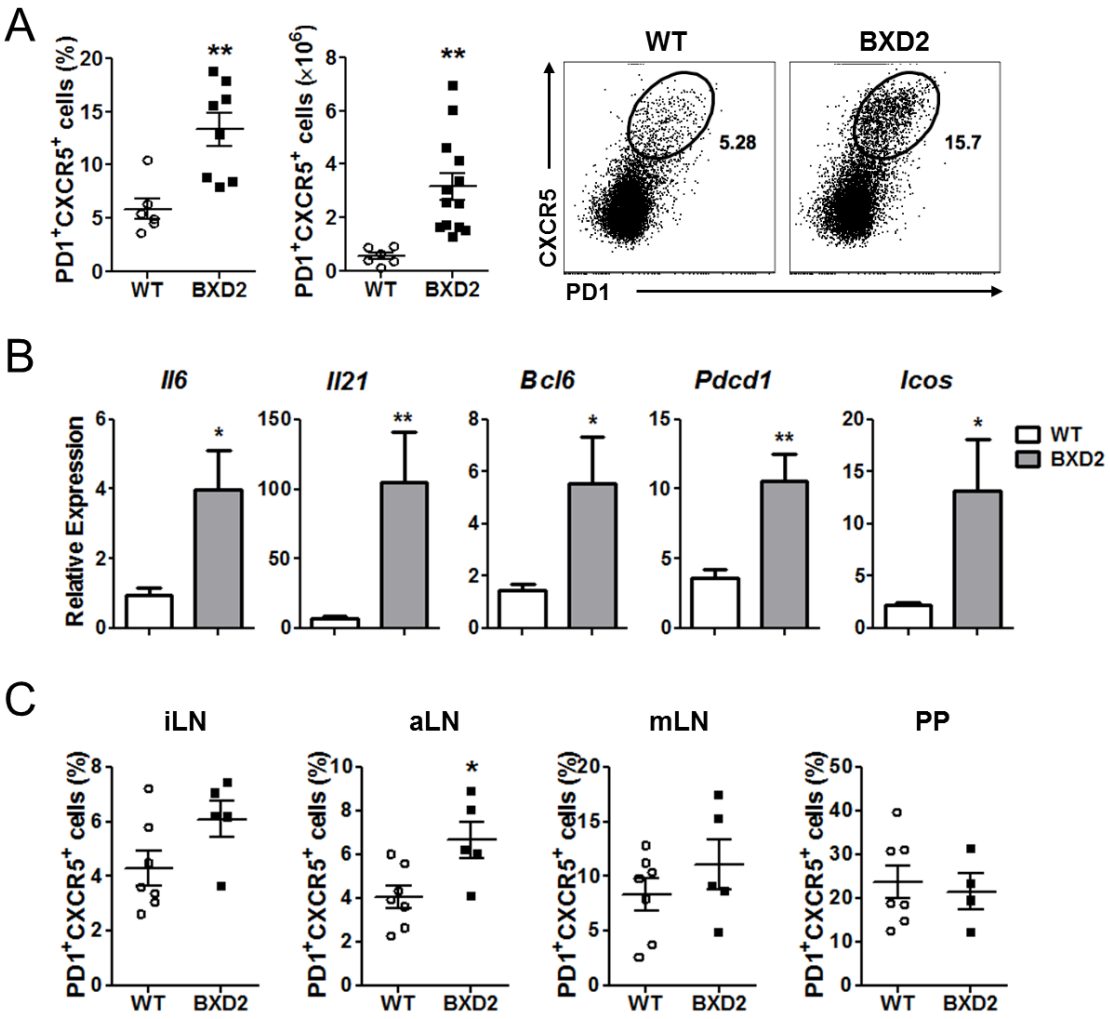
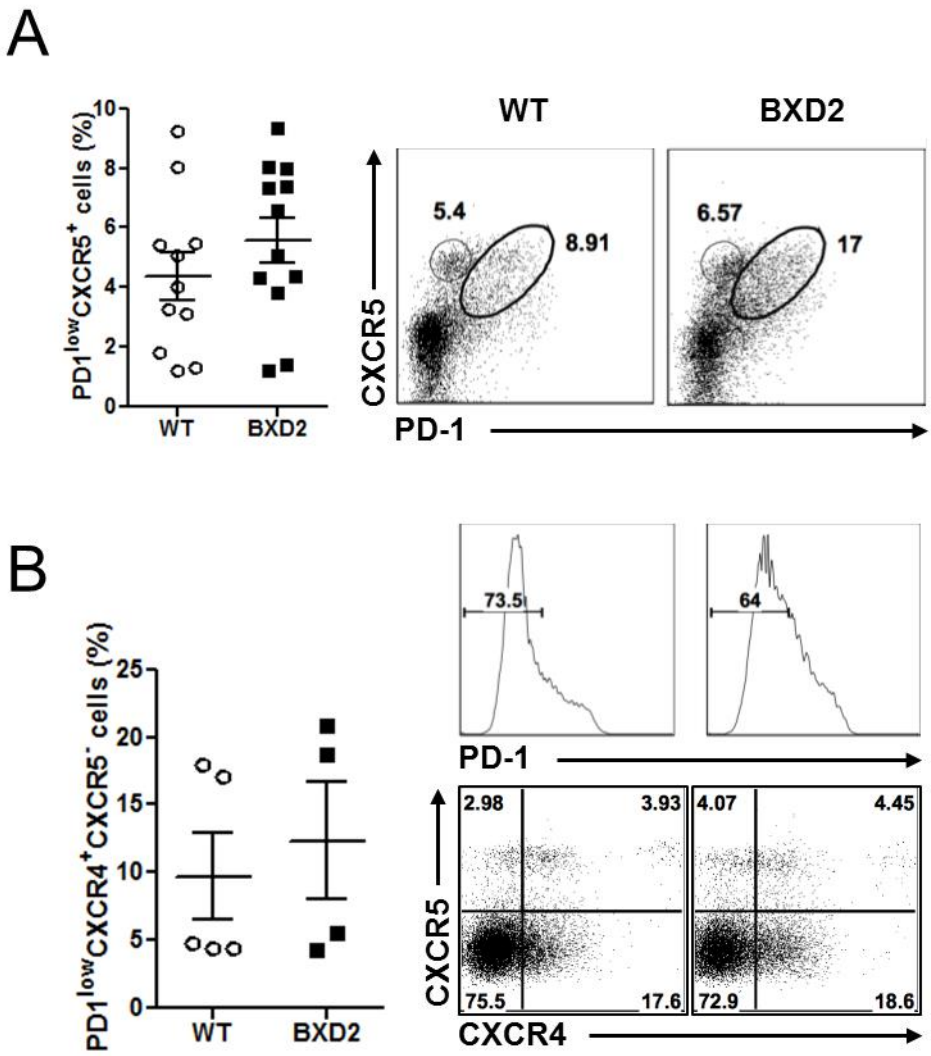
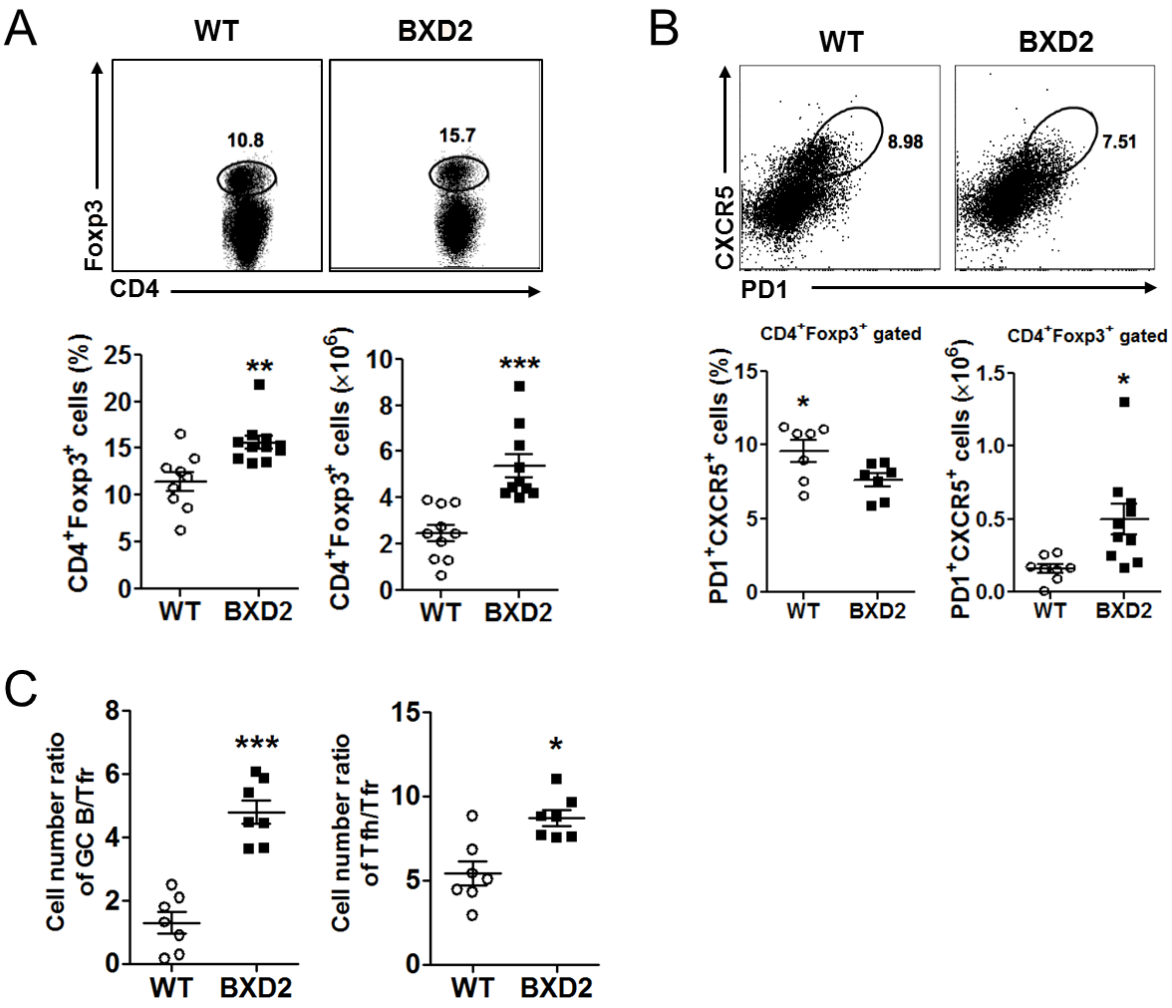


Figure 11. The frequency of extrafollicular T helper cells is similar between WT and BXD2 mice. (A) Flow cytometry analysis of PD-1^{low}CXCR5^{+} CD4^{+} T cells in the spleens of WT and BXD2 mice at the age of 3 months. (B) Flow cytometry analysis of PD-1^{low}CXCR4^{+}CXCR5^{-} CD4^{+} T cells in the spleens of WT and BXD2 mice at the age of 3 to 4 months. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.



$PD1^{+}CXCR5^{+}Foxp3^{+}$ Tfr cells have emerged as a specialized subset of regulatory T cells suppressing germinal center reactions *in vivo* [24-26]. Therefore, we determined if the frequency of Tfr cells, as well as the ratio of Tfh cells and germinal center B cells per Tfr cell in the BXD2 mice, differed from that of control mice. Although the frequency of total $Foxp3^{+}$ T cells was found to be higher in BXD2 mice than control mice (Figure 12A), we observed that the frequency of Tfr cells was slightly but significantly lower in the spleens of the former group (Figure 12B). The absolute number of Tfr cells, however, was higher in the spleens of BXD2 mice due to increased spleen cellularity (Figure 12B). As shown in Figure 4, 5 and 9, the numbers of germinal centers, germinal center B cells and Tfh cells were all significantly increased in the BXD2 mice. Accordingly, the ratio of germinal center B cells per Tfr cell was significantly higher in BXD2 mice than control mice (Figure 12C). Similarly, the ratio of Tfh cells per Tfr cell was also significantly greater in the former group (Figure 12C).

Figure 12. The ratio of Tfh/Tfr cells is increased in the BXD2 mice. (A) Flow cytometry analysis of the percentage and number of $CD4^+Foxp3^+PD\text{-}1^+CXCR5^+$ Tfr cells in the spleens of WT and BXD2 mice at the age of 6 months or older. (B) The percentage and absolute number of $Foxp3^+$ $CD4^+$ T cells in the spleens of WT and BXD2 mice. (C) The ratio of $Foxp3^-$ Tfh cells to $FoxP3^+$ Tfr cells in the mouse spleens and germinal center B cells to $FoxP3^+$ Tfr cells in the mouse spleen. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.



2.4. Correlation analysis between autoantibodies, germinal center B cells, Th17 and Tfh cells in BXD2 mice

Our results in Figure 7 to 11 showed no evident increase in Th17 cell responses in BXD2 mice, which are contradictory to previous studies of another group [117, 140, 147], suggesting that Th17 cell responses might not be associated with increased autoantibodies in the BXD2 mice. To address this possibility, we examined whether the levels of autoantibodies and the frequencies of germinal center B cells are correlated with the frequencies of Th17 or Tfh cells. Linear regression analysis showed no correlation between the frequencies of Th17 cells and those of germinal center B cells (Figure 13A). Similarly, no correlation was observed between the frequencies of Th17 cells and the levels of anti-dsDNA (Figure 13B) or between the numbers of Th17 cells and germinal center B cells (Figure 13C). In sharp contrast, both the frequencies of germinal center B cells and the levels of anti-dsDNA showed a clear positive correlation with the frequencies of Tfh cells (Figure 14A and 14B). Moreover, a strong correlation was observed between the number of Tfh cells and those of germinal center B cells (Figure 14C). In addition, the frequencies of Th1 cells appeared to be, with a lesser extent, also positively correlated with those of germinal center B cells and the levels of anti-dsDNA (Figure 15A and 15B). As expected, the levels of anti-dsDNA showed strong positive correlation with the frequencies of germinal center B cells (Figure 15C), while the frequencies of Tfr cells showed no correlation with those of germinal center B cells (Figure 15D). Together, these linear regression analyses clearly demonstrate Tfh cell responses, but not Th17 cell responses, showed a strong positive correlation with auto-reactive germinal center B cell responses in the BXD2 mice.

Figure 13. Th17 cells do not correlate with outputs of GC responses in BXD2 mice.

Linear regression analysis of the frequency of Th17 cells (A) with that of germinal center B cells, and Th17 cells (B) with dsDNA specific autoantibody levels. Linear regression analysis of the number of Th17 cells (C) with that of germinal center B cells. Pearson correlation coefficients (r^2) between the percent of T helper cell subset and germinal center B cells or those of T helper cell subset and dsDNA specific autoantibodies levels are indicated in each graph.

