Understanding Pathogenic and Physiological T Follicular Helper Cell Formation

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Dedicated to my mother

Statement of Originality

This thesis presents research undertaken in the Humoral Immunity and Autoimmunity Laboratory, Department of Pathogens and Immunity at the John Curtin School of Medical Research, Australian National University, Canberra, Australia.

The data presented in this thesis is my original work, performed under the supervision of Prof. Carola Vinuesa, with all contributions from others clearly indicated in the Preface and Figure Legend.

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Preface

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Chapter 3: Contributions from Dimitra Zotos, who performed the experiment on NP-KLH, Jennifer L. Marshall, who carried out the staining and scoring on spleen sections of *S. enterica* infection, Louis M. Tsai, who performed the experiment on HEL-OVA, Tyani D. Chan and Dr. Robert Brink, who carried out the experiment on *Il-21r*$^{-/-}$ mice, Roybel R. Ramiscal, who performed ELISA and bacterial counts on chimeric mice of *S. enterica* infection; and Dr. Robert Rigby, with whom I worked with to perform the work on the chimeric mice lacking Bcl-6 expressing T cells.

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List of Abbreviations

7AAD	7-aminoactinomycin D
ABTS	2'2-Azinobis [3-ethylbenzthiazoline sulfonic acid] diammonium salt
AID	activated-induced cytidine deaminase
AIH	autoimmune hepatitis
AITL	Angioimmunoblastic T-cell lymphoma
AML	acute myeloid leukemia
AMPK	AMP-activated protein kinase
ANA	antinuclear autoantibody
AP-1	activator protein-1
APC	antigen-presenting cell; allophycocyanin
ATF2	activating transcription factor 2
BAFF	B-cell activating factor of the TNF family
BATF	basic leucine zipper transcription factor ATF-like
BCG	Bacillus Calmette-Guerin
B-CLL	B cell-type chronic lymphocytic leukemia
Bcl	B-cell lymphoma
BCR	B cell receptor
Blimp-1	B lymphocyte induced maturation protein 1
BLyS	B lymphocyte stimulator
BTLA	B and T lymphocyte attenuator
CB	centroblast
CC	centrocyte
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD40L	CD40 ligand
cDNA	complimentary deoxyribonucleic acid
CFU	colony-forming unit
CM	central memory
CSF	colony stimulating factor
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
DC	dendritic cell
dsDNA	double-stranded DNA

EAE	Experimental Autoimmune Encephalomyelitis
EBI2	Epstein-Barr virus-induced gene 2
EFPB	extrafollicular plasmablast
ENU	N-ethyl-N-nitrosourea
ETS	E-twenty six
FDA	Food and Drug Administration
FDC	follicular dendritic cell
FoxP3	forkhead box P3
FSC	forward side scatter
GAS	IFN-γ-activated site
GC	germinal center
HEL	hen egg lysozyme
HIGM1	X-linked hyper-IgM syndrome
IFN	Interferon
IFN-γ	Interferon-gamma
IFN-γR	Interferon-gamma receptor
H & E	hematoxylin and eosin
ICOS	inducible T cell costimulator
Ig	Immunoglobulin
IL	Interleukin
iNOS	inducible nitric oxide synthase
IRF	Interferon regulatory factor
JAK	Janus kinase
JDM	Juvenile dermatomyositis
KLH	keyhole limpet haemocyanin
LCMV	lymphocytic choriomeningitis virus
mDC	myeloid dendritic cell
mAb	monoclonal antibody
Mac	macrophages
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
Mo	monocytes
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
NFAT	nuclear factor of activated T cells

NFκB	nuclear factor kappa B
NK	Natural killer
NP	(4-hydroxy-3-nitrophenyl) acetyl
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
P-body	processing body
PBS	phosphate-buffered saline
PBMC	Peripheral mononuclear cells
PCR	polymerase chain reaction
PD-1	programmed cell death-1
pDC	plasmacytoid dendritic cell
PI3K	phosphotidylinositol 3 kinase
PRR	pattern recognition receptors
PSGL-1	P-selectin glycoprotein ligand 1
RAG	recombination activation genes
rIFN-γ	recombinant Interferon-gamma
RNP	ribonuclear proteins
RORγt	retinoic-acid-receptor-related-orphan-receptor-γ-T
SAP	signaling lymphocytic activation molecule (SLAM)-associated protein
SHM	somatic hypermutation
SLE	systemic lupus erythematosus
SLEDAI	SLE Disease Activity Index
SLEC	short-lived effector
SOCS	suppressor of cytokine signaling
SRBC	sheep red blood cell
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Tfh	T follicular helper
Tfr	T follicular regulator
Th	T helper cell
TLR	Toll like receptor
TNF-α	Tumor necrosis factor-α
Treg	T regulatory
TYK	Tyrosine kinase

Abstract

T follicular helper (Tfh) cells localize to follicles where they provide growth and selection signals to mutated germinal center (GC) B cells, thus promoting their differentiation into high affinity long-lived plasma cells and memory B cells. T-dependent B cell differentiation also occurs extrafollicularly, giving rise to unmutated plasma cells that are important for early protection against microbial infections. Bcl-6 expression in T cells has been shown to be essential for the formation of Tfh cells and GC B cells but little is known about its requirement in physiological extrafollicular antibody responses. We use several mouse models in which extrafollicular plasma cells can be unequivocally distinguished from those of GC origin, combined with antigen-specific T and B cells, to show that the absence of T cell-expressed Bcl-6 significantly reduces T-dependent extrafollicular antibody responses. Bcl-6^{+} T cells appear at the T:B border soon after T cell priming and prior to GC formation, and these cells express low amounts of PD-1. Their appearance precedes that of Bcl-6^{+}PD-1^{hi} T cells, which are found within GC. IL-21 acts early to promote both follicular and extrafollicular antibody responses. In conclusion, $Bcl6^{+}$ T cells are necessary at B cell priming in order to form extrafollicular antibody responses and these pre-GC Tfh cells can be distinguished phenotypically from GC Tfh cells.

Overactivity of the GC pathway due to accumulation of Tfh cells causes autoimmunity, underscoring the need to understand the factors that control Tfh homeostasis. Here we have identified posttranscriptional repression of interferon-γ (*Ifng*) mRNA as a novel mechanism to limit Tfh cell formation. Using the *sanroque* lupus model, we have shown that decreased *Ifng* mRNA decay caused excessive IFN-γ signaling in T cells and led to accumulation of Tfh cells, spontaneous GC,

autoantibody formation and nephritis. Unlike ICOS and T-bet deficiency that failed to rescue several autoimmune manifestations, interferon-γ receptor (IFN-γR) deficiency completely prevented lupus development. IFN-γ blockade after disease onset reduced Tfh cells and autoantibodies, demonstrating that IFN-γ overproduction was required to sustain lupus associated pathology. Increased IFN-γR signaling caused Bcl-6 overexpression in Tfh cells and their precursors. This novel link between IFN-γ and aberrant Tfh formation provides a rationale for IFN-γ blockade in lupus patients with an overactive Tfh cell-associated pathway.

Chapter 1

Introduction

Chapter 1 – Introduction

Preamble

Vaccination is a preventive strategy used to induce a protective immunological response that allows rapid effector responses upon re-exposure to a pathogen, resulting in marked reductions in morbidity and mortality. Most approved human vaccines currently used are largely dependent on stimulating humoral immune processes. Modern day vaccination strategy is based on the capacity of antibody to neutralize protein antigens derived from infective pathogens, or the generation of protective antigen-specific B cell memory for long term antibody responses[1, 2]. Less attention has been given to T follicular helper (Tfh) cells which are essential in helping B cells to generate both antibody responses and immunological memory, a critical component of the protective immunity conferred by most human vaccines. Previous studies have demonstrated that the loss or dysregulation of Tfh cells dramatically alters the quality of antibody, leading to profound impairment of the immune response to infection. In humans, immunodeficiency disorders caused by genetic defects in CD40 ligand (CD40L), SH2D1A/SAP and inducible T cell costimulator (ICOS) lead to impaired Tfh production and defective memory B cell responses[3]. Therefore, understanding the cellular and molecular processes that regulate Tfh cell homeostasis is critical in the generation of optimal B cell responses, and in facilitating superior vaccination design and strategy.

Conversely, Tfh cells can be a fundamental driver of autoimmunity when aberrant accumulation occurs due to loss of regulatory mechanisms. This excess proliferation

in itself, or in conjunction with qualitative dysfunction, can lead to Tfh cell hyperactivity and accumulation. Recent growing evidence suggests that overactivity of Tfh cells can disrupt the GC milieu, leading to the production of pathogenic autoantibodies and subsequent autoimmune disease[4-6]. Cytokines appear to be central to this process because IFN-γ, TNF-α, IL-18, IL-21, IL-10, IL-17, and IL-6 have all been implicated in the dysregulation of Tfh cells and/or the pathogenesis of murine and human systemic lupus erythematosus (SLE)[7]. Therapies that specifically target dysregulated Tfh cell responses or cytokine signaling are urgently needed. Monoclonal antibodies directed against these cytokines are being trialled in the treatment of SLE even though the exact mechanism is unclear[8], it is imperative to understand their underlying mechanisms of action and therefore rationalise their use.

This thesis investigates two important aspects of Tfh cell biology that have fundamental implications in our understanding of Tfh cell function and regulation, and may in turn provide insights into how to better combat infection. It investigates priming of B cells by Tfh cells prior to differentiation to short-lived plasma cells or GC B cells. This thesis also investigates a novel molecular pathway to lupus development, linking excessive Interferon-gamma (IFN-γ) production with Tfh cell accumulation and autoantibody formation, thus providing a potentially potent framework for the prevention of Tfh and/or T helper type 1 (Th1)-mediated autoimmune disease.