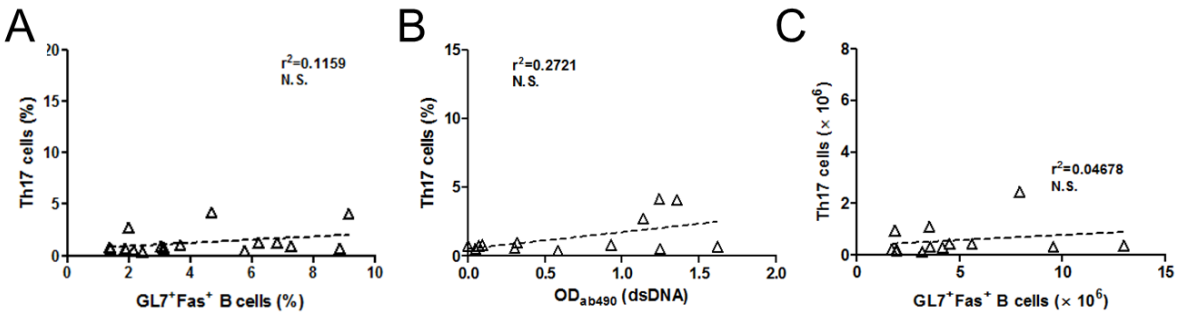


Figure 14. **Tfh cells positively correlate with outputs of GC responses in BXD2 mice.** Linear regression analysis of the frequency of Tfh cells (A) with that of germinal center B cells, and Tfh cells (B) with dsDNA specific autoantibody levels. Linear regression analysis of the number of Tfh cells (C) with that of germinal center B cells. Pearson correlation coefficients (r^2) between the percent of T helper cell subset and germinal center B cells or those of T helper cell subset and dsDNA specific autoantibodies levels are indicated in each graph.

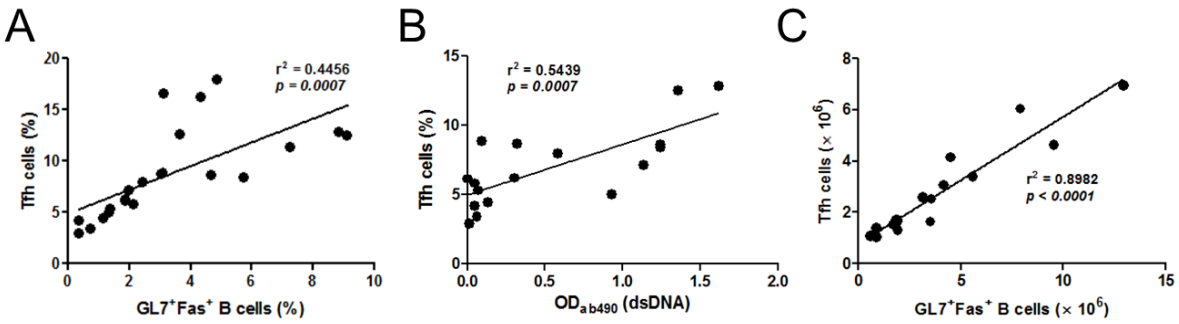
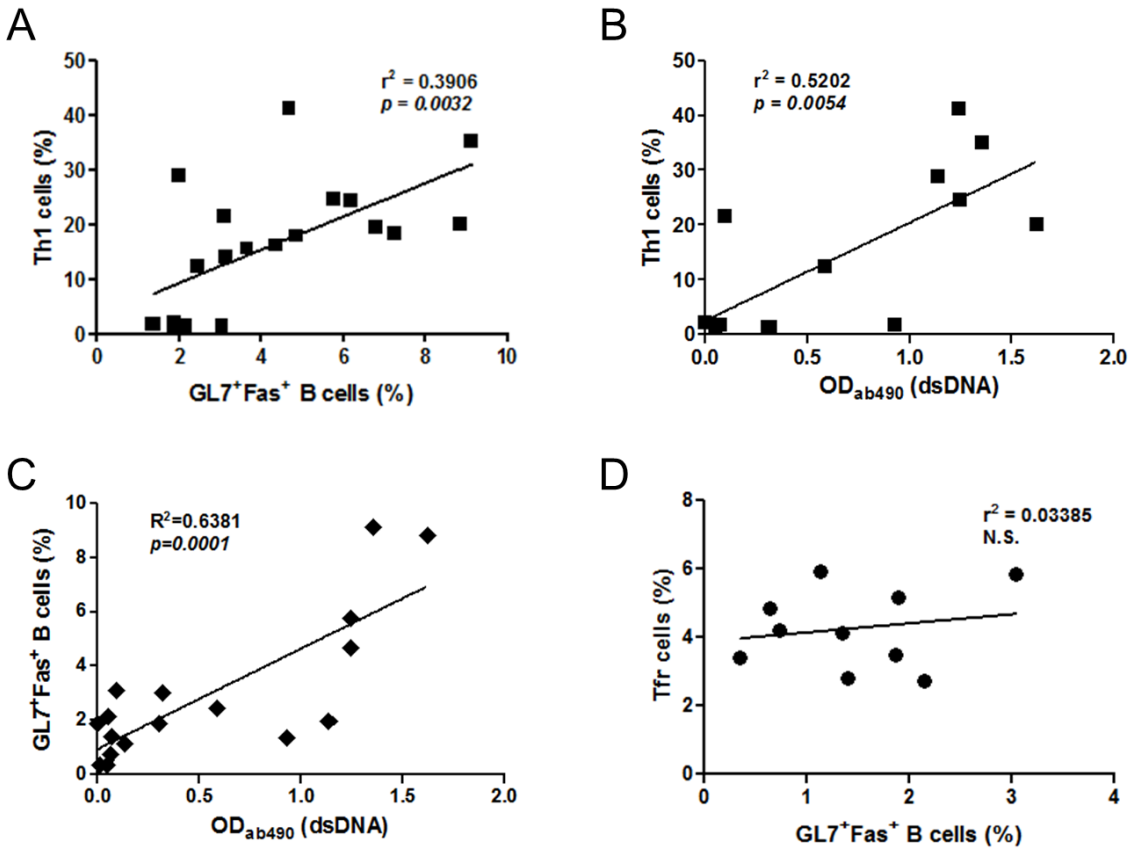


Figure 15. Linear regression analysis between Th1/Tfr cells and germinal center B cells. Linear regression analysis of the frequency of Th1 cells with GC B cells (A), Th1 cells with dsDNA specific autoantibody levels (B), Linear regression analysis of germinal center B cells with dsDNA specific autoantibody levels (C), the frequency of Tfr cells with germinal center B cells (D). Pearson correlation coefficients (r^2) between the percent of T indicated helper T cell subset and of germinal center B cells or those of Th1 cells or GC B cells and dsDNA specific autoantibodies levels are indicated at each graph.



2.5. $CXCR5^+CD4^+$ T cells, but not $CCR6^+CD4^+$ T cells, from BXD2 mice induce IgG production from naïve B cells in an IL-21-dependent, IL-17-independent manner

Although previous studies described a critical contribution of Th17 cell responses to the generation of autoimmune B cell responses in BXD2 mice, our findings showed no evident correlation between Th17 cells and autoantibody responses. To further determine the contribution of Th17 cells and Tfh cells to the generation of spontaneous germinal center B cell responses in the BXD2 mice, we sought to determine whether Th17 cells and Tfh cells isolated from BXD2 mice can trigger IgG production from naïve B cells. Expression of CCR6 and CXCR5 on $CD4^+$ T cells are reliable markers of Th17 and Tfh cells, respectively [9, 148, 149]. Hence, we purified $CCR6^+CD4^+$ T cells and $CXCR5^+CD4^+$ T cells from the spleens of 3~9 month-old BXD2 mice (Figure 16A). When re-stimulated with these cells, purified $CXCR5^+$ and $CCR6^+$ cells almost exclusively expressed IL-21 and IL-17, respectively (Figure 16A). Th17 cells are known to express IL-21 [150]; however, $CCR6^+CD4^+$ T cells from the BXD2 mice showed little expression of IL-21. Moreover, few $CXCR5^+CD4^+$ T cells from the BXD2 mice expressed IL-17 (Figure 16A). Consistent with these results, quantitative RT-PCR analysis showed that the $CXCR5^+$ population expressed significantly higher levels of *Il21*, *Bcl6* and *Ascl2* mRNA, while the $CCR6^+$ population expressed higher levels of *Il17a* and *Rorc* (Figure 16B). These results demonstrate the purified $CCR6^+$ and $CXCR5^+$ populations among the $CD4^+$ T cells represented Th17 cells and Tfh cells in the BXD2 mice. To determine the role of each $CD4^+$ T cell subset from the BXD2 mice on the activation and differentiation of B cells, the purified T cells were cultured with $B220^+GL7^-IgD^+$ naïve B cells isolated from BXD2 mice in the presence of anti-CD3

and anti-IgM for 7 days. As depicted in Figure 17A, we found that $CXCR5^+CD4^+$ T cells efficiently stimulated B cells to produce IgG even when the lower number of T cells was used (B:T cell ratio = 10:5). By contrast, $CCR6^+CD4^+$ T cells failed to do so, even when a higher number of T cells was used (B:T cell ratio = 10:10) (Figure 17A). To test the role of each helper T cell population on B cell expansion, we labeled B cells with CFSE before co-culturing them with T cells. The addition of $CXCR5^+$ $CD4^+$ T cells induced significant B cell proliferation while $CCR6^+$ $CD4^+$ T cells failed to do so (Figure 17B). Similarly, the addition of Th17 cells differentiated *in vitro* from naïve BXD2 T cells did not induce IgG production from co-cultured naïve B cells in our experimental setting (Figure 18A and 18B). To determine the role of IL-17 and IL-21 in this process, anti-IL-17 or IL-21R-Fc fusion protein was added into the culture. As shown in Figure 19, the addition of IL-21R-Fc almost completely abolished the production of IgG from naïve B cells co-cultured with $CXCR5^+CD4^+$ T cells of BXD2 mice, while the addition of anti-IL-17 showed no effect. These results strongly suggest that $CXCR5^+CD4^+$ T cells in the BXD2 mice were responsible for the enhanced autoantibodies and germinal center B cell responses through an IL-21 dependent, IL-17-independent mechanism, while $CCR6^+CD4^+$ T cells played little role in the activation of auto-reactive B cells in BXD2 mice in our experimental setting.

Figure 16. Isolated CXCR5$^+$CD4$^+$ and CCR6$^+$CD4$^+$ T cells from BXD2 mice present characteristics of Tfh-like and Th17 like cells, respectively. (A) CXCR5$^+$ CD4$^+$ T cells and CCR6$^+$ CD4$^+$ T cells were sorted and subjected to intracellular cytokine staining. (B) Quantitative RT-PCR analysis of Tfh or Th17 cell-related gene expression in the CXCR5$^+$ and CCR6$^+$ CD4$^+$ T cells from BXD2 mice. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

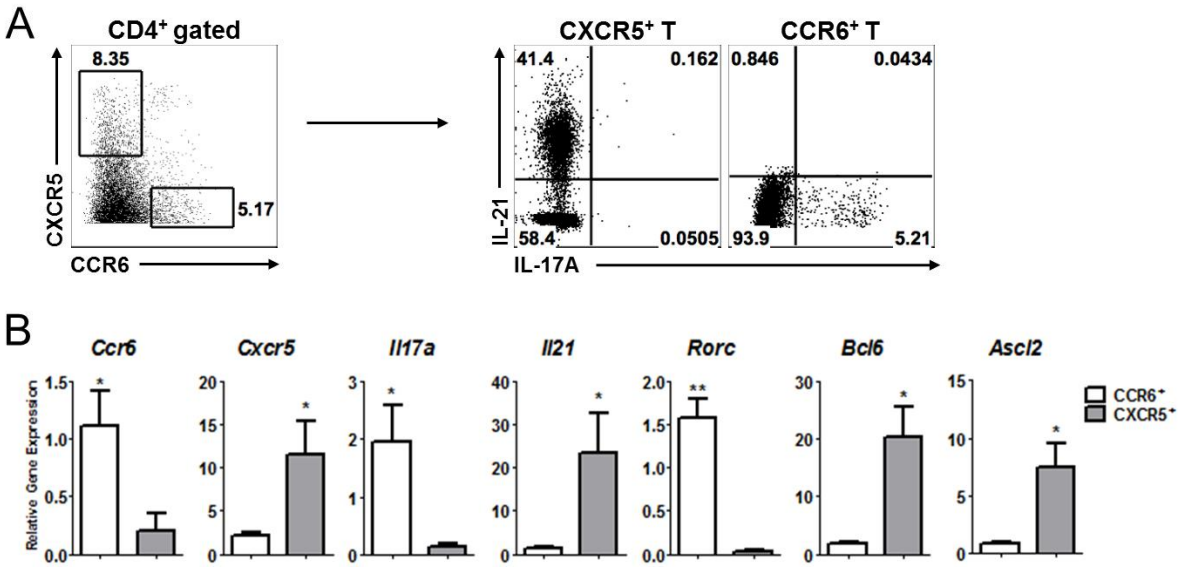


Figure 17. IL-21-producing CXCR5^{+}CD4^{+} T cells of BXD2 mice, but not IL-17-producing CCR6^{+}CD4^{+} T cells, provide B cell help for IgG production. (A) Naïve B cells (B220^{+}IgD^{+}GL7^{-}) from BXD2 mice were co-cultured with CXCR5^{+} or CCR6^{+} CD4^{+} T cells from BXD2 mice for 7days. The total IgG levels were measured by ELISA. (B) The proliferation of CFSE labeled naïve B cells (B220^{+}IgD^{+}GL7^{-}) from BXD2 mice obtained from (A) and determined by flow cytometry. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ###p < 0.001 comparing 10:10–10:5 ratio of B:T co-culture condition.