Thymus-dependent humoral response

Our immune system is divided into innate and adaptive immune systems. Innate immune responses are effective in providing rapid defence and a relatively non-specific response that target a very broad array of pathogens. This response is triggered when microorganisms are recognized by pattern recognition receptors (PRRs) on innate cells such as macrophages, neutrophils and dendritic cells (DC), which in turn elicit inflammatory and antimicrobial responses, and activate the adaptive immune response[9, 10]. The adaptive immune response is mediated by T cells and B cells with polyclonal antigen receptors of narrow specificities[11]. Antigens are substances, most frequently microbes that can trigger an immune response. Antigen-specific receptor recognition by T and B cells allows clonal selection of a specific population of lymphocytes that expresses an antigen receptor of a single specificity, in response to cognate antigen[12].

Upon infection or immunisation, antigen-presenting cells (APC) including DC and mature naive B cells, encounter antigen at sites of infection and in the blood, respectively. The antigen is then transported to the secondary lymphoid organs (lymph nodes, spleen, Peyer's patch, tonsils) where it is presented in the form of complexes of peptide and major histocompatibility complex (MHC) II or MHC I to T helper $CD4^+$ and cytotoxic $CD8^+$ T cells, respectively. PRRs recognition on DC also activates DC to produce cytokines and express cell-surface costimulatory molecules like CD80/86 and ICOSL. Subsequently, antigen-specific naïve $CD4^+$ T cells, that recognise antigenic peptides by their T-cell receptor and receive CD28 costimulatory signals from the ligands CD80/CD86, become activated to differentiate into one of several effector cell lineages depending on the type of infecting pathogens[9, 12-15]. The

helper T cells will then release cytokines, which influence the activity of many cell types including the APC that activated it[16, 17]. In response to priming stimuli described below, a small subset of naive T helper cells will clonally expand and proliferate to form Tfh effector cells that will migrate to the T-B border.

In the case of B cells, conventional B cells express unique B cell receptors (BCRs) that recognise only one particular antigen in its naive form. B cells take up the antigen through their BCR, process it and then present antigen complexed to MHC II molecules. In the case of protein antigens, T cell help is required for full activation and subsequent differentiation. Upon T-B cognate interaction, antigen-specific T cells that have previously been primed on the surface of DCs deliver CD40L and cytokines signals to B cells, which initiate B cell proliferation and differentiation[18, 19]. When an antigen and Pathogen-Associated Molecular Pattern (PAMP) are physically linked in a single particle such as in the Toll like receptor (TLR) ligands lipopolysaccharide or flagellin, B cells can directly bind to these molecules through co-engagement of BCR and PRR without T cell help. This type of antigen is known as a T-independent antigen[20].

B cells encounter antigen on the surface of follicular dendritic cells (FDCs), subcapsular sinus macrophages or lymphoid DC; they can also take up soluble antigen in B cell follicles[21-24]. In response to BCR cross-linking with antigens, they become activated and up-regulate CC-chemokine receptor 7 (CCR7; a receptor for CC-chemokine ligand 19 (CCL19) and CCL21 expressed by stroma cells in the T cell zone) that enables migration to the T-B border[25]. At this location, B cells can interact with cognate T cells that have been previously primed by DCs to form antigen-

specific cell clusters within 1 to 2 days of immunization[26-29]. As a consequence of priming by T cells at the T-B border, B cells upregulate activated-induced cytidine deaminase (AID) that initiates Immunoglobulin (Ig) class switching and is guided by different T cell-derived cytokines[30, 31]. As a consequence of isotype switching, B cells produce different classes of antibodies that activate specific modules of the innate system[32]. For example, IgG activates complement and opsonises pathogens to aid their phagocytosis by macrophages and neutrophils, whereas IgE activates mast cells and basophils[33, 34].

Activated B cells move to the outer follicle and differentiate into one of three alternate fates; short-lived extrafollicular plasmablasts (EFPBs), early memory B cells, or GC B cells, though the molecules and signals that determine these cell fate decisions are not entirely understood[35, 36]. Early memory B cells ($CD73^-$ IgM^+) can circulate in blood; they can form in a manner independent of B-cell lymphoma 6 (Bcl-6), ICOS and IL-21, and their significance for protective immunity is unclear[37, 38]. Both extrafollicular and follicular pathways are important for protective immunity. Extrafollicular plasma cells provide the first wave of rapid antibody production, whilst the GC B cells provide high affinity long-lived memory B cells and memory plasma cells that home to the bone marrow. Both memory B cell types can survive for long periods and together provide rapid and highly efficient antibody in the event of recurrent infection[39].

Notably, entry into follicles to seed GC reactions is only permitted for a small number (~3) of B cell clones that initiate the response[26, 40]. A pre-GC affinity-dependent T cell-mediated selection checkpoint was proposed to determine the GC versus EFPB

fate. Initially, it was thought that the decision would depend on the strength of the initial interaction between BCR and antigen, whereby B cells clones with high BCR affinity or more abundant epitopes favor the extrafollicular pathway, whereas decreasing BCR affinity or antigen density results in the GC pathway and B cell clones with weaker antigen affinity undergo affinity maturation in GCs[41]. Nevertheless, two separate subsequent studies showed that BCR affinity enhanced survival and proliferation rather than GC versus EFPB fate[42, 43]. Indeed, the expansion of IgG^+-switched EFPBs was supported by high antigen affinity: B cell clones that have the highest antigen affinity proliferated more rapidly and probably survived longer independently of IL-21R signaling[43]. Similar observations were made amongst GC B cells[42]. A separate study suggested high affinity B cells would capture more pMHC at the T-B border and thus outcompete lower affinity B cells for T cell help, become activated, proliferate and upregulate FAS, GL-7 and CCR6, and enter the GC. This direct competition for antigen would prevent B cells with relatively lower affinity from entering the GC, thus gradually eliminating these cells from the reaction[44]. Stable and long-lived interactions between B and T cells requiring SLAM-associated protein (SAP) signaling in T cells were shown to be essential to direct B cells to become GC B cells. By contrast, EFPBs were less dependent on SAP[6, 45].

The extrafollicular antibody response

Following immunisation with Thymus-dependent or Thymus-independent antigens, extrafollicular antibody responses occur either at the splenic bridging channels, also known as junction zones, at the boundary between the red pulp and T zones, and at lymph node medullary cords. B cells differentiating along this route upregulate

expression of B lymphocyte induced maturation protein 1 (Blimp-1), CCR7 and orphan G protein-coupled receptor Epstein-Barr virus-induced gene 2 (EBI2) and move to the T-B border[29, 46-48]. Here, antibody-secreting plasmablasts rapidly divide and expand to form extrafollicular foci and differentiate into low affinity unmutated switched (IgG^+, IgA^+ IgE^+) or unswitched (IgM^+) short-lived plasma cells[30, 49, 50]. However, somatic hypermutation has been observed in the extafollicular foci of lupus-prone MRL^{lpr} mice[50]. Although this initial wave of antibody response is destined to last only for few weeks, it is important to neutralize rapidly-dividing pathogens such as viruses[51].

Terminal differentiation of plasmablasts into plasma cells requires the upregulation of Blimp-1 and interferon regulatory factor 4 (IRF4), as well as the engagement with $CD11c^{hi}$ DCs that provides survival/growth signals[49, 52-54]. Blimp-1 induces CXC-chemokine receptor 4 (CXCR4) and represses CXCR5 and CCR7 resulting in the correct localisation of plasma cells[55]. Plasma cells secrete antibody for ~ 3 days, after which the majority undergo apoptosis *in situ*[49, 56].

The Germinal Center (GC) response

An important feature of the immune system's response to infection or immunisation is the generation of high affinity, class-switched antibodies that effectively neutralize protein antigens produced during inoculation. Once antigen activated B cell blasts have received T cell help, they further upregulate EBI2 and downregulate CCR7 in order to move to inter- and outer-follicular region before they can enter the follicle[57]. Those B cells adopting a GC B cell fate up-regulate Bcl-6 and then move to the

center of the follicle and grow as B cell blasts to fill the follicle. B blasts retain high expression of CXCR5 and CXCR4, as consequence of IRF8 and downregulation of EBI2 and CCR7, which in turn represses Blimp-1, thereby inhibiting B cell differentiation into plasmablasts and enabling correct positioning of GC B cells in the follicular dark zone that is located close to the T cell zones[46-48, 58-60]. Within the dark zone, B cell blasts become centroblasts (CBs) with oligoclonal BCR specificities and continue to express AID that drives somatic hypermutation (SHM). Through the process of SHM, random point mutations are introduced into Ig V region genes encoding the BCR. This process introduces stochastic changes in antibody affinity and specificity; as a consequence, some cells increase their affinity for the immunising antigen, whereas others may lose it or become self-reactive[61-63]. Using multiphoton live-imaging studies, GC dynamics are revealed as a bidirectional movement of CBs between dark and light zones, although the movement from light zone to dark zone is less prominent and only for a selected subset of cells[64-66, 68]. CBs that exit cell division become centrocytes (CCs) and move towards the light zone where they capture antigen held on FDCs via their BCR. The amount of antigen gathered has been proposed to be directly proportional to the affinity of their BCRs, leading to differential peptide-MHC density between high and low affinity B cells[67]. Subsequently, GC Tfh cells will select the CCs with highest peptide-MHC II levels, providing them with CD40L and cytokine signals such as IL-21 and IL-4, which are essential for survival, proliferation and differentiation[4, 39, 63, 68]. GC formation is abrogated with CD40L blockade even after the onset of the GC response[69]. CD40L deficiency also causes X-linked hyper-IgM syndrome (HIGM1) in humans and diminished GCs with impaired IgG production in mice[70, 71]. A small number of CCs can re-enter the dark zone for iterative cycles of proliferation and

selection to achieve enrichment in mutations leading to affinity maturation of the BCR[39, 68]. Selected B cells differentiate into either memory B cells or long-lived memory plasma cells that home to the bone marrow, whereas not selected self-reactive or low affinity GC B cells are thought to undergo apoptosis[72, 73].