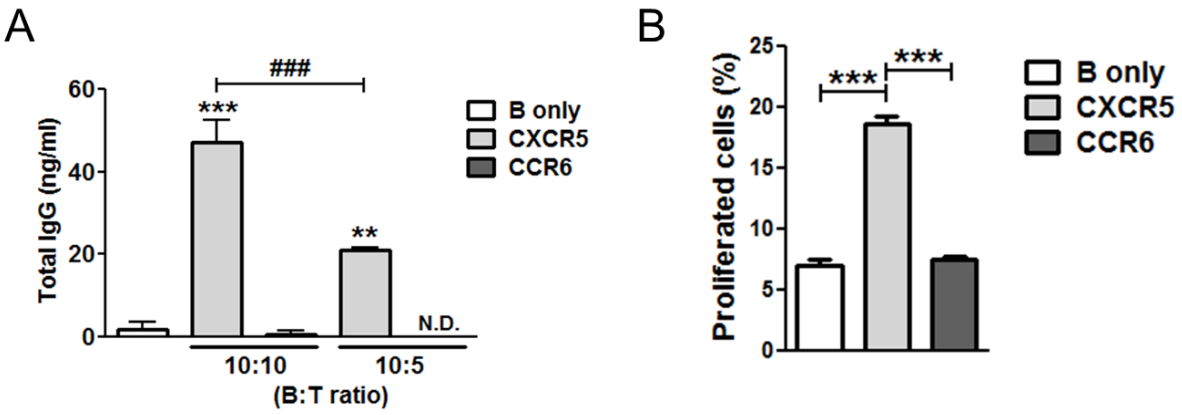


Figure 18. ***In vitro*** **differentiated Th17 cells do not provide B cell help for IgG production.** (A) Cytokines expression in *in vitro* differentiated Th17 cells 5 days after stimulation from naïve ($CD4^+CD25^-CD44^-CD62L^+$) $CD4^+$ T cells of BXD2 mice. (B) Naïve B cells ($B220^+IgD^+GL7^-$) from BXD2 were co-cultured with *in vitro* differentiated Th17 cells described in (A) for 7 days and the levels of total IgG were measured by ELISA. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

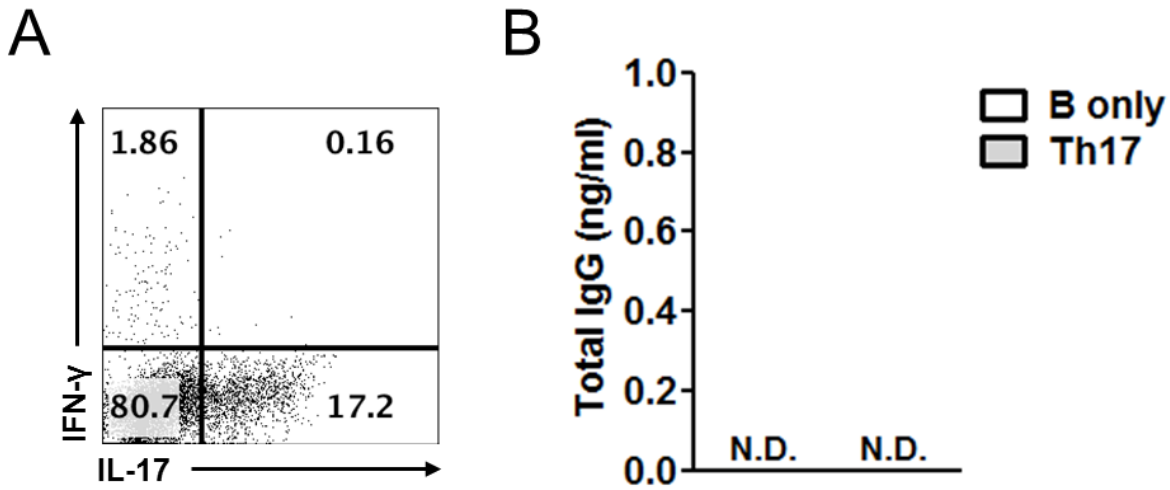
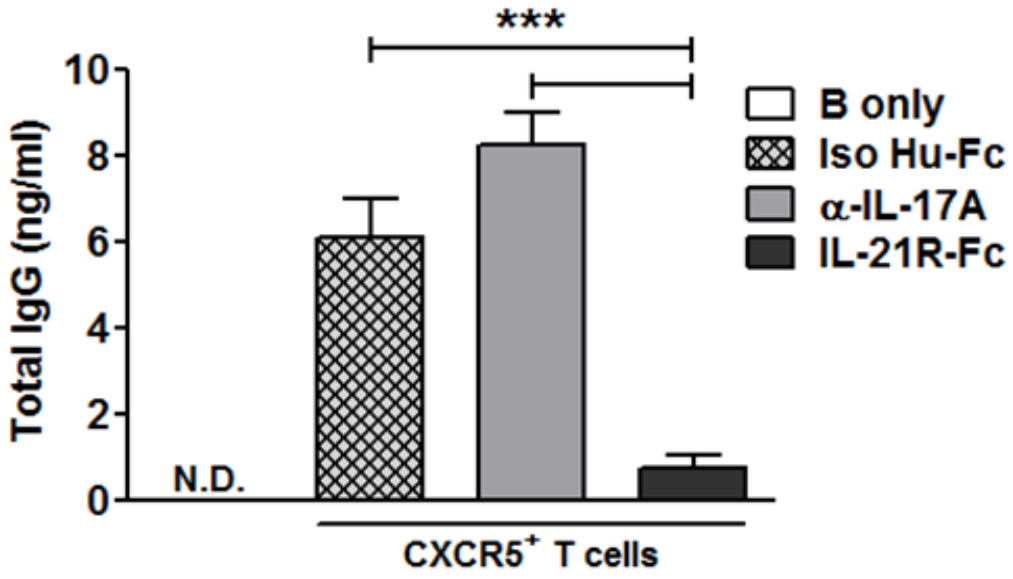


Figure 19. Blocking IL-21 signals in IL-21-producing CXCR5^{+}CD4^{+} T cells of BXD2 mice hampers the IgG. Different cytokine blocking reagents, isotype control antibody (Iso Hu-Fc), Rat anti-mouse IL-17A antibody (α-IL-17A), or recombinant mouse IL-21 receptor Fc chimera (IL-21R-Fc) were added (10 μg/ml, every other day) into the cell culture described in Figure 15 and the levels of total IgG were measured by ELISA. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.



3. Discussion

The role of Tfh cells and Th17 cells in the development of antibody-mediated autoimmune diseases such as lupus and arthritis has remained poorly understood. In this chapter, we sought to determine the correlation and contribution of Tfh and Th17 cells in the development of auto-reactive autoimmune B cell responses in the BXD2 mouse model of lupus. The role of IL-17 in the auto-reactive autoimmune B cell responses has been previously examined in the BXD2 animal model of lupus. The levels of IL-17 in the circulation and the frequency of Th17 cells in the spleen were shown to be elevated in the BXD2 mice [117]. The increased IL-17 enhanced the B cell chemotactic ability and stabilized the interaction of the germinal center B cells with nearby T cells [117, 139]. IL-17 also promoted the localization of Tfh cells in GC, but not the frequency and function of Tfh cells [140]. In contrast to these findings, our results demonstrated that Tfh cells are strongly associated with increased autoimmune B cell responses while Th17 cells play only a minor role in this process [142]. There were no clear differences in the IL-17 or Th17 cell related phenotypes; frequencies, genes expression levels, and protein level, between BXD2 mice and WT control mice (Figure 7 and 8). While the frequency of Tfh cells, the ratio of Tfh cells per Tfr cell, and Tfh related gene expression were all increased in BXD2 mice compared to control mice (Figure 10, 11 and 12). Using a co-culture system, we clearly demonstrated that IL-21, from Tfh-like cells ($CXCR5^+CD4^+$ T cells) rather than IL-17, from Th17-like cells ($CCR6^+CD4^+$ T cells) was essential for activating the production of IgG from naïve B cells (Figure 16 to 18). Thus, Tfh cells are responsible for the augmented autoantibodies and germinal center B cell responses through an IL-21 dependent mechanism in BXD2 mice.

Although the frequency of Tfh cells was increased in the BXD2 mice, the frequency of Tfr cells in the same mice were lower than that in control mice (Figure 10 and 12). In addition, the ratio of germinal center B cells per Tfr cell or Tfh cells per Tfr cell were all significantly increased in the BXD2 mice (Figure 12). As a consequence, spontaneous germinal center reactions and subsequent development of a lupus phenotype in this strain appeared due to decreased numbers of Tfr cells. A recent study showed that adoptive transfer of Tfr cells into young BXD2 mice decreased GC formation and reduced the number of autoantibody-producing B cells [119]. Therefore, transfer of Tfr cells might be a useful therapeutic approach for the treatment of autoimmune diseases. However, acquiring sufficient numbers of Tfr cells for this approach will be difficult. Alternative approaches that would provide similar results as transfer of Tfr cells may need to be developed. This issue will be addressed in Chapter 3.

CHAPTER 3

Application of Tfr like Cells for the Regulation of Auto-reactive B cell-mediated Autoimmune Disease Mouse Models

1 Rationale and Hypothesis

In chapter 2, we demonstrated that Tfh cells are integrally involved in the pathophysiology of auto-reactive autoimmunity in the BXD2 mouse model. Production of autoantibodies to self-antigen is a hallmark of variable autoimmune diseases such as SLE or RA or MS. Therefore, the regulatory mechanism is a precondition for preventing loss of self-tolerance in GC and autoantibody production.

Recent studies have identified Bcl-6^{+}CXCR5^{+}Foxp3^{+} Tfr cells, a distinct subset of Treg cells [24-26]. Tfr cells share both Tfh cells (Bcl-6, CXCR5, PD-1, and ICOS) and Treg cells (GITR, CTLA4, CD25, and Blimp-1) features and are specialized to suppress GC reactions *in vivo*. Although several groups have studied Tfr cells, the exact mechanisms by which Tfr cells inhibit GC B cell responses remain to be explored [116]. Due to their ability to specifically suppress GC B cell responses with little suppression of the other arms of adaptive immunity, the use of Tfr cells has been proposed as a novel approach for the treatment of autoantibody-mediated autoimmune diseases. However, the low frequency of the Tfr population has posed a major obstacle for the assessment of Tfr cells as a therapeutic for autoimmune disease.

For GC reaction, both Tfh cells and Tfr cells have to migrate to the B cell follicle. CXCR5-mediated migration of Tfr cells into B cell zone appears to be essential for their functional specificity [24]. As described above, Tfr cells share Treg cells properties and expression of CXCR5 and CXCR5-mediated migration into the B cell zone seem to be crucial for Tfr cell function. For these reasons, we hypothesized that the generation of Tfr-like cells by engineering Treg cells to express CXCR5, termed as ‘engineered Tfr cell (eTfr

cell)' would endow the eTfr cells to function as Tfr cells *in vivo*. Overexpression of CXCR5 on helper T cells has already been shown to increase the number of $CD4^+$ T cells in the follicle [60]. Therefore, we hypothesize that adoptively transferred CXCR5-transduced Treg cells would modulate humoral immune responses.

2. Results

2.1. Generation of CXCR5 expressing Treg cells

Recent studies identified $CXCR5^{+}Foxp3^{+}$ Treg cell subset, termed as Tfr cell, which specifically suppresses GC responses [24-26]. Previous studies have demonstrated the existence of Treg cells in the B cell follicles and GCs [104]. Treg cells also directly suppress B cell responses and auto-reactive B cells [105, 106]. Therefore, we hypothesized that adoptively transferred $Foxp3^{+}$ Treg cells which were retrovirally transduced with CXCR5 would migrate to germinal center B cell zones and impair the autoimmune humoral immune response.

To test this hypothesis, we first cloned mouse CXCR5 into a retroviral vector (RV) containing IRES and GFP. We transduced control RV-GFP or CXCR5-RV-GFP vector into Treg cells from Foxp3-IRES-mRFP (FIR) reporter mice, which expresses RFP in $Foxp3^{+}$ T cells. Thereafter, we call control RV-GFP transduce Treg cells as Control-transduced Treg and CXCR5-RV-GFP transduced Treg cells as CXCR5-transduced Treg cells. After five days of *ex vivo* expansion of Treg cells, GFP and RFP double positive cells were purified by flow cytometry (Figure 20A). First, we examined the CXCR5 expression on RV-transduced Treg cells. Figure 20B confirms that transduction with CXCR5-RV-GFP successfully induced the expression of CXCR5 on the surface of Treg cells. We next assessed whether CXCR5 overexpression affects the gene expression profile of the transduced Treg cells. Except in the case of the *Cxcr5* gene, expression levels of other genes related to either Tfh or Treg cells, were not affected following transduction (Figure 21). Therefore, overexpression of CXCR5 on $Foxp3^{+}$ Treg cells did not affect any off-target gene expression profiles.

Figure 20. Generation of CXCR5 overexpressing Treg cells. (A) Sorting strategy of isolating RV-transduced Treg cells. Treg cells from RFP-IRES-Foxp3 (RIF) mice were transduced by control-GFP or CXCR5-GFP retroviral vector. After 5 days of *ex vivo* expansion, $CD4^{+}GFP^{+}RFP^{+}$ cells were sorted by flow cytometry. (B) Flow cytometry analysis of CXCR5 expression from (A).

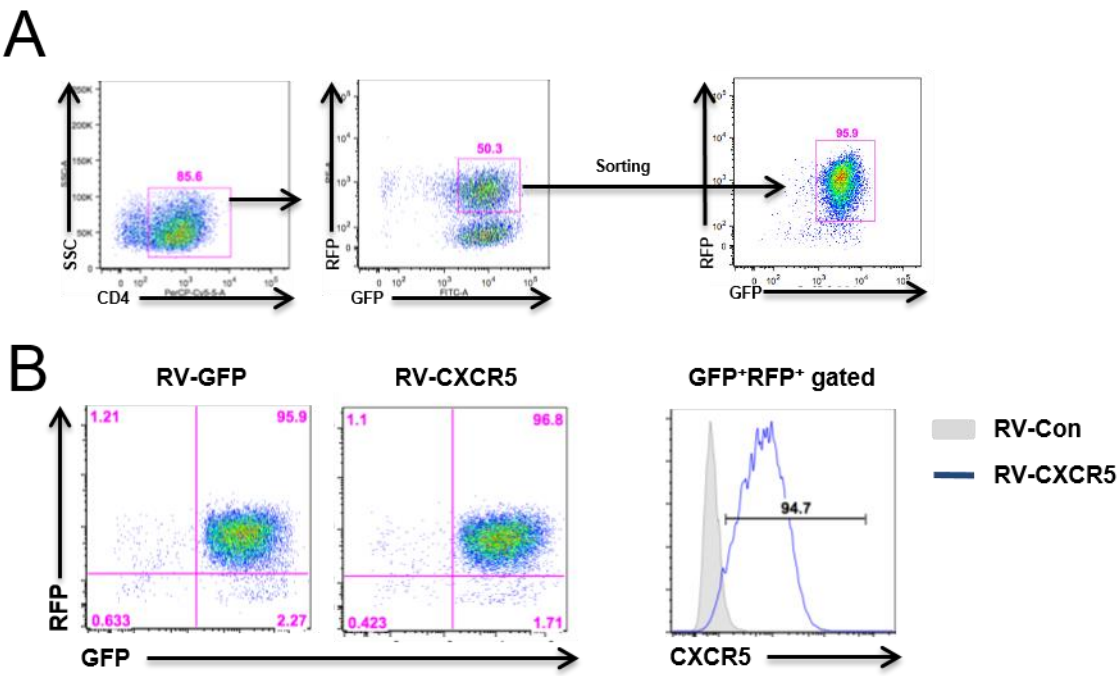
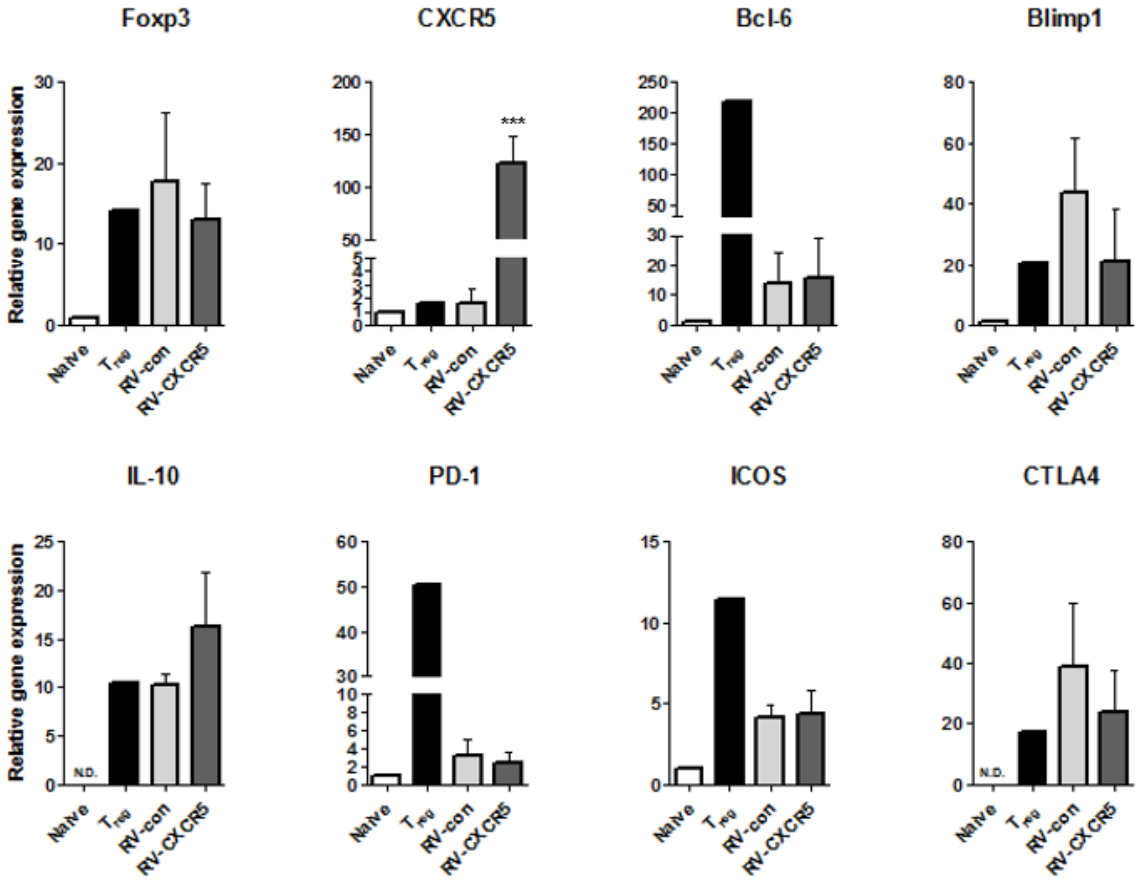


Figure 21. Retroviral transduction of CXCR5 on Treg cells does not change the Treg genes expression profiles. Quantitative RT-PCR analysis of Tfh cell and Treg cell related genes from Control or CXCR5-transduced Treg cells. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.



2.2. Functional analysis of CXCR5-transduced Treg cells *in vitro*

We observed that there were no critical gene expression profile changes in CXCR5-transduced Treg cells other than *Cxcr5* gene. We then addressed whether CXCR5 overexpression affects the suppressive role of Treg cells. To examine the suppressive function in RV-transduced Treg cells, we performed an *in vitro* Treg suppression assay. Conventional $CD4^+$ T responder cells (Tresp) were stained with proliferation dye (eFlour670) and cultured with $CD4^+$ T cell-depleted splenocytes plus either Control or CXCR5-transduced Treg cells at different ratios. We found no difference between Control and CXCR5-transduced Treg cell's suppressive capacity (Figure 22). Thus, overexpression of CXCR5 on Treg cells does not affect Treg cell's suppressive function.

We next examined whether the CXCR5 overexpressed on the Treg cells is functional. CXCL13 is the known ligand for CXCR5 and is critical for B cell homing to follicles and formation of GC [151]. To determine whether CXCR5-transduced Treg cells respond to CXCL13, we employed an *in vitro* chemotaxis assay (cell migration assay) using a transwell system (Figure 23). Control-transduced Treg cells migrated into the bottom well at a minimal level (around 10% of input cells). However, CXCR5-transduced Treg cells successfully migrated into the bottom well at a much higher level. As CXCL13 concentration was increased, the migration of the CXCR5-transduced Treg cells increased. Thus, overexpression of CXCR5 on Treg cells enabled them to migrate in response to CXCL13 while not affecting their gene expression profile or their ability to suppress conventional CD4 responder proliferation.

Figure 22. CXCR5-transduced Treg cells do not lose their suppressive ability. Analysis of cell proliferation dye-labeled responder T cells (Tresp, $CD4^{+}CD25^{-}$ T cell). Tresp cells and Control or CXCR5-transduced Treg cells were co-cultured with anti-CD3 and irradiated T cell-depleted splenocytes for 3 days. Cell proliferation was measured by flow cytometry. The results shown are representative of three independent experiments.

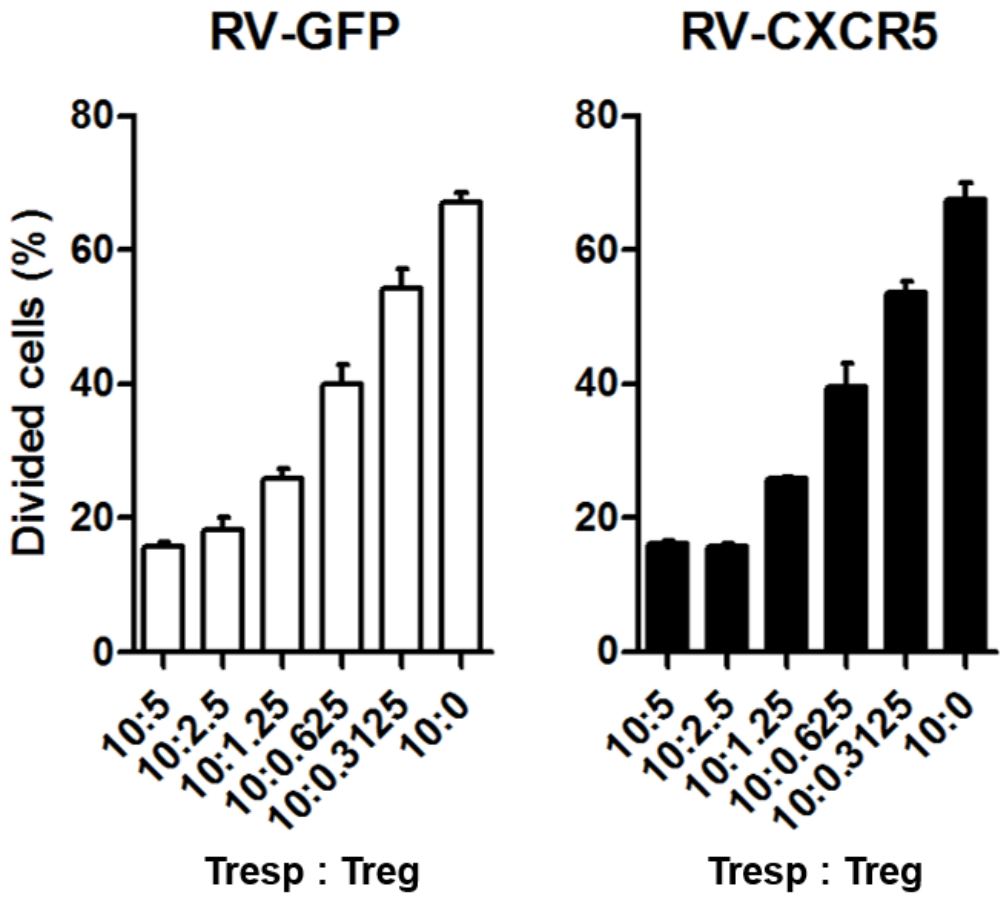
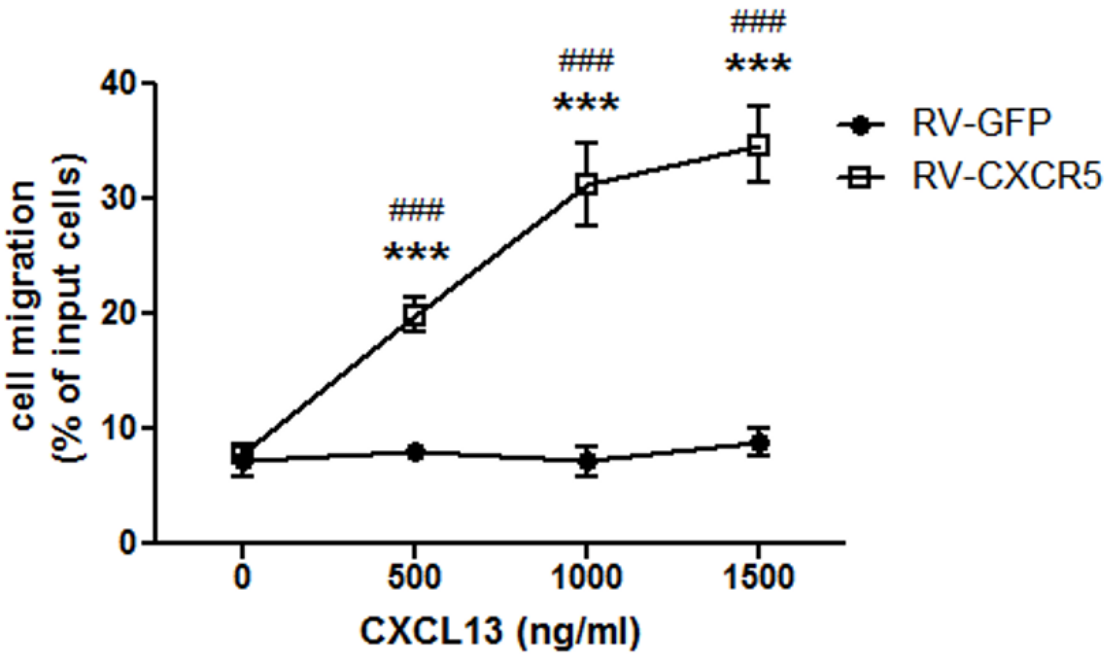


Figure 23. CXCR5-transduced Treg cells can migrate in a CXCL13 dependent manner. Transwell migration assay of Control and CXCR5-transduced Treg cells in the presence of CXCL13. CXCR5-transduced Treg cells migrated dependent on CXCL13 concentration. The results shown are representative of three independent experiments. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared with different concentration of CXCL13 in the same group. #p < 0.05, ##p < 0.01, ###p < 0.001 compared with Control and CXCR5-transduced Treg cell in the same concentration of CXCL13.



2.3. Functional analysis of CXCR5-transduced Treg cells *in vivo*

As shown in previous experiments, CXCR5-transduced Treg cells have both suppressive and migratory capacity *in vitro*. We next sought to determine whether these transduced cells would migrate into the GC area and their ability to suppress a GC response *in vivo*. It was reported previously that CXCR5 overexpressing conventional $CD4^+$ T cells from Ovalbumin (OVA) specific TCR receptor (OT-II) expressing mice preferentially migrated into B cell follicles compared to control-RV transduced OT-II cells [60]. Additionally, lentivirally transduced CXCR5 on thymus-derived natural Treg (nTreg) cells from $CD4^+$ T cell specific NFAT2 deficient mice rescued Tfr cell-mediated function *in vivo* [110]. Therefore, we expected that CXCR5-transduced Treg cells will migrate to the GC area and regulate GC responses *in vivo*.

To verify *in vivo* function of CXCR5-transduced Treg cells, we designed adoptive co-transfer experiment based on previous publications [107, 152]. Tfh cells from 4-hydroxy-3-nitrophenylacetyl-conjugated keyhole limpet hemocyanin (NP-KLH) immunized B6SJL congenic mice (CD45.1) and RV-infected Treg cells were transferred into TCRβ-deficient mice (Figure 24). Due to the lack of TCR signals, TCRβ-deficient mice do not have T cells. Therefore, this approach allowed us to determine if Tfh cells and CXCR5 overexpressing Treg cells could functionally regulate GC responses separately from differentiation [107]. We adoptively co-transferred FACS sorted 50,000 of Tfh cells from NP-KLH immunized B6SJL mice and 25,000 of Control or CXCR5-transduced Treg cells into TCRβ KO mice. We subcutaneously immunized recipient mice 1 day later with NP-KLH and analyzed GC reactions and titers of NP-specific IgG and IgM at day 10 after immunization. We measured

the proportion of GC B cells, PCs, and Tfh cells from draining lymph nodes (dLN) (Figure 25). Unfortunately, the results from the transfer experiments were inconsistent. GC B cells and PCs were decreased in the first experiment (Figure 25A), but not in the second experiment (Figure 25B). As was the case with the GC B cell frequencies, contradictory results were observed in the antibody measurement of the two independent experiments (Figure 26). In the first experiment, levels of NP-specific general affinity IgM and IgG were decreased in CXCR5-transduced Treg cell recipients (Figure 26A). In the second experiment, levels of NP-specific both high and general affinity IgM were decreased while those of IgG were increased in CXCR5-transduced Treg cell recipients (Figure 26B).

Figure 24. A schematic diagram of adoptive co-transfer experiment using immunized Tfh and RV-transduced Treg cells. Congenic B6SJL mice were immunized with NP-KLH on Day 0. On Day 2, Treg cells were isolated from RIF mice and infected with Control or CXCR5-RV, followed by cell expansion. On Day 7, FACS-sorted 50,000 of Tfh (PD-$1^{+}CXCR5^{+}CD4^{+}$) cells from NP-KLH immunized B6SJL mice and 25,000 of $GFP^{+}RFP^{+}$ Treg cells were cotransferred into TCRβ KO mice followed by immunization of recipient mice with NP-KLH. 10 days later, sera were collected and draining lymph nodes were obtained for further studies.