Which are the T cells that prime B cells?

I have described above how $CD4^+$ T cells proliferate in the T cell zone within 2 days of antigen recognition and priming by DC and how a subset moves to the T-B border where they can interact with B cells. This interaction is key to B cell differentiation into either follicular or extrafollicular pathways, and for isotype switching, via provision of cell surface ligands and cytokine signals[30, 31, 38, 74]. When the work in this thesis commenced, it had become clear that Bcl-6-expressing Tfh cells were essential to initiate and sustain a GC B cell response. However, it was unclear which T cell type primed B cells at the T-B border, initiated Ig isotype switching and induced an extrafollicular antibody response. Since IFN-γ and IL-4 are required for the IgG isotype switching to IgG2a (IgG2c in C57BL/6) and IgG1, respectively[31, 75], it had been suggested that Th1 and Th2 cells are the T cell subsets responsible for priming B cells to become EFPBs. In some autoimmune mouse models such as MRL^{lpr}, $CD4^+$ T cells had been found increased in numbers at the extrafollicular sites and responsible for sustaining (and thus potentially also priming) extrafollicular responses[76]. In MRL^{lpr} mice, which produce autoantibody predominantly from chronic autoreactive extrafollicular plasma cells, $CD4^+$ T cells that localise to extrafollicular sites and provide help to B cells are analogous to Tfh cells with the exception of reduced expression of CXCR5. They downregulate P-selectin glycoprotein ligand 1

(PSGL-1), require ICOS to upregulate CXCR4, and mediate IgG antibody responses via CD40L and IL-21[76]. In non-autoimmune prone mice, Thymus-dependent extrafollicular antibody responses require CD40L/CD40 signaling at the initial sites of T-B cell contact, and this pathway is also ICOS dependent[77, 78].

It had been suggested that "pre-GC Tfh" cells might play roles to initiate both GC and extrafollicular antibody responses[79]. Nevertheless, it remained to be formally tested whether the T cells positioned at the T-B border that prime B cells for physiological extrafollicular antibody responses, are bona-fide Tfh cells had not been tested. One of the aims of my thesis is to clarify this point of contention.

Checkpoints for the regulation of antibody production.

B cell responses require tight regulation to prevent autoimmunity. Pathogenic autoantibodies are mainly high affinity class-switched IgG antibodies and can arise at a high frequency during T cell-dependent responses to foreign antigen. They are mainly the by-products of SHM and antigen-driven selection in GCs[80-85], but they can also be generated extrafollicularly in T cell-dependent and T cell-independent responses[50, 86-88]. Failure to remove self-reactive B cells often results in an increased number of autoreactive plasma cells and memory B cells that can live for many years[5]. These autoreactive B cells can potentially lead to autoimmune diseases with end organ injury. Systemic autoimmune diseases include SLE and rheumatoid arthritis[89-92].

The quality and quantity of antibody is tightly controlled by the integration of diverse signals in each step of B cell development. There are a few important central and peripheral checkpoints in place to eliminate self-reactive GC B cells. It is estimated that up to 55% of emergent early immature human B cells are self-reactive and large numbers of them are removed from the repertoire via receptor editing, clonal deletion, immunological ignorance or anergy during B cell maturation in the bone marrow or at the transition into mature naïve B cells in the periphery[93-100]. The avidity of the BCR for the autoantigen determines the fate of autoreactive B cells, whereby stronger BCR signals invoke deletion or receptor editing, while weaker signals induce anergy[104].

Autoreactive B cells that escape initial tolerance mechanisms will be subjected to further screening for self-reactivity in the periphery. These peripheral checkpoints include deletion and anergy, but not receptor revision[101-103]. Anergic peripheral B cells fail to respond to antigenic or mitogenic stimulus[105, 106], although provision of T cell help can result in their activation and recruitment into an antibody response[107, 108]. In some cases, fully functional autoreactive B cells can be auto-antigen ignorant[109, 110]. In healthy individuals, these autoreactive B cells are recruited into the circulating mature naïve B cell pool and may enter GC; but typically remain quiescent and do not exit GCs as memory B cells[111]. However, in SLE patients, these cells are often the precursors of cells that produce pathogenic antibodies[91, 92, 112]. SLE patients can still produce high titers of self-reactive autoantibodies during clinical remission even in the absence of clinical signs and symptoms, strongly suggesting that these patients fail to maintain B cell tolerance regardless of their disease status[91, 92, 113]. The proapoptotic protein Bim has also been shown to be

important for the maintenance of anergic B cells: loss of Bim can lead to the survival of autoreactive B cells due to reduced requirement for BAFF[125]. Combined deficiency of Bim and FAS also results in early-onset SLE[122] and overexpression of Bcl-XL interferes with the apoptosis of autoreactive B cells[126-128].

In the periphery, when homeostasis is perturbed by a change in the internal environment such as infection, stressed and damaged host tissues, autoreactive B cells can be activated by TLR ligands (both TLR7 and TLR9 ligands) together with BCR in response to autoantigens, even in the absence of T cell help. For example, hypomethylated CpG-containing DNAs derived from apoptotic cells or necrotic debris can form complexes with anti-DNA IgG autoantibodies. These immune complexes can then bind to rheumatoid factor B cells resulting in activation of autoreactive B cells[114-117]. This required B cell-intrinsic expression of MyD88 suggesting that TLR signals direct to B cells are important[114-117]. Signals from the innate system can also help to determine the fate of autoreactive B cells. For example, excessive expression of the receptor for B-cell-activating factor (BAFF) - a key B cell survival factor produced by stromal and myeloid cells - can enable self-reactive B cells to compete for limiting amounts of BAFF and allow them to survive in the follicular and marginal zone niches[118, 119]. Indeed, BAFF overexpression leads to autoimmunity in mice and SLE patients show increased levels of BAFF[120, 121]. Overexpression of genes encoding BCR activating signals such as ETS-family transcription factor SPIB, the B cell co-receptor CD45, and GTPases that can enhance B cell activation, can also lead to overt autoreactive responses[123, 124].

Within GC, mature autoreactive B cells can also arise as a consequence of SHM of BCR during affinity maturation and inappropriate FDC- and Tfh-cell derived signals/selection[5, 122]. Because of limiting follicular niches, self-reactive B cells can also be excluded based on competition for antigen presented by specialised antigen-presenting cells in the follicles including macrophages, FDCs, and B cells themselves[66, 68, 132, 133]. Interactions between B cells and FDC will provide selection signals at the time of antigen encounter. When GC B cells are exposed to soluble self-antigens, they are eliminated by apoptosis *in situ*[69, 134, 135]. There is also growing evidence showing that posttranslational modification of antigens during apoptosis can generate new self-antigens by altering immunologic processing and presentation. These self-antigens may play a role in the initiation of autoimmune disease because they are not encountered during central tolerance processes in the bone marrow[129-131].

Recent advances in *in vivo* imaging technology that allow tracking of individual GC B cells for prolonged periods of time showed that the selection of centrocytes is limited by competition for small numbers of Tfh cells[68]. Only those GC B cells that are successful in forming stable cognate interactions with Tfh cells will be provided with proliferation, survival and differentiation signals through CD40L ligation and cytokines like IL-4 and IL-21[68, 136]. This suggests that tight regulation of Tfh cell numbers is also crucial for T-cell mediated selection. Also, non-antigen-specific and self-reactive GC B cells appear to be kept in check by $CD4^+$ $FoxP3^+$ T follicular regulatory cells (Tfr) and Qa-1 restricted $CD8^+$ T regulatory (Treg) cells which are capable of suppressing Tfh-mediated antibody production[137-142]. Alterations in the number and function of these specialized follicular regulatory cells have been

implicated in autoantibody production: a five-fold increase in Tfh cell numbers in Qa-1 mutant mice (mutation of Qa-1 molecules disrupt the inhibitory interaction between $CD8^+$ Treg cells and Qa-1^+ Tfh) was associated with lupus-like autoimmune disease as a consequence of excess T cell help[138]. The depletion of Tfr also leads to the expansion of non-antigen specific B cells and GC Tfh cells; the latter are sufficient to drive antibody production by anergic B cells[139, 143]. Taken together, multiple intrinsic and extrinsic factors control B cell signaling thresholds, and can increase the likelihood that autoreactive B cells escape tolerance and cause autoimmune disease.

T follicular helper cells

Under the influence of cytokines produced by the innate immune system, and depending on the type of antigen, after priming by DCs, naïve T cells will undergo transcription factor mediated differentiation into distinct effector T helper subsets in order to coordinate the immune response[16, 17, 144]. Each of the T cell subsets has distinct gene expression programs under the control of specific transcription factors, including the expression of signature cytokines, cell surface receptors and homing receptors that facilitate the migration to non-lymphoid sites of inflammation, and regulatory factors that mediate specific effector function[145]. These T cell subsets also have specific roles in immune system. Th1 cells, which produce IFN-γ, are critical to activate macrophages and other cell types to provide protection against intracellular pathogens[146]; Th2 cells via IL-4, IL-13, and IL-5 production control the function of eosinophils, basophils and the mucosal epithelia, are required for the clearance of helminths[147]; whilst IL-17 producing Th17 cells have been known to have important

functions in protection against extracellular bacteria and fungi such as *Staphylococcus aureus* and *Candida albicans*[148-150]. A special cell type, the Treg cells, are responsible for maintaining immune system homeostasis and preventing autoimmunity[151].