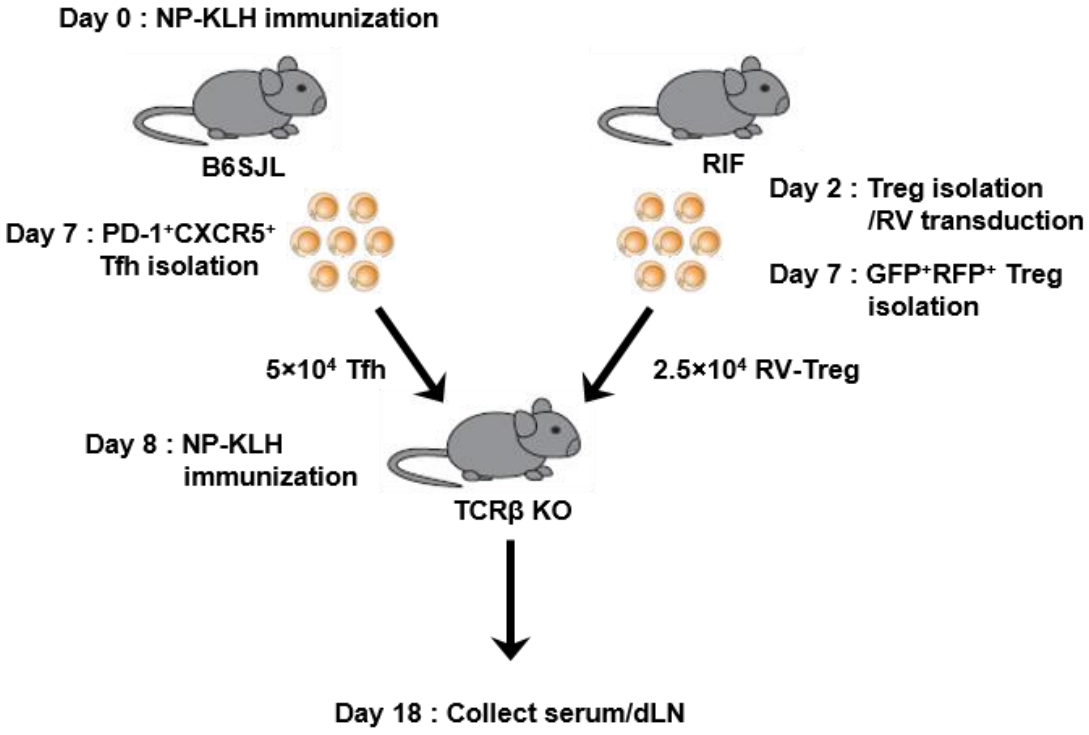


Figure 25. GC responses in mice co-transferred with Tfh and RV-transduced Treg cells are inconsistent. Two independent cotransfer experiments (A and B) of GC responses (GC B cells, PCs, and Tfh cells) from dLN of TCRβ KO recipients. (A) The frequencies of GC B cells and PCs were statistically decreased in CXCR5-transduced Treg cells recipients. (B) No significant changes in GC B cell responses were detected. The frequency of Tfh cells slightly increased in CXCR5-transduced Treg cells recipients in both experiments (A and B). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

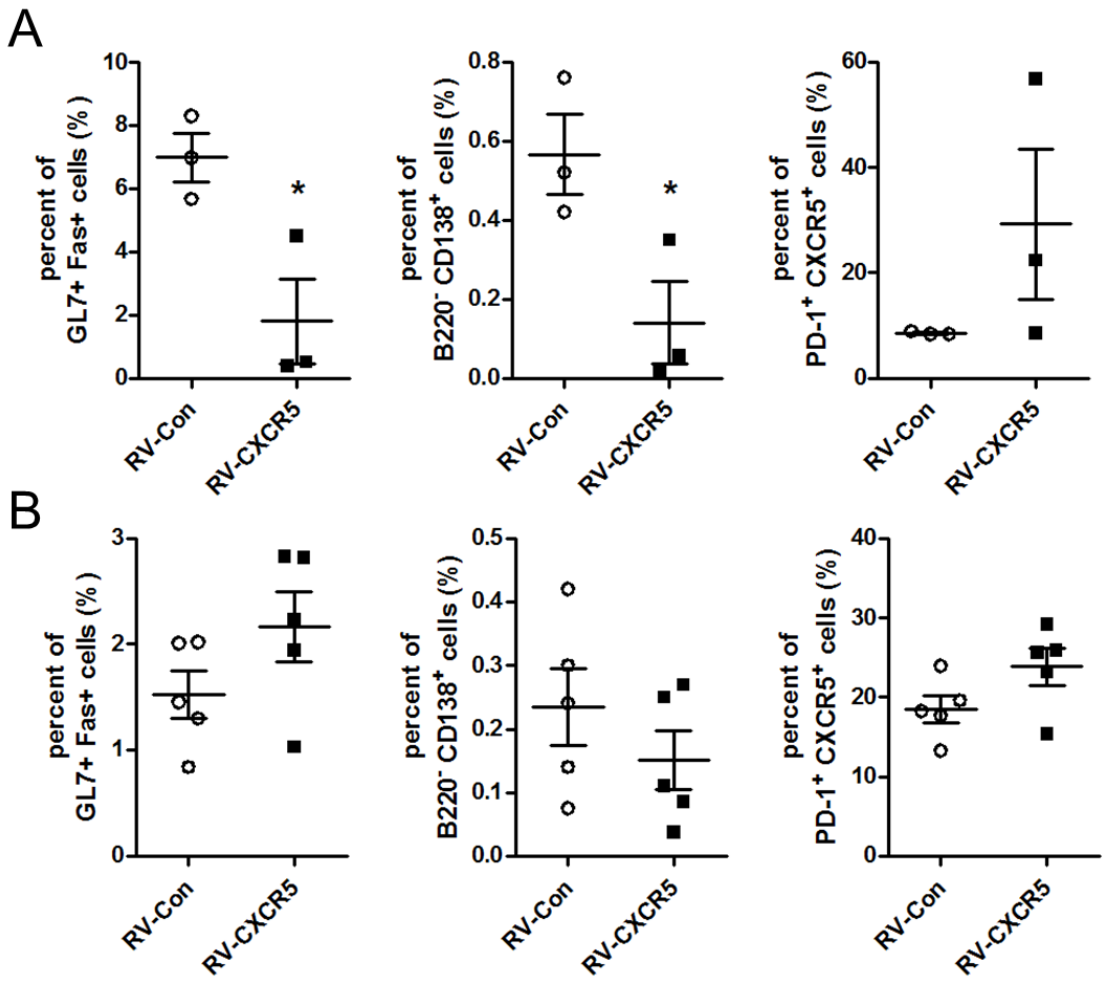
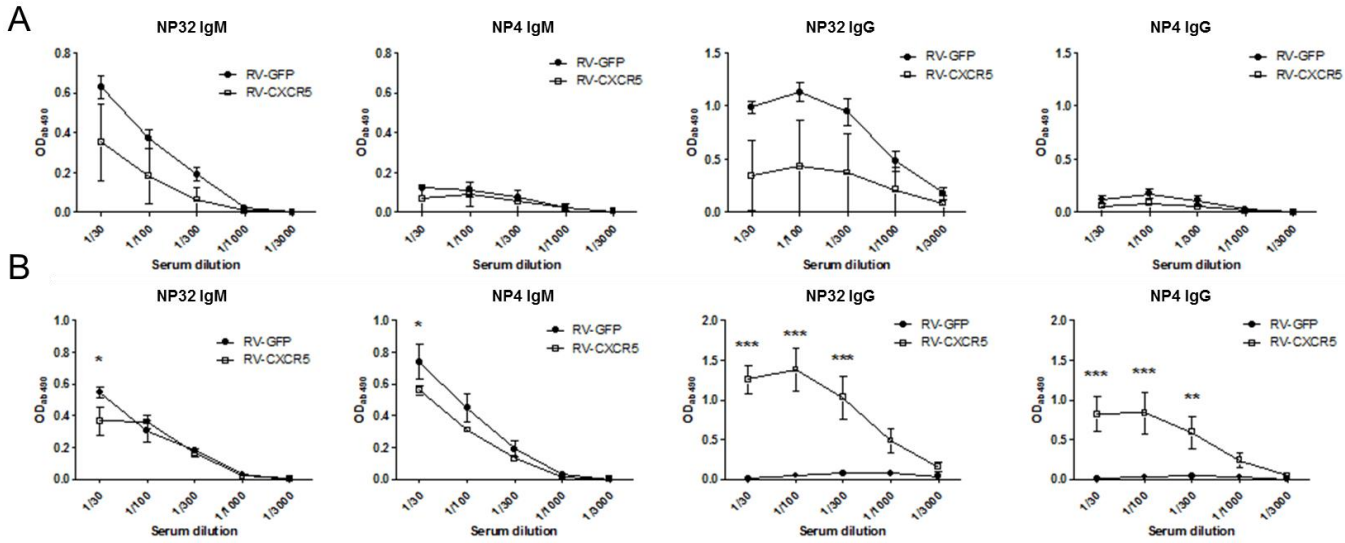


Figure 26. NP-specific immunoglobulin levels in mice co-transferred with Tfh and RV-transduced Treg are inconsistent. Two independent cotransfer experiments (A and B) of NP-specific immunoglobulin levels (IgM and IgG, NP4 for high affinity and NP32 for general affinity) in the sera from TCRβ KO recipients. (A) Both IgM and IgG levels were decreased in CXCR5-transduced Treg cell recipients. No statistical difference between the groups. (B) IgM levels were decreased in CXCR5-transduced Treg recipients while IgG levels were increased in CXCR5-transduced Treg recipients. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.



We reasoned that the small numbers of transferred cells used in the above-mentioned experiment could be the cause of the inconsistent results. Therefore, we modified the experimental design by using another transfer model from a recent study [110]. Instead of Tfh cells from NP-KLH immunized mice, we transferred naïve $CD4^+$ T cells and also increased the number of transferred to 500,000 cells (Figure 27). 5 days after *ex vivo* expansion of RV-transduced Treg cells, FACS sorted naïve $CD4^+$ T cells ($CD4^+CD25^-CD44^-CD62L^+$) from congenic B6SJL mice and GFP^+RFP^+ Treg cells were adoptively transferred into TCRβ KO mice.

First, we compared RV-transduced Treg cell stability after cell transfer. As shown in Figure 28, there was no difference in the frequency of transferred Treg cells between Control-transduced Treg cell recipients and CXCR5-transduced Treg cell recipients. As we increased the number of transferred cells (bottom panels), we observed increased frequencies of live donor GFP^+RFP^+ Treg cells in the dLNs. Therefore, we next examined the role of CXCR5-transduced Treg cells in GC reactions in this experimental setting. The frequencies of GC B cells and Tfh cells were slightly decreased in CXCR5-transduced Treg cell recipients group, but the differences were not statistically significant (Figure 29A and 29C). The frequency of PCs was comparable between Control-transduced Treg recipients and CXCR5-transduced Treg cell recipients (Figure 29B). Levels of NP-specific both high and general affinity IgM were increased in sera from CXCR5-transduced Treg cell transferred group (Figure 30A). However, NP-specific high and general affinity IgG levels were comparable between Control-transduced Treg recipients and CXCR5-transduced Treg cell recipients (Figure 30B). Our results indicated that CXCR5-transduced Treg cells in these

experiments did not show stronger suppressive function of antigen-specific GC reactions *in vivo*.

Figure 27. A schematic diagram of modified adoptive co-transfer experiment using naïve CD4$^+$ T cells and RV-transduced Treg cells. On Day 0, Treg cells were isolated from RIF mice and infected with Control or CXCR-RV, followed by cell expansion. On Day 5, 5,000,000 of FACS-sorted naïve CD4$^+$ T (CD4$^+$CD25$^-$CD44$^-$CD62L$^+$) cells from B6SJL mice and 500,000 of GFP$^+$RFP$^+$ Treg cells were transferred into TCRβ KO mice followed by immunization of recipient mice with NP-KLH. On Day 16, sera were collected and draining lymph nodes were obtained for further studies.

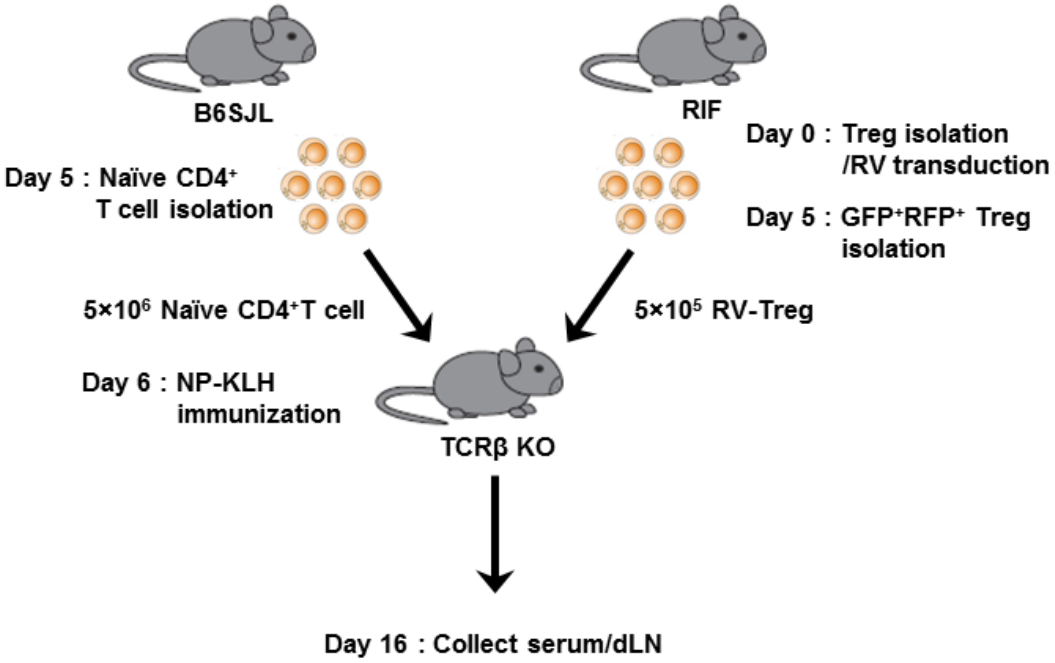


Figure 28. Comparison of transferred RV-transduced Treg cells in different experimental designs. The frequencies of RV-transduced Treg cells from Figure 23 (upper panel) and Figure 26 (bottom panel) in the draining lymph nodes of recipients.

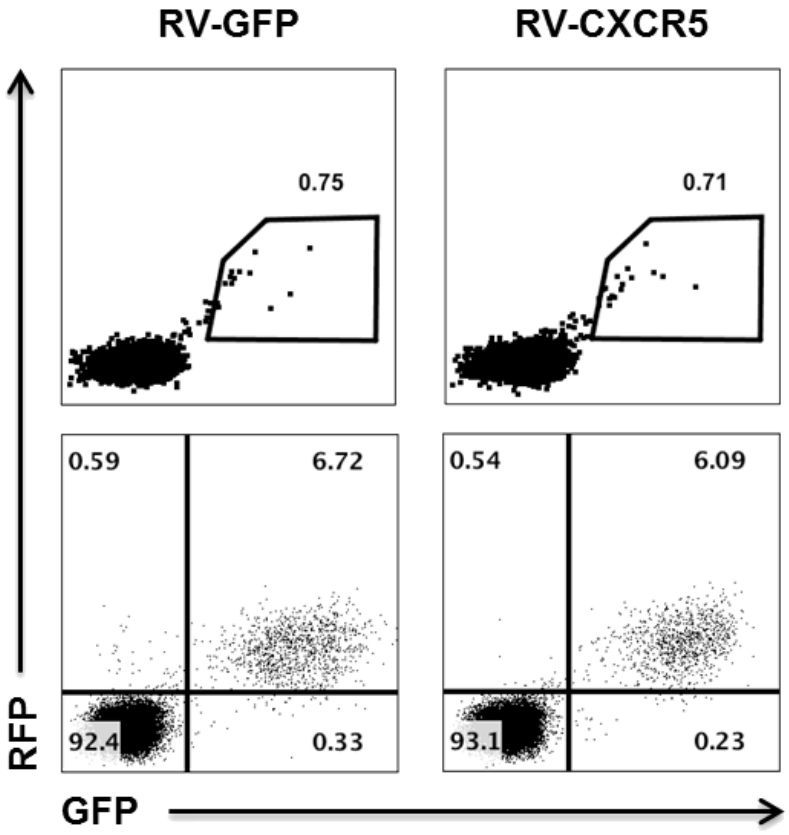


Figure 29. GC responses in mice co-transferred with naïve CD4$^+$ T cell and RV-transduced Treg cells. GC responses (GC B cell, PC, and Tfh cells) from dLN of TCRβ KO recipients. The proportion of GC B cells (A) and Tfh cells (C) were slightly decreased in CXCR5-transduced Treg cell transferred group, but the data were not statistically significant. The frequency of PCs (B) was comparable between the groups. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

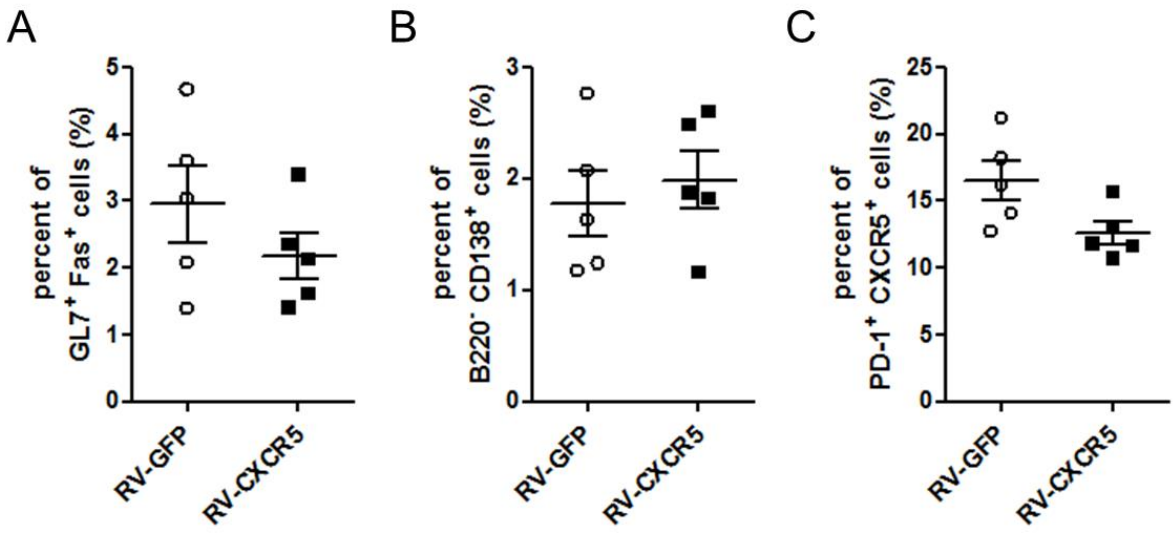
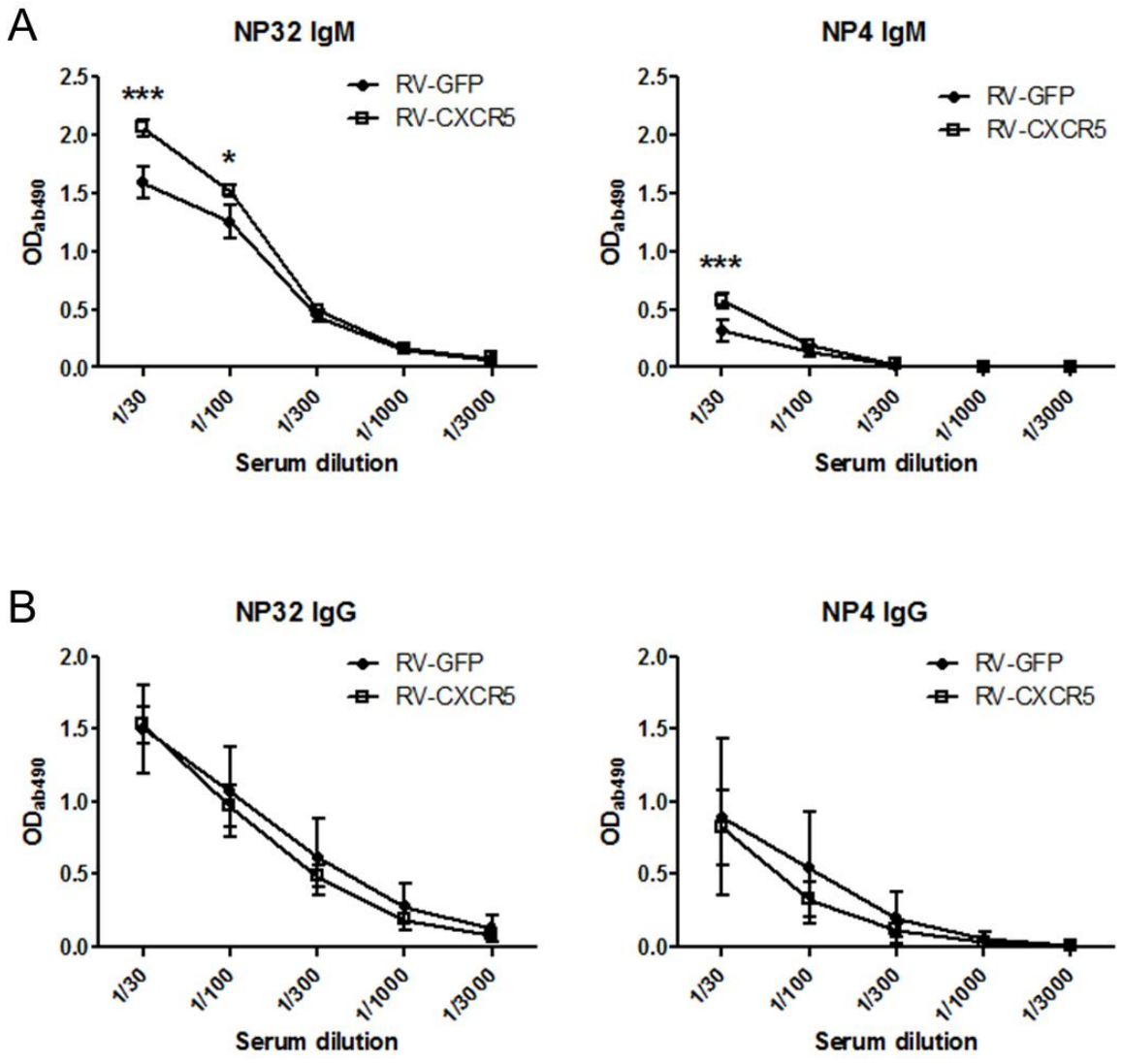


Figure 30. NP-specific immunoglobulin levels in mice co-transferred with naïve CD4$^+$ T cells and RV-transduced Treg cells. NP-specific immunoglobulin levels (IgM and IgG, NP4 for high affinity and NP32 for general affinity) in the sera from TCRβ KO recipients. (A) NP-specific both high and general affinity IgM levels were higher in CXCR5-transduced Treg cell transferred group. (B) NP-specific both high and general affinity IgG levels were comparable between the groups. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.



3. Discussion

Abnormal generation and function of Tfh cells contribute to the pathogenesis of autoimmune diseases. Therefore, regulating aberrant Tfh cell activity is a key mechanism to relieving autoantibody-mediated autoimmune diseases. For the treatment of autoantibody-mediated autoimmune lupus, many non-biological reagents are currently being used. However, many side effects and non-specific immunosuppression have been reported in clinical studies. The use of Tfh specific regulatory Treg cell subset, Tfr cell, has been proposed an alternative autoimmune disease immunotherapy. Owing to the rare number of Tfr cells in normal conditions, Tfr cells are limited in possible therapeutic applications. Numerous studies demonstrate that CXCR5 is the key chemokine receptor for the migration of immune cells into GC area [39-41]. In addition, direct regulation of auto-reactive B cells by Treg cells has been reported [104-106]. Therefore, to examine an alternative to the Tfr cell limitations, we generated CXCR5 overexpressing Treg cells by retroviral transduction and examined their suppressive roles in GC responses.

Our results demonstrated that overexpression of CXCR5 did not affect Treg gene expression profile (Figure 21). Except *Cxcr5*, other Tfh or Treg cell representative genes such as *Foxp3, Bcl6, Prdm1*, *Pd1*, *Icos*, and *Ctla4* expression levels were not altered. Moreover, these CXCR5-transduced Treg cells did not lose their suppressive activity *in vitro* (Figure 22). Even after retroviral transduction, CXCR5-transduced Treg cells had similar suppressive properties as Control-transduced Treg cells. CXCL13 dependent chemotactic migration is mediated by CXCR5 expression and signaling. This feature is also shown in CXCR5-transduced Treg cells (Figure 23). Using *in vitro* transwell system, we demonstrated

that CXCR5-transduced Treg cells migrated in response to CXCL13 in a dose-dependent manner. The *in vitro* suppressive and migratory functions of CXCR5-transduced Treg cells appeared therefore promising as a possible cellular therapeutic for autoimmune diseases. To test the immunosuppressive effects of these transduced Treg cells, we used two different *in vivo* adoptive transfer experiments in T cell-deficient mice.

Unfortunately, the results from these experiments were not consistent, suggesting that our experimental designs will need to be further refined. For example, the initial Tfh cell co-transfer experiments were not reproducible. Since the number of transferred cells was relatively small (50,000 of Tfh cells and 25,000 of RV-transduced Treg cells), we suspected that was the main cause of inconsistent results. Next we modified the experiment and tested the role of CXCR5-transduced Treg cells. We showed the decrease of GC B cell and Tfh cells in CXCR5-transduced Treg cell recipients, but again we did not observe meaningful results. When we checked the transferred cell stability, we could clearly detect Foxp3 expression in RV-transduced Treg cells (Figure 28). Accumulating studies strongly support that the expression of *Foxp3* gene is correlated with their suppressive capacity in T cells [17]. We did demonstrate a stable $Foxp3^{+}$ population in the recipients, so they likely are providing a suppressive role. However, to clearly demonstrate the suppressive function of these CXCR5-transduced Treg cells, we will need to find the optimal condition of transfer or perhaps employ more suitable animal model.

CHAPTER 4

General discussion and future directions

1. General discussion

1.1. Tfh cells in antibody-mediated autoimmune diseases

Autoimmune diseases contain more than 100 different types of disorders characterized with various pathogenesis [1]. However, many of their pathogenic mechanisms are not clearly defined. In the United States alone, more than 50 million Americans suffer from autoimmune diseases with an estimated annual cost of autoimmune related diseases is more than $100 billion [1]. Multiple genetic and environmental factors are known to trigger the breakdown of immune tolerance to self-antigens and lead to autoimmune diseases [115]. The generation of auto-reactive antibodies against self-antigens including nuclear components, organ-specific antigens, and soluble factors, is a hallmark of autoantibody-mediated autoimmune diseases [115]. As Tfh cells principal role is to support GC B cell responses, accumulated studies in both mice and humans strongly support that abnormal generation and/or activation of Tfh cells are associated with the pathogenesis of autoantibody-mediated autoimmune diseases. Understanding the role of Tfh cells is important for developing treatment of antibody-mediated autoimmune diseases. In this regard, we aimed to study the role of Tfh cells and Tfr cells in animal model of autoimmune lupus in the present study.

1.2. BXD2 mice as an animal model of antibody-mediated autoimmune lupus

To appropriately study autoantibody-mediated autoimmune diseases, we need a suitable and representative animal disease models. There are many mouse autoimmune disease models (Table 4). Among them, the BXD2 strain is the most optimal mouse

autoimmune disease model. First of all, BXD2 mice spontaneously develop autoimmune lupus, arthritis and glomerulonephritis which are common symptoms in patients with autoimmune diseases [118]. Second, unlike Fas-deficient MRL/*lpr* mice, BXD2 mice do not have significant gene deficiency while having several autoimmune loci, related to autoimmune phenotypes [118]. Interestingly, IL-17 drives autoantibody-mediated autoimmune responses by promoting spontaneous GC formation in the BXD2 mice [117]. Therefore, we employed BXD2 mice as an autoimmune disease model for experimental T cell-mediated therapy.

1.3. Tfh cell, rather than Th17 cell, mediates auto-reactive GC responses in BXD2 mice

We first inspected the general phenotypes of BXD2 mice. As described in previous studies, BXD2 mice developed auto-reactive B cell responses in comparison to age-matched wild-type mice, including increased number of GCs, proportion and number of GC B cells, autoantibodies titers in sera, and proportion and number of Tfh cells [142]. In contrast to another study, the percentage of IL-17 positive $CD4^+$ T cells, levels of IL-17 in the circulation, and Th17 cell related genes expression were not increased in our investigations of BXD2 mice. Based on these observations, we re-investigated the mechanism of auto-reactive GC reaction in the BXD2 mice. Our results showed that Tfh cells, rather than Th17 cells had positive correlations with the GC responses including the proportion of GC B cells, and autoantibody levels. Moreover, we clearly proved that IL-21, not IL-17 was essential for initiating the production of IgG from naïve B cells. Including our study, many other studies suggest that IL-21 has a critical role in inducing terminal B cell differentiation and a potential role for further development of autoimmune diseases [141]. Our results also bolstered the

role of Tfh cells in the development autoimmune lupus. IL-21 is induced by IL-6, which is also critical for Th17 cell differentiation, and Th17 cells also express IL-21 [150]. Therefore, targeting IL-21 might be effective for suppressing Tfh as well as Th17 responses. There are several on-going clinical trials using blocking IL-21 for RA and SLE [141]. Therefore, regulation of IL-21 and its signaling pathway would be effective therapeutics not only for the autoantibody-mediated autoimmune diseases, but also other autoimmune diseases, in which Tfh and Th17 cells are simultaneously involved in disease onset and progression, such as RA.

Increased levels of IL-17 in the circulation in patients with SLE support the role of IL-17 in auto-reactive B cell responses [153]. However, other studies have argued that there is no strong correlation between levels of IL-17 and auto-reactive disease, even IL-17 levels were increased in SLE patients [154-156]. Our studies with BXD2 mice study demonstrated no correlation between Th17 cell/IL-17 and the generation of autoimmune B cell responses. Therefore, the pathologic role of IL-17/Th17 cells in auto-reactive GC responses is remains somewhat controversial and warrants further investigation.

The differences in the results of our study and that of the Mountz laboratory regarding examining of Th17 cells in autoimmune B cell responses in BXD2 mice is presently not clear. One potential explanation is that differences in diet or gut microbiota may result in different levels of Th17 responses in the two studies. All mice in chapter 2 were purchased from Jackson Laboratory, known to be SFB free [143], and maintained in a barrier facility. However, the proportion of Th17 cells in the mesenteric lymph node and Peyer's patches of BXD2 mice was similar to that of control mice even after 4 weeks of co-housing (Figure 9). Therefore, the difference of Th17 cell immunity between previous studies and our study is

not likely because BXD2 mice retained less Th17-associated gut microbiota than control mice in our study. Instead, we observed an increase of IFN-γ-producing Th1 cells, not IL-17-producing Th17 cells in BXD2 mice (Figure 7). Th1 and Th17 cells reciprocally regulate each other and IFN-γ is also associated with autoimmune diseases [157]. *Sanroque* mice have excessive IFN-γ and sustain lupus-associated pathology [126]. These results may explain why BXD2 mice have less Th17 cell immunity and IFN-γ mediated auto-reactive autoantibodies generation in BXD2 mice in our experiments. Further study will be necessary to determine the role of Th1/IFN-γ in auto-reactive GC responses in BXD2 mice.