This decade has brought about the discovery of a new effector subset, Tfh cells. Tfh cells are regarded as a separate lineage from Th1, Th2, and Th17 cells due to their high expression of Bcl-6, which dictates their selective follicular homing ability and distinct cytokine secretion pattern[136, 152-154]. These cells are specialised in assisting GC B cell affinity maturation through the provision of survival and selection signals in GC. Murine Tfh cells, that reside in the GCs, are characterized by their high expression of CXCR5, programmed cell death-1 (PD-1), ICOS, B and T lymphocyte attenuator (BTLA), CD40L, CXC-chemokine ligand (CXCL13), SAP, IL-21, and decreased expression of CCR7, PSGL-1 and CD127[4, 155-157]. Human Tfh cells share biomarkers with murine Tfh cells and a proportion of human Tfh cells also express CD57[158, 159]. There are also memory-type Tfh cells that reside in the lymph node, which are $CXCR5^+$ $ICOS^{lo}$, $CD69^+$ [160], and recirculating central memory (CM) cells in peripheral blood ($CCR7^+$ $CD45RA^-$ $CD4^+$ TCM) that are $CXCR5^+$ $CXCL13^+$ $ICOS^{hi}$ and secrete IL-10[161, 162]. These memory-type Tfh cells with accelerated antigen-recall ability could regulate B cell responses.

Kinetics of Tfh cell differentiation and formation

Naïve T cells, which are $CCR7^{hi}$ will migrate to T zones in response to CCL21 gradient on the follicular stromal cells. In the T zone, antigen-specific T cells

encounter antigen presented by DCs and are subsequently primed. Within hours of DC priming in conjunction with signals from the T cell receptor (TCR) and costimulatory molecules including CD28 and ICOS, some antigen-specific naïve T cells will upregulate Bcl-6 as early as first cell division, downregulate CCR7 and move to T-B border[48, 163]. Recent studies have shown that the decision by a naïve T cell to polarize into a Bcl-6 expressing Tfh cell or Blimp-1-expressing non-Tfh cell occurs at the time of T cell priming by DC, after the first or second cell division, and precedes T-B interaction[59, 163-165]. This is also supported by studies showing human DCs can induce Bcl-6 expression in T cells *in vitro* under the influence of IL-12[166, 167]. DC-derived instructive signals to commit into a Tfh cell fate were largely dependent on the type of antigen and ICOSL upregulation. It has been shown that targeting peptide to specific receptors (Clec9A) on $CD8^+$ DCs that enhance and prolong presentation on MHC class II promoted Tfh cell development[168]. Early ICOSL-derived signals from DCs also favor Tfh cell formation by inducing Bcl-6 upregulation[164]. B cells have been shown not be required for the initial upregulation of Bcl-6 mRNA/protein, but interaction with B cells was important to sustain – and possibly enhance - Bcl-6 expression, important for the maintenance of Tfh-related gene expression including CXCR5, PD-1 and IL-21[59, 163, 165, 169-171].

Bcl-6 expressing $CD4^+$ T cells will prime B cells within 1 to 3 days of antigen encounter by forming a stable and prolonged interaction with cognate B cells. Co-engagement involving SAP between T and B cells triggers signals that are important to stabilise T-B interactions and to sustain Bcl-6 expression[45, 172]. Interaction with B cells coincides with a second wave of Bcl-6 expression in Tfh cells at around their fifth cell division[59, 165]. Taken together, sustained and high levels of Bcl-6 expression

are required to coordinate a stepwise signaling program that facilitates Tfh cell entry and persistence in GC, resulting in a distinct and stable GC Tfh cell population. At the late phase of the GC response, Tfh cells will gradually downregulate Bcl-6, terminate proliferation and upregulate IL-7 receptor[59]. Although their fate is not completely elucidated, it is possible that some leave as effector or memory Tfh cells[162] and others die by apoptosis.

The role of Bcl-6

B cell lymphoma-6 (Bcl-6) expression by B cells has been shown to be important for the proliferation, differentiation and survival of GC B cells[173, 174]. Bcl-6 deficiency abrogates GCs and affinity maturation[175-177]. Bcl-6 expression in T cells is sufficient to drive the differentiation of Tfh cells, to initiate and sustain GCs and, as we show in this thesis, extrafollicular plasma cells responses[136, 152, 153, 178]. A prominent function of highly and stably expressed Bcl-6 is first to facilitate the initial Tfh and B cell interaction at the T-B border by downregulating CCR7 and upregulating CXCR5, SAP and CXCL13. Bcl-6 expression also directs follicular entry of Tfh cells by further upregulation of CXCR5 that responds to a CXCL13 gradient expressed by stromal FDCs and Tfh cells themselves. Upregulation of CXCR4 and downregulation of EBI2 may also contribute to the trafficking of Tfh cells into the follicle[48, 136, 152, 153, 179-181]. Bcl-6 does not regulate the expression of IL-4 and IL-21[169, 179], rather the opposite: IL-21 produced by T cells themselves promotes and enhances Bcl-6 expression upon T cell activation.

Bcl-6 is a transcriptional repressor: it represses expression of other lineage transcription factors such as T-bet, GATA3, RORγt and Blimp-1, thereby inhibiting the differentiation of other $CD4^+$ effector T cell subsets[3, 136, 152, 153]. Blimp-1 and Bcl-6 have been shown to mutually repress each other; the balance of these two transcription factors will determine whether a primed T cell follows a Tfh or an effector cell fate[3, 152]. Indeed, 20% of virus-specific T cells had fully differentiated and polarized to Bcl-6^+ $CXCR5^+$ Tfh while the remaining cells displayed a Blimp-1^+ $CXCR5^-$ IL-$2R\alpha^+$ non-Tfh effector phenotype by 3 days after lymphocytic choriomeningitis virus (LCMV) infection[164].

Development and maintenance of Tfh cells

Tfh development is a multi-stage process requiring multiple signals from DCs, B cells and Tfh cells themselves. Studies have identified the requirements of DC and B cell-derived ICOSL signals[164, 182-185], phosphotidylinositol 3 kinase[186-188] (PI3K), SAP[6, 45, 172], IRF4[189]; IL-6[153, 185, 190, 191], IL-21[184, 185, 190, 192], IL-12[166, 167], IL-27[193], basic leucine zipper transcription factor ATF-like (BATF[194, 195]), Maf[179] (c-Maf in mice), the strength of TCR binding[196, 197], CD80 expression on B cells[198], TCR avidity of antigen[199] and Type 1 IFNs[191] for development and optimal function of Tfh cells. Conversely, negative regulators such as plasma cells[36] and IL-2 signaling via a constitutively active form of Signal transducer and activator of transcription 5 (STAT5), have been shown to suppress Tfh cell differentiation by up-regulating Blimp1 and repressing CXCR5, c-Maf, Bcl-6, BATF and IL-21[200-202].

ICOS has been shown to promote Tfh differentiation by coupling with the cytoplasmic YFMF motif to induce PI3K activation, probably through the p110δ isoform of PI3K, and these signals are critical for the induction of Tfh cytokines such as IL-21 and IL-4, but not required for Bcl-6 expression, downregulation of CCR7 or Tfh cell entry into the follicles[186, 187]. Interestingly, a research study by Choi et al. showed that ICOS is upstream of Bcl-6 and is critical for the initial upregulation of Bcl-6; sequential ICOS signals are important for Tfh cell differentiation[164]. ICOS can also induce c-Maf, which regulates optimal production of IL-21, in turn regulating the induction and/or expansion of Tfh cells[184, 203]. A study analyzing human Tfh showed that coexpression of BCL-6 and MAF could induce the expression of ICOS, CXCR4 and PD-1[179]. However, it is unclear whether MAF alone can upregulate ICOS. Mice deficient in ICOSL selectively on B cells and treated with anti-ICOSL had a reduced Tfh cell population[164, 185].

Cytokines such as IL-6, IL-12, IL-21 and IL-23 can induce IL-21 expression in human tonsils and in human peripheral and cord blood. However, only IL-12 appears to sustain CXCR5 and ICOS expression[166,167]. IL-6 and IL-21 itself have been shown to induce IL-21 production in mice[185, 204-207]. IL-27 has also been shown to induce IL-21 production via the STAT3 signaling pathway and promote the survival of Tfh cells in mice[193]. Furthermore, ICOS and priming by B cells are required for the induction and optimal production of IL-21 in mice[169, 184, 185, 203]. Kroenke et al. showed that MAF was important for the ability of human Tfh cells to express IL-21[179]. IL-21, a key cytokine from Tfh cells, had long been known to be an important cytokine for B cell differentiation and expansion[208, 209]. It was then shown to be an important regulator for Tfh homeostasis to maintain optimal Tfh cell numbers and formation[184, 185, 192, 205].

Subsequent studies completed the picture by showing that IL-21 also acts within GCs to promote GC B cell survival – which could also have a positive effect in Tfh cell homeostasis, and affinity maturation[192, 210-212]. Differential requirements for IL-21 in Tfh differentiation shown by the different studies are likely to result from differences in the type and dose of infecting agents leading to differential strength and chronicity of TCR signaling[192, 211, 213].