A previous study demonstrated that IL-21 suppresses Tfr cell development in BXD2 mice [119]. Similar to this previous report, our present investigation showed that the frequency of Tfr cells was decreased while that of Tfh cells was increased in BXD2 mice (Figure 10 and 12). Moreover, the ratio of GC B cell per Tfr cell and Tfh cell per Tfr cell were increased in BXD2 mice. This imbalance between Tfh and Tfr cells likely contributes to the observed autoimmune lupus in BXD2 mice, indicating that this aberrant immune homeostasis leads to the development of autoimmune diseases. Our findings indicate that reconstituting balance between Tfh cells and Tfr cells, either decreasing Tfh cells or increasing Tfr cells, might be the key to developing new treatments for autoantibody-mediated autoimmune diseases.

1.4. Use of Engineered Tfr-like cells is as a novel cellular therapeutics for the treatment of antibody-mediated autoimmune diseases

As described in Table 2, immunosuppressive chemical agents are generally being used to relieve the autoimmune inflammatory symptoms in patients with SLE. However,

many of these agents report side effects or only have partial specificity [2]. For example, due to their systemic immune suppressive function, non-biological reagents also downregulate normal, unnecessary immune functions.

On the other hand, Tfr cells control GC reactions by suppressing either Tfh cell or GC B cells and prevent the emergence of auto-reactive B cells [24, 25, 116]. Also, transfer of Tfr cells greatly reduced the auto-reactive B cell responses in BXD2 mice [116, 119]. These results indicated that Tfr cells have GC response-specific suppressive function and efficiently regulate auto-reactive B cell responses and autoantibody productions. Therefore, Tfr cells are a novel candidate for the treatment of autoantibody-mediated autoimmune diseases. However, this approach has a significant hurdle to overcome in that it will be difficult to obtain enough Tfr cells for effective therapy.

To overcome this drawback, we hypothesized that enforcing CXCR5 expression on the more abundant $Foxp3^+$ Treg cells will facilitate robust migration of Treg cells to the GC area, thereby suppressing auto-reactive reactions in the GCs. Several studies have demonstrated that Treg cells regulate GC responses [116] and expression of CXCR5 enables cells to migrate into B cell follicles [39-41, 60]. We generated CXCR5-transduced Treg cells and expanded these cells *ex vivo* to acquire sufficient number of cells for investigation.

Overexpression of CXCR5 specifically on Treg cell did not affect the expression of other genes related to Tfh cells or Treg cells (Figure 21). Likewise, retroviral transduction of CXCR5 on Treg cells did not impair the quality of suppression *in vitro* (Figure 22). Most importantly, the CXCR5 expression on Treg cells empowered these cells to migrate in a CXCL13 dependent manner during an *in vitro* transwell assay (Figure 23). A recent study

with conventional $CD4^+$ T cells also indicated that expression of CXCR5 enhances cell migration toward GC area [60]. Collectively, these *in vitro* experimental results strongly support the possibility of this cell as a drug candidate for diseases dependent on Tfh cell activity. We next tested the *in vivo* the possible immunomodulatory function of CXCR5-transduced Treg cells in adoptive transfer experiments. We designed two different adoptive transfer experiments based on several references [107, 110, 152]. To exclude the role of Tfh or Tfr cells from the host mice, we used TCRβ KO mice which have no endogenous T cells in all adoptive transfer experiments.

The co-transfer experiments with NP-KLH immunized Tfh cells and RV-transduced Treg cells yielded data that was highly variable and inconsistent (Figure 24). Since we transferred only 50,000 of Tfh and 25,000 of RV-transduced Treg cells into the recipients, we assumed that increasing number of transferred cells would provide an experimental system that can define the function of eTfr cells *in vivo*. We thus transferred naïve $CD4^+$ T cells, instead of immunized Tfh cells and increased the numbers of transferred cells by transferring 5 million of naïve $CD4^+$ T cells and 500,000 of RV-transduced Treg cells into TCRβ KO mice. Even in this setting, we still did not observe clear differences between GC responses of Control and CXCR5-transduce Treg cell recipients. We successfully demonstrated the migration of CXCR5-transduced Treg cells in response to CXCL13 *in vitro*, and a recent *in vivo* study with conventional $CD4^+$ T cells [60]; however, it is unclear whether CXCR5-transduced Treg cells efficiently migrated into B cell zone at this stage. Therefore, it is necessary to determine whether CXCR5-transduced Treg cells have *in vivo* migration capacity to CXCL13 gradient into B cell zone by using immunohistological

examination. However, we did detect reliable numbers of $GFP^{+}RFP^{+}$ Treg cells in the recipients (Figure 28, bottom). Since the expression of Foxp3 in T cells is critical for their suppressor activity [17], transferred Treg cells might have a suppressive function. Moreover, ectopic expression of Foxp3 in conventional T cells induced the conversion of conventional T cells into Treg-like cells and had suppressive function [158-160].Therefore, we may generate Tfr-like cells from Tfh cells by overexpressing Foxp3. This method is relatively simpler and cost-effective than generating eTfr cells from CXCR5-transduced Treg cells. Nevertheless, Foxp3 is a transcription factor, and this transcription factor will induce many changes in other genes expressions while transduction of CXCR5 did not affect other genes profile. Therefore, we believe that generation of eTfr cells from CXCR5-transduced Treg is more reliable system than from Foxp3-transduced Tfh cells. Thus, improving the quality and quantity of eTfr cells are also critical issue in our future study for the development of Tfr cell-based immunotherapy.

Numerous studies have performed adoptive transfer experiments of Treg including nTreg or peripheral Treg (pTreg), and these cells are about sufficient to suppress the auto-reactive B cell responses [4, 18, 161]. As we first hypothesized, if this transferred cells successfully migrated into GC area, perhaps, if with further optimization of this transfer condition, we will observe better and more consistent suppression in the GC responses.

As described above, we designed *in vivo* experiments based on the publications which used T cell or immune cell-deficient mice [107, 110, 152]. Although using TCRβ KO mouse has some benefits in our experimental setting, this strain provides incomplete T cell immunity. Therefore, this imperfect condition of host mice may cause the conversion of

transferred eTfr cells, either non-Treg cell (loss of Foxp3 expression) or other T helper subsets. This might be another reason why we could not gain consistent results in both experimental settings. Therefore, use of alternative mouse model for the *in vivo* experiment might be necessary to address functional aspects of eTfr cell *in vivo*. There are two different conditional depletion of $Foxp3^+$ Treg cell mice [162, 163]. Utilizing this mice, we can focus on the function of transferred cells while minimalizing the inadequate T cell immune responses.

1.5. Conclusion

Overall, our current study demonstrated a crucial role for Tfh cells in auto-reactive B cell responses in BXD2 mice and from these findings proposed a possible alternative immune cell-based therapeutic approach for autoimmune disease by using CXCR5-transduced Treg cells. *In vitro*, the CXCR5-transduced Treg cells behaved as hoped with both chemoattractant and immunosuppressive properties. We still feel that with continued effort in enhancing the transfer procedures that the therapeutic use of CXCR5-transduced Treg cells will be validated *in vivo* with the BXD2 mice.

2. Future Directions

2.1. Investigate the role of Th1 cells/IFN-γ in the development of autoimmune diseases

Our present study successfully showed the roles of Tfh cells and IL-21 in the BXD2 mouse model of autoimmunity. An increase of Th1 cells, instead of Th17 cells was also observed. The role of IFN-γ in the development of autoimmune disease has also been

reported [126, 157]. Therefore, IFN-γ, secreted by Th1 cells may have a role in autoimmune disease onset. However, we could not explain the mechanism behind the increase of Th1 cells in the BXD2 mice. Therefore, we propose in future investigations to determine the role of Th1 cell responses in the generation of auto-reactive GC responses in BXD2 mice. We will investigate whether excessive IFN-γ signaling affects in auto-reactive GC responses in BXD2 mice. By blocking IFN-γ signaling, we will also address whether this blocking will relieve the autoimmune phenotypes in BXD2 mice.

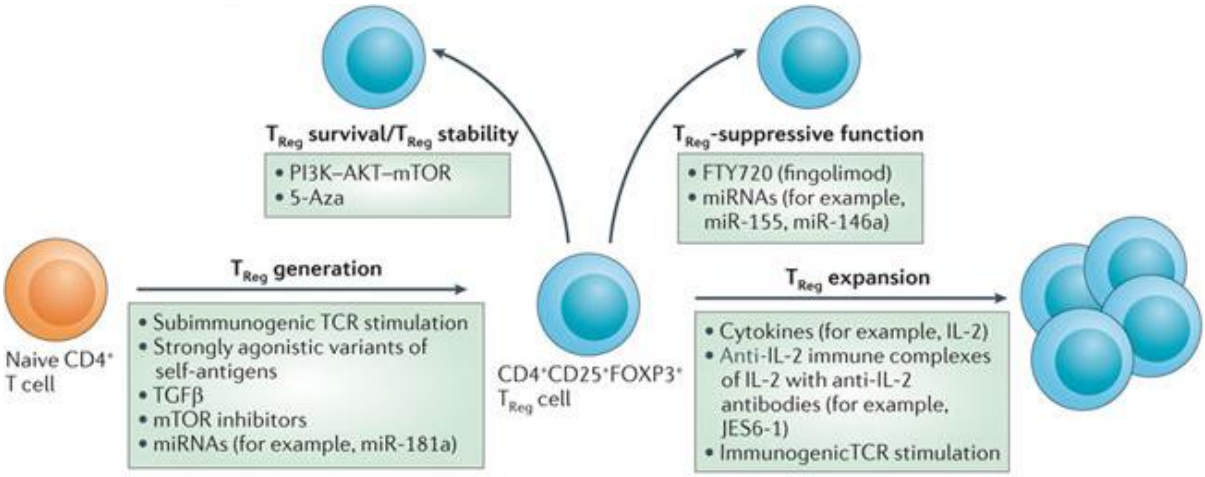
2.2. The ways to improve the quantity and quality of eTfr cells

Multiple studies strongly supported the idea that the application of Treg cells as an immunosuppressor in many autoimmune diseases and accordingly we successfully demonstrated the function of eTfr cells *in vitro*. Nevertheless, we did not show the function of eTfr cells *in vivo*. Since our current experimental design is not optimized, we will first address this problem. Future studies will need to test different conditions of adoptive transfer; for example, increase the ratio of RV-transduced Treg cells (naïve T:RV-Treg = 10:1 to 5:1 or higher) in adoptive transfer experiments.

In adoptive transfer experiments, the number and the quality of transferred cells are critical for the outcome, and these issues will likely need to be addressed in future studies. A recent paper demonstrated that adoptive transfer of Tfr cells inhibited Tfh cells and suppressed GC development and autoantibody production in an animal autoimmune disease model [119]. To get a sufficient number of Tfr cells, they isolated Tfr cells from IL-21-deficient BXD2 mice, which increased the frequency of Tfr cells. However, there still remains the problem of obtaining enough number of Tfr cells for human therapeutics.

Expression of Foxp3 in T cells is critical for their suppressor activity [17]. Additionally, maintaining expression of Foxp3 is essential for the continued suppressive quality of Treg cells. In addition, IL-2 signaling is also involved in Treg cell expansion and homeostasis [164]. Thus, there are many ways to manipulate the number and quality of Treg cells for future studies (Figure 31).

Figure 31. General strategies for the manipulation of Treg cells in autoimmunity.



Adapted with permission from von Boehmer H and Daniel C, *Therapeutic opportunities for manipulating T(Reg) cells in autoimmunity and cancer.* Nature reviews. Drug discovery, 2013. **12**(1): p. 51-63.

Either *ex vivo* or *in vivo* expansion of eTfr cells are shown potential ways to increase the yield of eTfr cells. When we expanded the eTfr cells *ex vivo*, we used a relatively low-dose mouse IL-2 (50 U/ml). However, other studies demonstrated that a high dose of IL-2 can promote the proliferation of Treg cells *ex vivo* [18, 165-167]. Moreover, the administration of IL-2 together with an IL-2-specific monoclonal antibody (JES6-1) leads to rapid expansion of $Foxp3^+$ Treg cells *in vivo* [164, 168, 169]. Since IL-2 inhibits Th17 differentiation [170], this may prevent transferred eTfr cells from conversion to other T helper subsets.

Increase the eTfr cell survival and stability may be another way to improve either quantity or quality of eTfr cells. Treatment of PI3K-AKT-mTOR inhibitors or DNA methyltransferase 1 inhibitors such as 5-aza-2-deoxycytidine (5-Aza) enhance the Treg cell survival and stability [18, 164, 171, 172]. Alternatively, by administration of fingolimod (FTY720) or by targeting miRNAs such as miR-155 and miR-146a, the suppressive function of Treg cells can be augmented [30, 164, 173-176].

Several studies demonstrated that antigen-specific Treg cells are more efficient in inhibiting pathogenic immune responses compared to polyclonal Treg cells in many disease model systems [177]. Therefore, induction of strong agonistic TCR signals, such as self-antigen or immunogen, under subimmunogenic conditions are required to generate stable Treg cells [164]. Since subimmunogenic conditions may not activate the pathway involving PI3K-AKT-mTOR, this enables cells to induce stable Foxp3 expression [164]. Instead, generating genetically targeted Treg cells using antigen-specific TCR transgenic Treg cells or chimeric antigen receptor (CAR) may enhance the suppressive activity of Treg cells. CAR^+

cells can generate the desired numbers of cells with targeted specificity without the need co-receptor engagement or HLA restriction.

Alternatively, pTreg cells can be utilized, instead of nTreg cells, for generating eTfr cells. Unlike thymus-derived nTreg cells, pTreg cells can be differentiated from conventional $CD4^+$ T cells under variable stimulatory conditions in the periphery, and have a distinct suppressive mechanism compared to the nTreg cells [178]. In addition, pTreg cells can be easily differentiated from naïve $CD4^+$ T cells by IL-2 and TGF-β stimulation. Since pTreg cells are being used to control autoimmunity in various animal models of autoantibody-mediated autoimmune diseases [179], albeit Tfr cells are originated from nTreg cells [24, 25], we may utilize pTreg cells for generating eTfr cells. Moreover, since a greater number of naïve $CD4^+$ T cells is available than nTreg cells, generating a large number of eTfr cells from pTreg may be easier than from nTreg cells.

2.3. Regulation of IL-21/IL-21 signals will provide the synergistic effect on eTfr cell-based immunotherapy

IL-21, a major cytokine of Tfh cells, has a direct role in GC responses and is associated with autoimmune disease development [141]. IL-21 has a suppressive role in Tfr cell differentiation and function [119]. Moreover, many on-going autoimmune disease clinical trials are based on blocking IL-21 and its signal pathway [141]. Therefore, if eTfr cell based immunotherapy were perfected, combining two different treatments, eTfr cell transfer and blocking IL-21/IL-21 signaling should have a synergistic effect on alleviating autoantibody-mediated autoimmune diseases.

CHAPTER 5

Materials and Methods

This chapter is based on and reproduced from the following journal article. As per PLoS policy, no permission is required to reprint: Kim YU, Lim H, Jung HE, Wetsel RA, and Chung Y (2015) Regulation of autoimmune germinal center reactions in lupus-prone BXD2 mice by follicular helper T cells. PLoS ONE 10(3): e0120294. doi:10.137/journal.pone.012294

1. Mice

C57BL/6, Foxp3-IRES-mRFP (FIR), B6SJL (CD45.1), *Tcrb*$^{-/-}$ (TCRβ KO), and BXD2 mice were purchased from Jackson Laboratory. All mice were maintained in the specific pathogen-free facilities at the vivarium of the Institute of Molecular Medicine, the University of Texas Health Science Center at Houston. All animal experiments were performed using protocols approved by Institutional Animal Care and Use Committee of the University of Texas at Houston.