There have also been some conflicting results regarding the ability of IL-21 and IL-6 to work together or independently to induce Tfh development in mice. IL-6 or IL-21 alone, which signal through phosphorylation of STAT3, had been shown to be capable of inducing Bcl-6 expression and contribute to formation of Tfh cells in mice immunized with keyhole limpet haemocyanin (KLH) in CFA[185], but not in some experimental models such as (4-hydroxy-3-nitrophenyl) acetyl (NP)-CGG in alum or LCMV infection[170]. A subsequent study showed that IL-6 combined with IL-21, but not the individual cytokines, could promote Bcl-6 expression through phosphorylation of STAT3 during LCMV infection[190]. Interestingly, a study showed that IL-6 produced by irradiation-resistant cells is important for Bcl-6 upregulation and enhanced Tfh cell response at late but not early stages of LCMV chronic infection[214]. The reasons accounting for all these discrepancies again may be due to differences in the stage of the infection, the type and route of immunization including adjuvant used, the presence of replicating viruses or chronic viral persistence. Overall, these studies have suggested that IL-6 and IL-21 may be functionally partially redundant with respect to the upregulation of Bcl-6 and consequently Tfh cell development. In some circumstances, IL-6 may compensate for the lack of IL-21 since they both signal via STAT3, The role of STAT3 in Tfh cell development does not appear to be redundant

as showed by the defect of Tfh cell formation in STAT3-deficient mice and STAT3-deficient $CD4^+$ T cells in human[185, 215]. However, the latter result may also be confounded by the consequences of defective STAT3 signaling in B cells. Importantly, the above results also suggest that there are IL-6 and IL-21-independent mechanisms for Tfh cell development.

Roles of Tfh cells in physiological immune responses

The main known function of GC Tfh cells is to facilitate antigen-specific selection of GC B cells by providing growth and survival signals via IL-21 and CD40L[3, 62, 66, 68]. Tfh cells also maintain GC niches via the secretion of IL-21, a requirement for sustaining maximal Bcl-6 expression in both Tfh and GC B cells, which in turn promotes proliferation and affinity maturation of GC B cells as well as their transition to plasma cells and memory B cells[192, 211, 216]. IL-21 also provides survival signals to Tfh cells in an autocrine manner[184, 185, 192]. Tfh cells also produce substantial amounts of IL-4 that, in concert with IL-21, maintains GC B cell survival and selection[77, 209]. IL-4 is also known to be important for isotype switching to IgG1[217]. Expression of the inhibitory receptor PD-1 on Tfh cells is required for the selection and survival of GC B cells; deficiency of PD-1 led to reduced numbers of antigen-specific long-lived bone marrow (GC-derived) plasma cells. Deficiencies in PD-1 or any one of its ligands PD-L1 and PD-L2 only minimally alter Tfh cell numbers, while the formation of plasma cells was considerably diminished in the combined absence of both PD-L1 and PD-L2[218]. Interestingly, another study showed an increase in antigen-specific antibody responses to infection and immunisation as a result of expansion of Tfh cell in PD-1 deficient mice and upon PD-L1 (B7-H1) blockade on B cells[219]. The

discrepancy between these two studies remained unresolved but again might be related with different immunization models.

Roles of Tfh cells in pathological immune responses

Given the pivotal importance of the Bcl-6 gene in GC B cell development, an aberrant regulation of Bcl-6 expression has consistently been associated with B-cell lymphomas. More recently, a peripheral T cell lymphoma, Angioimmunoblastic T-cell lymphoma (AITL) has been shown to derive from the malignant transformation of Tfh cells[220]. Human AITL is the second most common subtype of peripheral T-cell lymphoma, displaying some autoimmune disease features including lymphadenopathy, hepatosplenomegaly, skin rash and hypergammaglobulimia[221, 222]. AITL contain infiltrates of immunoreactive $CD4^+$ T cells that are analogous to Tfh cells, characterized by the overexpression of Tfh associated genes such as CXCL13, BCL-6, ICOS and PD-1 though the neoplastic T cells only account for a small fraction of the lymphoid infiltrate[220, 223-226].

Tfh overactivity also represents a new mechanism of autoimmunity. Since the first description of Tfh accumulation linked with systemic lupus in *sanroque* mice[227], aberrant accumulation of Tfh cells has been shown to cause, exacerbate, or be associated with several human and mouse diseases such as SLE, Type 1 diabetes, arthritis, hepatitis, Sjogren's syndrome and others (reviewed in[5, 7, 157]). Mouse models have been used to investigate Tfh-mediated autoimmunity, including the *sanroque* model used in this thesis, which is characterized by the presence of spontaneous GC reactions and pathogenic autoantibodies mediated by aberrant regulation of Tfh

cells. Previously, we showed that *sanroque* mice develop a Tfh cell-driven lupus-like syndrome caused by a mutation in a novel immune regulation gene, *Roquin* (discussed further in the section below). Similarly, another murine lupus model, B6.Sle1.Yaa mice, is also characterized by increased expression of Tfh-associated genes including *Icos*, *Pdcd1*, *Cxcr5*, *Il21*, and increased IFN-γ-secreting cells[228].

IL-17-producing Tfh cells have been shown to promote spontaneous GC formation in autoimmune BXD2 mice by modulating CXCL12, resulting in the accumulation of GC B cells and increased production of pathogenic anti-DNA IgG, anti-histone IgG, IgG and anti-BiP IgM autoantibodies[229]. In addition, abundant extrafollicular Tfh-like cells have been found outside GCs in MRL^{lpr} mice[76]. Tfh cells have also been shown to induce autoimmune hepatitis (AIH) with severe T-cell infiltration and increased production of antinuclear antibodies that leads to fatality in neonatal thymectomy NTx-PD-1 deficient mice[230].

In humans, Tfh cells are associated with several autoimmune diseases including SLE and juvenile dermatomyositis. A subset of SLE patient shows expansion of circulating Tfh cells ($CXCR5^+$ $IL21^+$ $CD4^+$ $ICOS^+$) that correlates well with the diversity and quantity of autoantibodies, as well as the severity of glomeluronephritis[231-233]. Patients with juvenile dermatomyositis (JDM) also displayed blood $CXCR5^+$ Tfh subsets skewed towards Th2- and Th17-type cells and these subsets were able to induce naïve B cells to produce antibodies via IL-21. The extent of skewing correlated with disease severity and frequency of circulating plasmablasts[158].

Systemic Lupus Erythematosus (SLE)

SLE is a complex multi-organ disease characterised by significant clinical heterogeneity, and is driven by different lymphocyte effector cells including T cells and B cells as well as antigen-presenting cells such as myeloid cells. In this systemic autoimmune disease, the immune system recognises self-nuclear antigens resulting in inflammatory lesions within several organs; when taken in the context of side effects related to non-specific immunosuppressive drugs used to control the disease, a vast burden of illness is represented. Some of the clinical manifestations affect multiple organs, which include the skin, kidneys, heart, central nervous system, lungs, heart, gastrointestinal tract and joints in SLE patients. In SLE, autoreactive B cells become activated and produce autoantibodies against nuclear antigens and their interacting proteins. Typically these are antibodies against dsDNA, RNA, ribonuclear proteins (RNPs) and histones and tend to be somatically mutated and class-switched (IgG)[112, 234, 235]. Autoantibody-autoantigen immune complexes activate complement and are deposited in various tissues leading to tissue inflammation and injury[234, 235].

SLE predominantly affects women of childbearing age with a nine to one female gender bias. Both hormonal and X-chromosome factors have been proposed to account for this difference. Amongst the latter is the observed aberrant activation of immune response genes on the normally silenced X chromosomes such as CD40L, caused at least in part by DNA demethylation [424-425]. A role for genes in the X-chromosome is also supported by the data showing higher incidence of SLE in men with Kleinfelter's syndrome (47,XXY) compared to men in the general population and protection amongst women with Turner's syndrome (XO), suggesting a close

correlation between X chromosome gene dose and SLE[425-426]. The estimated prevalence of SLE is between 12-64 per 100,000 in European populations and 1 per 2000 in US populations. The prevalence in non-Caucasians is two to four-fold higher than in the Caucasian population[236, 237]. The diagnosis of SLE is not solely determined by the presence of antinuclear antibodies, although it is present in 99% of patients. Rather, the American College of Rheumatology criteria stipulate 4 of 11 clinical or biochemical criteria to be present for the diagnosis of SLE[238, 239] (**Appendix 1.1**).

Given the disease's complexity and the clinical heterogeneity of SLE patients, the development of new therapeutic strategies in SLE has been difficult. The marked improvements in the management of lupus that have been associated with improved survival are largely attributable to the advances in medical management of lupus-associated life threatening conditions such as renal impairment. Recent clinical trials, based on robust preclinical effects in animal models, have tested rituximab (anti-CD20), infliximab (tumor necrosis factor inhibitor), tocilizumab (IL-6 inhibitor) and abatacept (costimulation blocker), but have all met with limited success due to lack of clinical efficacy and, in some instances, a high adverse event rate[240]. The first drug that has been approved by US Food and Drug Administration (FDA) for the last 50 years is belimumab (a monoclonal antibody that targets BAFF) which targets moderately active lupus and is the only drug that showed significant clinical benefit in phase 3 clinical trials, though this benefit was marginal[240].

The pathogenesis of SLE

The pathogenesis of SLE remains unclear. However, the current view is that genetically susceptible individuals exposed to diverse environmental and hormonal risk factors, trigger activation of both innate and adaptive immune responses, resulting in loss of tolerance to ubiquitous self-antigens[234, 236, 237, 241, 242]. Three major pathways have been identified in SLE pathogenesis: aberrant clearance of nucleic-acid-containing debris and immune complexes, excessive innate immune pathway activation by TLR and type I IFNs, and abnormal T and B lymphocyte activation[234]. Several genetic loci have also been implicated in disease etiology[243]. In this thesis, we will focus on the discussion of failure of regulation and excessive activation of the adaptive immune system.

In SLE patients, the regulatory mechanisms for self-reactive T and B cells may be genetically defective or environmentally altered. 20-50% of mature naive B cells circulating in the peripheral blood of untreated SLE patients produce self-reactive autoantibodies many years before the clinical onset of SLE, implying that breakdown in B cell tolerance checkpoints leads to clonal expansion of autoreactive B cells[91, 113]. Another mechanism of B cell autoreactivity is enhanced BCR signaling due to increased signals from CD19, intracellular TLRs and BAFF receptor, leading to B cell hyper-activation and survival[244]. Defective GC selection by Tfh or FDCs also results in spontaneous GC formation and permits autoreactive B cells to differentiate into pathogenic memory and plasma cells. This may be the consequence of excess Tfh cell help[5, 245, 246]. Loss of GC tolerance may also be the result of defective B cell signaling, defective regulatory cell function, or inadequate clearance of apoptotic

cellular debris, as described above; this has been illustrated in human SLE and in lupus-prone mouse models[5, 234, 245].