2. Cell isolation and culture

Total B cells and $CD4^+$ T cells were obtained using anti-CD45R and anti-CD4 MACS columns (Miltenyi Biotech). $B220^+GL7^-IgD^+$ Naïve B cells, and $CD4^+CD25^-CD44^-CD62L^+$ naïve T cells from mouse spleen and peripheral lymph nodes were sorted by the FACSAria II (BD Biosciences). Treg cells from FIR mice were obtained using Treg isolation kits and stimulated Treg cells used Treg expansion kits (all from Miltenyi Biotech), according to the manufacturer's protocols with a small modification (50 U/ml of mIL-2, instead of 1000 U/ml).

Primary T and B cells were cultured in RPMI 1640 medium (Lonza) supplemented with 10% FBS, 55 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 units penicillin-streptomycin (all from Gibco), and 10 μg/ml Gentamicin (Sigma-Aldrich). 293T cells were cultured in DMEM medium (Lonza) supplemented with 10% FBS 4.5g/l glucose, 2mM L-glutamine, and 100 units penicillin-streptomycin.

For *in vitro* Th17 cell differentiation, sorted $CD4^+CD25^-CD44^-CD62L^+$ naïve T cells were stimulated for 5 days with plate-bound of anti-CD3(1 μg/ml) and anti-CD28 (1 μg/ml), in the presence of 2 ng/ml recombinant human TGF-β, 10 ng/ml recombinant mouse IL-6 (all purchased from Peprotech), 40 ng/ml recombinant mouse IL-23 (R&D systems), 10 μg/ml anti-mouse IFN-γ (clone XMG 1.2, BioXcell) and 10 μg/ml anti-mouse IL-4 (clone 11B11, BioXcell).

3. CXCR5 cloning and retroviral transduction

Mouse CXCR5 cDNA PCR fragment was prepared using iProof High-Fidelity DNA polymerase (Biorad), with CXCR5 cloning primer sets (Forward 5'-ATCGAGATCTATGAACTACCCACTAACCCTGGAC-3' and Reverse 5'-ATCGCTCGAGCTAGAAGGTGGTGAGGGAAGTAGC-3'). After *Bgl II* and *Xho I* (all from New England Biolabs) enzyme digestion, the mCXCR5 fragment was ligated into the unique *BglII* and *XhoI* site of RV-GFP vector using T4 ligase (Invitrogen). 10 μg of pCL-Eco packaging vector with 10 ug of control or CXCR5-RV-GFP vector were co-transfected into the 293T cells using calcium phosphate/chloroquine (100 μM, Sigma) method. 24 hours after Treg cells stimulation and retroviral transfection, cells were infected with retroviruses CXCR5-RV-GFP or control empty vector with 8 ug/ml of polybrene (Sigma). 4 days after the infection, GFP and RFP double positive cells were FACS sorted for further approaches.

4. *In vitro* Treg suppression assay

Cell proliferation dye eFluor670 (eBioscience, 5 μM) labeled responder $CD4^+$ T cells (Tresp, 1×10^5) from congenic B6SJL mice were co-cultured with indicated ratio of

GFP^+RFP^+ FACS sorted retroviral transduced Treg cells in a U-bottom 96-well plate in the presence of 0.5 μg/ml of anti-CD3 and irradiated (3000 rad) T cell-depleted splenocytes (1×10^5) for 3 days. Proliferation of the T_{resp} cells was measured by eFluor670 dilution among the $CD4^+CD45.1^+$ population by flow cytometry.

5. *In vitro* cell migration assay

3×10^5 GFP^+RFP^+ sorted retroviral transduced Treg cells were rested at 37 °C for 2 hours in complete RPMI media before a migration assay. Cells were placed in the upper chamber (Corning, Polycarbonate, 6.5mm diameter, 5 μm pore size) containing 100 μl of complete RPMI media with the lower chamber containing 600 μl complete RPMI media with various concentrations of CXCL13 (PeproTech). After 4 hours of incubation, cells from the lower well were collected and examined with a FACS Calibur for 1 minute at 60 μl/min of flow rate in triplicates. Migration index was calculated as follows: ((number of migrated cells/number of input cells)*100)

6. Adoptive transfer studies and keyhole limpet hemocyanin immunization

B6SJL mice were subcutaneously (s.c.) immunized at the tail base with 100 μl of 4-Hydroxy-3-nitrophenylacetyl hapten-conjugated keyhole limpet hemocyanin (NP-KLH, 0.5 mg/ml, Biosearch Technologies) emulsified in CFA (Sigma). Seven to ten days later, $CD4^+PD\text{-}1^+CXCR5^+$ Tfh cell population from draining lymph nodes of immunized mice were isolated by flow cytometry. 5×10^4 of Tfh cells from B6SJL and 2.5×10^4 of control or CXCR5-transduced Treg cells were adoptively cotransferred into *Tcrb-/-* mice. Or $3\sim5 \times 10^6$ of naïve $CD4^+$ T cells from B6SJL mice were isolated by flow cytometry and 5×10^5 of

control or CXCR5-transduced Treg cells were adoptively cotransferred into *Tcrb-/-* mice. The next day, the recipient mice were s.c. immunized with NP-KLH in CFA. Seven to ten days later, lymphoid cells from draining lymph nodes of recipient mice were collected and analyzed for further studies.

7. Flow cytometry

For cell phenotype analysis, lymphoid cells isolated from mouse spleens, peripheral lymph nodes, and Peyer's patches were obtained and stained with PerCp-Cy5.5-conjugated anti-CD4 (clone GK1.5, BioLegend), APC-conjugated anti-CD45R/B220 (clone RA3-6B2, BioLegend), Pacific blue-conjugated anti-CD45.1 (clone A20, BioLegend) or CD45.2 (clone 104, BioLegend), PE-conjugated anti-CD138 (clone 281-2, BioLegend), Alexa488 anti-GL7 (clone GL7, BD Pharmingen, San Jose, CA), PE-conjugated anti-CD95 (Fas, clone 15A7, eBioscience), FITC-conjugated anti-CD279 (PD-1, clone J43, eBioscience), Brilliant-Violet-421-conjugated anti-CD279 (clone 29F.1A12, BioLegend), PE-conjugated anti-CD184 (CXCR4, clone L276F12, BioLegend), Biotin-conjugated anti-CD185 (CXCR5, clone L138D7, BioLegend), PE or APC-conjugated Streptavidin (BioLegend), Alexa647-conjugated CD196 (CCR6, clone 140706, BD Pharmingen).

For intracellular cytokine staining, lymphoid cells isolated from mouse spleens, peripheral lymph nodes, and Peyer's patches were stimulated with PMA (100 ng/ml, Sigma-Aldrich) and ionomycin (1 μM, Sigma-Aldrich) in the presence of Brefeldin A and Monensin (all from eBioscience) for 4 hours, followed by staining with PerCp-Cy5.5-conjugated anti-CD4. The cells were then resuspended in permeabilization buffer (eBioscience) for 30 min at 4°C, followed by staining with PE-conjugated anti-IL-17A (clone TC11-18H10.1,

BioLegend), Alexa488-conjugated anti-IFN-γ (clone XMG1.2, eBioscience). For IL-21 staining, after permeabilization, cells were stained with Alexa488-conjugated anti-IL-17A (clone TC11-18H10.1, BioLegend), recombinant mouse IL-21R-Fc Chimera (R&D Systems), followed by APC or PE-conjugated human Fc IgG (R&D Systems).

For $Foxp3^+$ cell detection, after surface staining, cells were incubated in Foxp3 staining buffer (eBioscience) for 30 min, followed by Alexa488-conjugated anti-Foxp3 (clone 150D/E4, eBioscienece). The stained cells were analyzed by FACSAria II flow cytometer (BD Bioscience, San Jose, CA), and the data were analyzed using FlowJo software (TreeStar, Ashland, OR).

8. Immunohistochemical Analysis

Mouse spleens or draining lymph nodes were fixed in 4% paraformaldehyde embedded in Tissue-Tek O.C.T compound (Sakura Finetek, Torrance, CA), and cut into 6 μm sections. The slides were incubated for 30 min with Image-iT FX Signal Enhancer (Life Technologies). After washing, slides were incubated with BlockAid Blocking Solution (Life Technologies) for 30 min, and then stained with Biotinylated Peanut Agglutinin (PNA, Vector Laboratories, Burlingame, CA), and hamster anti-mouse CD3e (clone 145-2C11, BioXcell). The slides were further incubated with Pacific Blue-conjugated anti-IgD (clone, 11-26c.2a, BioLegend), DyLight649-conjugated anti-Hamster IgG (clone Poly4055, BioLegend), and DyLight594-conjugated Streptavidin (Pierce Biotechnology). The slides were mounted with Fluoromount-G (SouthernBiotech). Images were acquired with a Nikon TE2000E fluorescence microscope equipped with a Photometrics Coolsnap HQ2 camera.

9. *In vitro* co-culture assay

$B220^{+}GL7^{-}IgD^{+}$ Naïve B cells, $CD4^{+}CXCR5^{+}$ and $CD4^{+}CCR6^{+}$ T cells from BXD2 mouse spleen were sorted by the FACSAria II (BD Biosciences). For *in vitro* antibody production assay, sorted 1×10^5 Naïve B cells and diverse ratio of $CXCR5^{+}$ or $CCR6^{+}$ CD4 T cells or *in vitro* differentiated Th17 cells were co-cultured in the presence of 2 μg/ml soluble anti-CD3e (clone 145-2C11, BioXcell) and anti-IgM (AffiniPure $F(ab')_2$ Fragment Goat anti-IgM, μ chain specific, Jackson ImmunoResearch) for 7 days.

For cytokine blockade, 10 μg/ml of recombinant human Fc-G1 (Human Fc-G1, BioXCell), anti-IL-17A (clone 50104, R&D Systems) or recombinant mouse IL-21R-Fc Chimera (R&D Systems) was added into the B and T cell co-culture every other day. Seven days later, the levels of murine IgG in the culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA).

For B cell proliferation assay, FACS-sorted 1×10^5 naïve B cells labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester, Invitrogen) were co-cultured with 5×10^4 $CXCR5^{+}$ or $CCR6^{+}$ CD4 T cells as described above. The percent of divided cells was determined by flow cytometry.

10. ELISA

To measure the levels of antibodies to dsDNA and histone in circulation, serum was collected from the wild type or BXD2 mice at various time points and was measured by ELISA as described previously [118]. Briefly, ELISA plates (Greiner Bio-one or Nunc) were coated with 0.01% of poly-L-lysine (Sigma-Aldrich) for 1 hour prior to coating with 5 μg/ml

of calf thymus dsDNA (Sigma-Aldrich) and 5 μg/ml histone (from calf thymus, Sigma-Aldrich) overnight at 37°C. The plates were blocked for 2 hours with PBS-Tween 20 (PBST) plus 3% milk. Sera were diluted in PBS with 1% of FBS, were transferred to the plates and were incubated for 2 hours at room temperature. Then the assay was performed with HRP-conjugated total IgG detection antibody.

To measure the levels of IgG produced by B cells *in vitro*, total murine IgG was quantified in culture supernatants with a total IgG capture antibody (Donkey anti-mouse IgG (H+L), Jackson ImmunoResearch) and HRP-conjugated total IgG detection antibody.

NP-specific IgM and IgG antibodies in sera obtained from NP-KLH immunized mice were measured by ELISA. ELISA plates were coated with 5 μg/ml of NP4-BSA (for high affinity) or NP32-BSA (for general affinity, all from Biosearch Technologies). The plates were blocked for 2 hours in PBS with 10 % FBS. Serum samples were added in a three-fold serial dilution in PBS with 1% FBS, followed by HRP-conjugated IgM or total IgG detection antibody (Goat anti-mouse IgM or IgG, SouthernBiotech).

11. Quantitative real-time RT-PCR

Total RNA was extracted from splenocytes (1×10^6 cells) or retrovirus transduced cells (3×10^5 cells) with TRIzol (Invitrogen) and reverse transcribed using amfiRivert reverse transcriptase (GenDepot) according to the manufacturer's protocol. Gene expression was measured with iTaq-SYBR Green Supermix (Bio-Rad Laboratories) and the ABI-PRISM 7900 detection system (Applied Biosystems). Data were normalized to expression of the β-actin gene. The primer pairs used in quantitative RT-PCR are described in Table 5.

12. Statistical analysis

Data were analyzed with GraphPad Prism 5 (GraphPad). Statistics was calculated with the two-tailed Student's t-test. For correlative analyses among the percent of $GL7^{+}Fas^{+}$ germinal center B cells, the percent of T helper cell subsets, the levels of dsDNA specific autoantibodies, linear-regression analysis was performed. p- values below 0.05 were considered statistically significant.

Table 5. List of real-time PCR primers

Gene	5’ primer	3’ primer
Ifng	GATGCATTCATGAGTATTGCCAAGT	GTGGACCACTCGGATGAGCTC
Il17	CTCCAGAAGGCCCTCAGACTAC	GGGTCTTCATTGCGGTGG
Il6	TATGAAGTTCCTCTCTGCAAGAGA	TAGGGAAGGCCGTGGTT
Il4	AGATCACGGCATTTTGAACG	TTTGGCACATCCATCTCCG
Il10	ATAACTGCACCCACTTCCCAGTC	CCCAAGTAACCCTTAAAGTCCTGC
Il21	TCATCATTGACCTCGTGGCCC	ATCGTACTTCTCCACTTGCAATCCC
Tbx21	CAACAACCCCTTTGCCAAAG	TCCCCCAAGCAGTTGACAGT
Rorc	CCGCTGAGAGGGCTTCAC	TGCAGGAGTAGGCCACATTACA
Bcl6	AGGCCTCCTTCCGCTACAAG	CAAATGTTACAGCGATAGGGTTTCT
Foxp3	GGCCCTTCTCCAGGACAGA	GCTGATCATGGCTGGGTTGT
Icos	CGGCAGTCAACACAAACAA	TCAGGGGAACTAGTCCATGC
Pdcd1	CCGCCTTCTGTAATGGTTTGA	GGGCAGCTGTATGATCTGGAA
Ctla4	ACTCATGTACCCACCGCCTA	GGGCATGGTTCTGGATCAAT
Prdm1	ACATAGTGAACGACCACCCCTG	CTTACCACGCCAATAACCTCTTTG
Cxcr5	ACTCCTTACCACAGTGCACCT	GGAAACGGGAGGTGAACCA
Ccr6	CCTCACATTCTTAGGACTGGAGC	GGCAATCAGAGCTCTCGGA
Ascl2	CGCTGCCCAGACTCATGCCC	GCTTTACGCGGTTGCGCTCG
Id3	TGCTACGAGGCGGTGTGCTG	AGTGAGCTCAGCTGTCTGGATCGG
Actb	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG

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