Studies have also shown that the aberrant T cell activation implicated in SLE development may be due to faster T cell calcium influx, oxidative stress caused by mitochondrial dysfunction, decreased CD8 cytotoxic activity and increased expression of CD40L and CD44 on T cells[234]. Furthermore, the strongest genetic risk allele for SLE has been mapped to the MHC region, suggesting that aberrant MHC presentation may lead to excessive T cell activation and subsequent immune responses[247].

The sanroque model of SLE

Sanroque mice were discovered as a new model of lupus in the N-ethyl-N-nitrosourea (ENU) murine screening program for immune regulatory genes. *Sanroque* mice display systemic lymphadenopathy, splenomegaly, hypergammaglobulinemia with high IgG1, IgG2a (IgG2c in C57BL/6), IgG2b, IgG3, IgM and IgE, autoimmune thrombocytopenia, elevated high affinity anti-DNA antibodies in the serum and glomerulonephritis with deposition of IgG complexes in the kidney. *Sanroque* mice (homozygous for the "san" allele of Roquin, *Roquin*$^{san/san}$) develop a lupus-like systemic autoimmunity due to a homozygous mutation in the *Roquin* gene, in which there is "T" to "G" substitution in methionine at position 199 (M199R) leads to an amino acid change to arginine. The M199R mutation is located in a novel protein domain termed ROQ, which so far has only been identified in *Roquin* and its paralogue *Mnab*. Microarray analysis revealed that *Roquin*$^{san/san}$ mice

express elevated Tfh cell signature genes including PD-1, CXCR5, CXCL13, IL-21, SAP, CD40L, ICOS and Bcl-6. This is also accompanied by large GCs with accumulation of excessive numbers of Tfh cells that can already be observed in the first weeks of life[227].

Haploinsufficiency of Bcl-6 ameliorates spontaneous GC formation, pathogenic autoantibody (dsDNA IgG) production and renal pathology in *sanroque* mice[6]. SAP-deficiency also abrogates spontaneous Tfh and GC formation, autoantibody production (dsDNA IgG) and renal pathology, but not hypergammaglobulimia, lymphadenopathy and splenomegaly in *sanroque* mice[6]. The adoptive transfer of *sanroque* Tfh cells drives spontaneous GC formation in recipient mice, suggesting that the disease is Tfh-cell driven[6]. Collectively, these data suggest that the *Roquin*^{san} mutation induces accumulation of Tfh cells in a T-cell intrinsic manner. This, in part results from defective posttranscriptional degradation of *Icos* mRNA by mutant ROQUIN[248, 249]. Taken together, Tfh cell accumulation drives aberrant positive selection of autoreactive B cells in the GCs and lupus.

Studies from our lab also revealed that *sanroque* mice have other Tfh-mediated pathologies. For example, 50% of heterozygous *sanroque* mice (*Roquin*$^{san/+}$ mice) develop Tfh cell-driven, AITL-like tumors with hypergammaglobulinemia by the age of 6 months[250]. *Roquin*$^{san/san}$ mice also develop autoimmune diabetes in a susceptible genetic background. Furthermore, the *Roquin*^{san} mutation dramatically increases the progression to type 1 diabetes in TCR^{+} HEL^{+} transgenic mice: 100% of *Roquin*$^{san/san}$ TCR^{+} HEL^{+} mice develop diabetes by 8 weeks of age characterised by ectopic GC containing large numbers of Tfh cells in the pancreas, accompanied by peri-insulitis

with islet destruction and exocrine pancreatitis. The pathology is IgG anti-islet antibody-mediated - triggered by transfer of purified anti-islet-expressed antigen (HEL) IgG probably as a consequence of excessive Tfh activity[251]. Also, transfer of short-lived effector (SLEC)-like $CD8^+$ T cells from *Roquin*$^{san/san}$ mice triggers autoimmune diabetes in a diabetes susceptible model, RIP-mOVA mice, whereas wild-type T cells failed to do so[252].

ROQUIN (gene symbol *Rc3h1*) belongs to the RING-type E3 ubiquitin ligase protein family and, upon stress, localizes to cytosolic stress granules where RNA metabolism is regulated. It contains a RING domain (consensus for the E3 ubiquitin ligase protein), a novel ROQ domain (RNA-binding and stress-granule-localising), a CCCH zinc-finger domain (RNA binding) and a proline-rich-region domain (important for interacting with SH3 domain-containing proteins). Both ROQ and CCCH domains enable ROQUIN to bind to and regulate mRNA: they mediate ROQUIN's binding to the 3'-untranslated region (3' UTR) of *Icos* mRNA leading to its repression[248, 253]. Homozygosity for *Roquin*^{san} causes overexpression of *Icos* in *sanroque* mice through the impairment of *Icos* mRNA degradation. *In vitro*, this has been show to be due to ROQUIN's ability to promote mRNA decapping in the processing (P)-bodies. This process is mediated by ROQUIN's interaction with the helicase Rck and enhancer of decapping Edc4 which in turn associate with the decapping complex Dcp1:Dcp2 to mediate mRNA degradation independent of miRNAs[253]. Whether *Roquin* also regulates other mRNAs by the same mechanism remains elusive. Halving the gene dose of *Icos* ameliorated the hypercellularity (splenomegaly and lymphadenopathy) of *sanroque* mice. *Sanroque Icos*$^{+/-}$ mice still had elevated levels of ICOS on the surface of T cells, but the levels were lower than those of *sanroque Icos*$^{+/+}$ mice.

Given that *Roquin*$^{san/san}$ *Icos*$^{-/-}$ mice still developed splenomegaly, as shown in this thesis for the first time, we hypothesised that additional dysregulated ROQUIN mRNA targets may be required for the development of lupus in *Roquin*$^{san/san}$ mice. Increased expression of IL-21 was previously excluded as a candidate to cause the phenotype, given that elimination of IL-21 neither dampened the GC and Tfh accumulation, nor the onset of autoimmunity[6].

Another conundrum was the recent demonstration that loss of *Roquin* caused a phenotype distinct to that of *Roquin*$^{san/san}$ mice[254]. Complete knockout of *Roquin* results in perinatal lethality, whilst the ablation of *Roquin* in hematopoietic cells results in the expansion of myeloid cells (eosinophils and macrophages) and ICOS^{+} T cells population, in particular the short-lived effector CD8^{+} T cells. However, *Roquin* deficiency itself was not sufficient to cause autoimmunity as the knockout model demonstrates intact T cell development in the thymus with normal CD4^{+} T cell homeostasis and no spontaneous GCs. The reason why the *sanroque* phenotype fails to be recapitulated by *Roquin* knockout has not been fully explained, but we have unpublished observations pointing to two non-mutually exclusive possibilities that are discussed in the final chapter (Chapter 6).

Cytokines as regulators of Tfh cells and SLE

Cytokines and their networks are implicated in the innate and adaptive immune response, driving inflammation and pathogens neutralization. Other than their function in normal immune responses, there is overwhelming data showing that dysregulation of certain cytokines and activation of various signaling pathways by these cytokines contributes to SLE pathogenesis (reviewed in[255, 256]) and/or dysregulation of Tfh number and function (reviewed in[7, 157]; **Figure 1.1**). However, there is only a handful of studies that link cytokines, aberrant regulation of Tfh and GCs and SLE together.

Of interest, cytokines produced by Tfh appeared to contribute to SLE development. This is not surprising, however, given their role in facilitating GC B cell formation and maturation. IL-21, the predominant cytokine secreted by Tfh cells, has markedly increased levels of production from T cells, including Tfh cells, in BXSB.Yaa mice and SLE patients[216, 257, 258]. IL-21 deficiency or blockade (soluble IL-21R-Fc) has been shown to ameliorate disease development and progression. Symptoms dampened include hypergammaglobulinemia, autoantibody production, renal disease, monocytosis, expansion of splenic T and B cells including marginal zone B cells, and also premature morbidity in BXSB.Yaa and MRL^{lpr} mice[258-261]. IL-21 deficiency, conversely, has no effect on *sanroque* mice (unpublished observation).

Another cytokine, IL-17, is produced by Tfh cells and has been shown to promote spontaneous GC formation in autoimmune BXD2 mice by modulating CXCL12, a chemotactic migration factor of B cells that results in the retention of B cells in GCs, and increased production of pathogenic anti-DNA IgG, anti-histone IgG, anti-BIP IgG

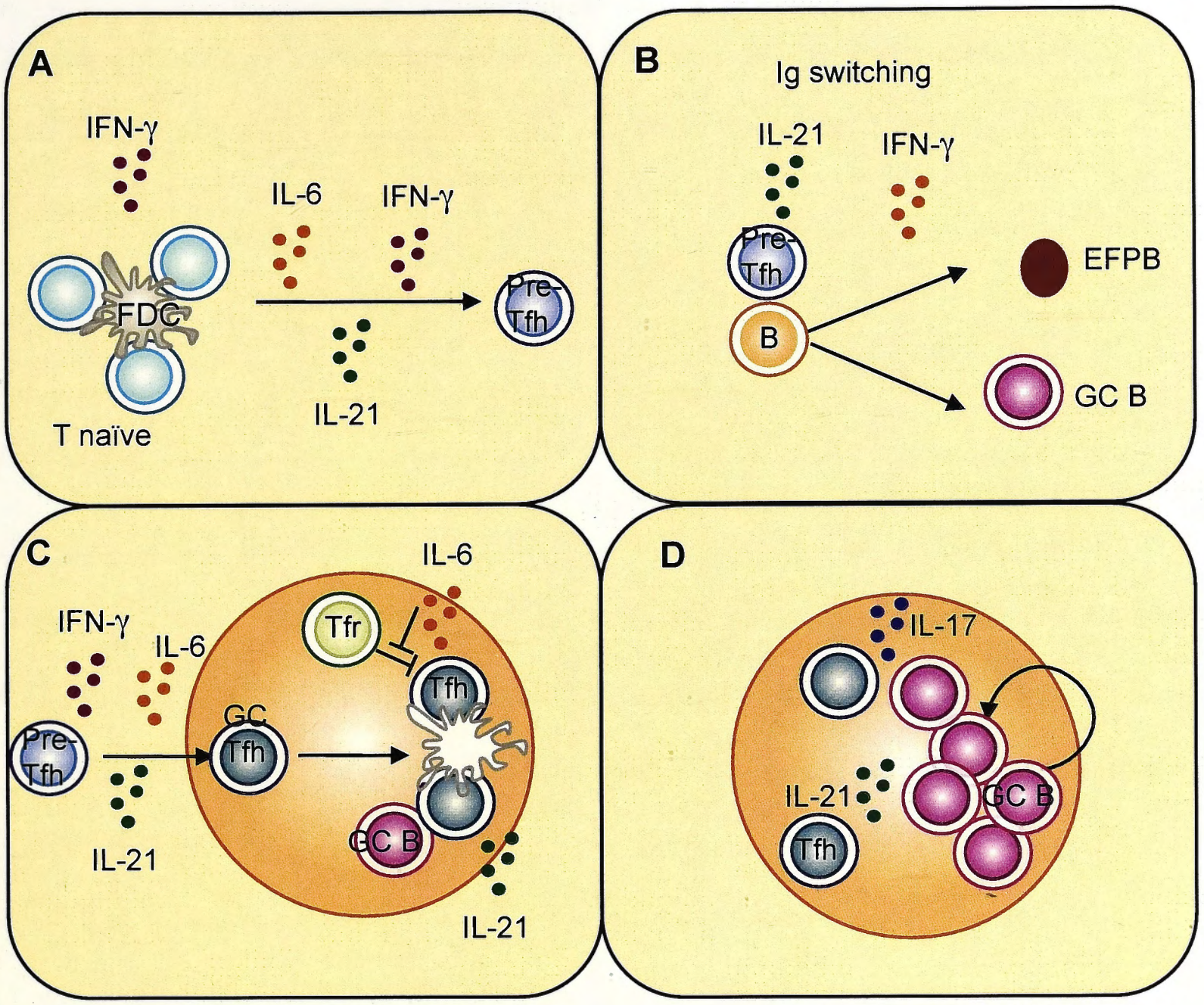


Figure 1.1. Cytokine-mediated dysregulation of Tfh and GC responses (the pathogenic effects of IFN-γ were taken from this thesis).

A)**Tfh formation:** IFN-γ promotes the proliferation of early effector T cells. IFN-γ, IL-21 and IL-6 are able to induce Bcl-6 expression[184, 185, 190, 192].

B) B cell priming & Ig switching: At the time of T cell priming, IL-21 produced by Tfh cells promotes B cell activation and differentiation along the extrafollicular and GC pathways. IFN-γ can induce isotype switching to IgG2a, the most pathogenic isotype, in mice[185, 192, 211].

C) Accumulation of Tfh cells and suppression of Tfh: Excessive IFN-γ, IL-6 and IL-21 promote the accumulation of Tfh cells that sustain spontaneous GC responses. Both IL-6 and IFN-γ can induce Bcl-6 overexpression in GC Tfh cells and/or their precursors. IL-6 can repress Treg cell function[423], thus it is possible that it also represses Tfr cells that normally limit aberrant Tfh and GC B cell accumulation[184, 185, 190, 192].

D) GC B cell retention and survival: IL-21 produced by Tfh cells acts directly on GC B cells to sustain Bcl-6 expression, thus promoting their growth and survival[185, 192, 211]. IL-17 excess produced by Tfh cells in autoimmune-prone BXD2 mice can promote GC B cell retention in GC[229], which may lead to more rounds of somatic mutation and more self-reactive GC B cells.

and IgM autoantibodies[229]. Other cytokines such as IL-2, type I interferons, IL-4, IL-6, IL-12 and IL-27 have all been independently shown to have important roles in Tfh or SLE (reviewed in[7, 157, 255, 256]).

Interferon-γ

Interferon-γ (IFN-γ) is the only member of the type II class of interferons and plays a central role in innate and adaptive immunity. IFN-γ is produced by innate cells including plasmacytoid dendritic cells (pDCs), myeloid dendritic cell (mDCs), γδ T cells, Natural killer (NK) cells, NK T cells and macrophages (Mac) under the influence of IFNα/IFNβ, IL-12, IL-18, IL-2, and IL15; in contrast, it is inhibited by TGFβ and IL-6. IFN-γ is upregulated upon cytokine stimulation, including IL-2, IL-12, IL-18 and/or IFN-γ itself produced by NK cells, DCs and other APC, activated CD8 T cells and CD4 T cells[262, 263]. IFN-γ is the signature cytokine of Th1 cells. The Th1 differentiation program is driven by the transcription factor, T-bet, which is necessary for full commitment to IFN-γ production[217, 264]. The biologically active form of IFN-γ is a 34 kDa homodimer which exerts its biological response through binding the IFN-γ receptor (IFN-γR), which is composed of a heterodimer of two membrane-spanning proteins (IFN-γR1 and IFN-γR2) that signal through the Janus kinase (JAK)-STAT pathway[265] (**Figure 1.2**).

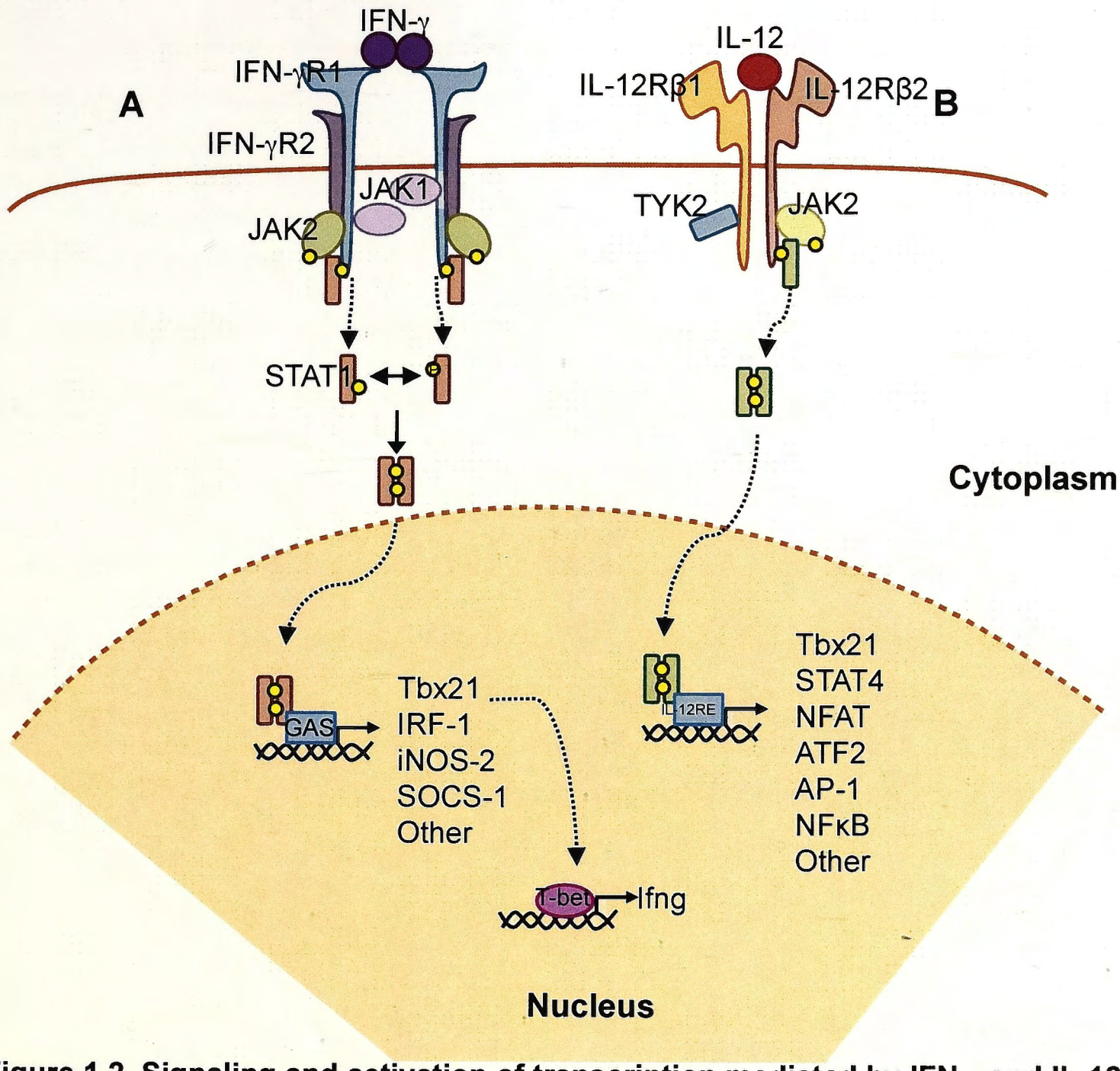


Figure 1.2. Signaling and activation of transcription mediated by IFN-γ and IL-12.

A) IFN-γ signaling is initiated by the binding of the IFN-γ homodimer, which directs the activation of two IFN-γR1 chains and two IFN-γR2 chains. Receptor subunit assembly then leads to phosphorylation and activation of two receptor-associated Janus kinases (JAKs) (i.e. JAK 1 and JAK2). These JAKs phosphorylate a tyrosine residue on the IFN-γR1, which directs the SH2 domain-dependent recruitment and activation of STAT1. Activated STAT1 molecules then dissociate from the receptor and form homodimer complexes. Subsequently, phoshorylated STAT1 homodimers translocate to the nucleus and bind to IFN-γ-activated site (GAS) enhancer elements in the promoters of IFN-γ-stimulated genes, thereby switching on gene transcription.

B) Binding of IL-12 to the IL12Rβ1 and IL-12Rβ2 chains induces phosphorylation of Tyrosine kinase (TYK) 2 and JAK2 and results in the binding of STAT4 to the cytoplasmic tail of IL-12Rβ2. Phsphorylated STAT4 molecules then dimerise and translocate to the nucleus and bind to the promoter region of IL-12-induced genes (those with an IL-12 response element, IL-12RE) and drive the transcription of IL-12 responsive genes.

AP-1, activator protein-1; ATF2, Activating transcription factor 2; iNOS, inducible nitric oxide synthase; IRF, interferon regulatory factor; NFAT, nuclear factor of activated T cells, NFκB, nuclear factor κB; SOCS, suppressor of cytokine signaling

Regulation of IFN-γ production

In humans, IFNα and IFNβ in conjunction with IL-18 and IL-12 can drive acute IFN-γ secretion in Th1 cells via the STAT1/STAT4 signaling pathway[266-271]. In mice, IL-12R is able to induce effective and sustained STAT4 activation[269, 272] (**Figure 1.2**), but type I IFNs do not induce STAT4[273-275]. Upon stimulation with TLR4 and TLR7/8, human DCs can also induce naïve $CD4^+$ cells to produce IFN-γ and IL-10[276]. Another cytokine, IL-27, secreted by virally-infected cells, activated Mac and DCs, can drive proliferation of Th1 cells and induce IFN-γ production by synergizing with IL-12 and IL-18 to activate both STAT3 and STAT1 proteins, in turn inducing T-bet expression[263, 277]. Notably, IL-27 has been shown previously to support Tfh formation via induction of IL-21[193].

IL-10 serves as a feedback regulator for IFN-γ production. Acute ablation of Treg cells – a prominent cellular source of IL-10 - leads to an increase in IFN-γ-producing Th1 cells, indicating that Th1 effector function was limited by the Treg cells[278]. IL-10 is produced abundantly by Treg cells and is known to directly inhibit IFN-γ production by T cells and indirectly suppress IFN-γ by inhibiting Mac and DCs, but not B cells, from stimulating Th1 cells to produce IFN-γ[279-281]. Th1 cells themselves can also secrete IL-10 in response to intracellular protozoa infection, suggesting a self-regulatory role to limit the IFN-γ pathogenic potential[282, 283]. IL-27 and IL-6 can also induce Th1 cells to produce IL-10 via STAT1/STAT3 and STAT3 alone signaling pathways respectively, suggesting that these cytokines also exert a feedback regulatory role on Th1 cells to prevent unwanted chronic inflammatory responses[284].

Physiological and pathogenic roles of IFN-γ

IFN-γ exerts multiple responses against certain viral, intracellular bacterial (mycobacteria, Listeria, *Salmonella*) and protozoan (Toxoplasma and Leishmania) infections. It also has pro-inflammatory, immunoregulatory and anti-tumour effects via the regulation of various genes. Its important functions in the immune system include enhancing antigen presentation through up-regulation of both MHC-I and MHC-II expression, activating Mac by increasing phagocytosis, leukocyte trafficking at sites of inflammation including leukocyte-endothelial interactions, and promoting NK cell activity[263].

In this thesis, I will focus on the role of IFN-γ in regulating $CD4^+$ T cell function. The cytokine milieu is crucial for $CD4^+$ T cell differentiation: the main function of IFN-γ on $CD4^+$ T cells is to mediate the differentiation, maturation and activation of Th1 cells against infections, while also suppressing Th2 and Th17 cell differentiation, probably via T-bet[264]. IFN-γ also induces T-bet expression in Th1 cells and promotes Ig class switching to IgG2a (IgG2c in C57BL/6) in mice, though a recent report suggested that IFN-γ is not required for isotype switching or B cell antibody production in humans[75, 158]. It also antagonises the effect of IL-4 on switching to IgG1 and IgE[30, 31, 285]. Furthermore, IFN-γ also exerts pro-apoptotic effects, increasing effector cell apoptosis and restoring the balance of the T cell population expanded by antigenic stimulation, consequentially suppressing autoimmunity[262, 286]. IFN-γ also appears to play intriguing anti-inflammatory roles in certain disease models: in the context of experimental allergic encephalomyelitis (EAE), IFN-γ induces FoxP3 expression in $CD4^+$ T cells and also promotes the conversion of non-Treg cells to Treg cells, thereby limiting tissue damage associated with overt inflammation[287].

IFN-γ production is tightly controlled by Treg cells: ablation of Treg cells leads to increased IFN-γ production that rapidly triggers autoimmune diabetes[288]. Interestingly, IFN-γ and IL-27 promote a specialised Treg subset, which is T-bet$^+$ CXCR3$^+$, and appears to be critical to limit Th-1 cell-mediated inflammation[289, 290]. Notably, Tfr also secrete IL-10, which limit Tfh and GC numbers, they may also play a role in the regulation of IFN-γ production within GCs[137, 139].

Relationship between IFN-γ and Tfh cells

Although Tfh cells can produce IFN-γ during inflammatory conditions, infection, and in the context of autoimmunity[152, 291], human tonsil and mouse GC Tfh cells generally produce less IFN-γ than non-Tfh cells including Th1 cells in both human tonsils and mice, suggesting a critical need for limiting IFN-γ production in normal GC responses[136, 166]. Low levels of IFN-γ in Tfh cells may be due to elevated expression of Bcl-6, wherein Bcl-6 antagonizes Blimp-1 required for the differentiation of non-Tfh effector subsets including IFN-γ-producing Th1 cells[152, 292]. Bcl-6 can also directly repress both *Ifng* and *Tbx21* transcription, the latter also leading to partial downmodulation of IFN-γ[136, 153].

One of the proposed mechanisms to regulate the balance between Tfh and Th1 cells is through the T-bet-Bcl-6 axis in which both IL-2 and IL-12 signaling have been shown to contribute to the phenotype and function of Tfh and Th1 cells. In fully developed Th1 cells, T-bet has shown to repress IFN-γ transcription by physically recruiting Bcl-6 to the *Ifng* locus, repressing its activity in the late Th1 differentiation stages and thereby limiting IFN-γ production[136, 153]. IL-2 availability at the time of T

cell priming *in vivo* also appears to be a critical factor influencing whether a developing effector T cell elects a Th1 or Tfh cell fate. When there are limiting amounts of IL-2, Th1 cells increase their Bcl-6/T-bet ratio, allowing excess Bcl-6 to repress Blimp-1, subsequently favoring a Tfh cell-like profile[293]. IL-2 directly inhibits Tfh differentiation via STAT5, which competes with STAT3 for binding to the Bcl-6 locus, and promotes the expression of *Prdm1*, the gene encoding Blimp-1[201, 202].

Early coexpression of Bcl6 and T-bet in a transition stage before the cell fate decision is made, is supported by work showing that T-bet may be essential for both the Th1 and Tfh differentiation pathways at the initial stage[294]. This notion is supported by the fact that IL-12/STAT4 pathway is shared by both Tfh and Th1 cells. STAT4 can bind directly to regulate Tfh related genes such as Bcl6 and IL-21[294]. At the initial phase of IL-12 mediated Th1 cell differentiation downstream of STAT4, Tfh and Th1 cells share a transient stage where uncommitted $CD4^+$ T cells exhibit a Tfh-Th1-like phenotype ($IL21^+$ IFN-γ^+ $Bcl6^+$ T-bet^+). The subsequent induction of T-bet represses Bcl-6, resulting in full differentiation of Th1 ($IL21^+$ IFN-γ^+ $Bcl6^-$ T-bet^+) cells at late stages of Th1 differentiation. Th1 cells use T-bet and STAT4 signaling to limit Bcl-6 expression, which favors a Th1 phenotype and limits Tfh cell formation[294]. This paper suggests that there are threshold levels of T-bet and Bcl-6 expression that need to be reached, similar to the Bcl-6 and Blimp-1 regulatory axis dictating Tfh or Th1 cell phenotype, thus controlling the levels of IFN-γ in normal conditions. In this thesis, I will describe a novel consequence of the action of excessive IFN-γ on Tfh cells: to promote Tfh cell accumulation and cause autoimmunity in a manner that operates independently of T-bet.

The other IFN family - type I interferons - has also been shown to act as an adjuvant on DCs to selectively support the differentiation of lymph node resident Tfh cells. Upon activation by antigens in conjunction with TLR3 (pI:C) or TLR4 (LPS) agonists, type I interferons (IFNα/IFNβ) support the differentiation of Tfh cells by enhancing CD86 expression on DCs and B cells to strengthen their T cell stimulatory capacity. In addition, autocrine and paracrine IFN-I signalling also induces IL-6 production from $CD11c^+$ DCs[191]. IL-6 signalling via STAT3 in T cells appears to be required for Tfh differentiation[185].

Relationship with SLE

Aberrant secretion of IFN-γ has been shown to mediate the progression of several autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and diabetes. Increased serum levels of IFN-γ correlated with the development, disease activity and progression of SLE in patients. Several studies have shown high levels of IFN-γ in serum or kidney sections of SLE patients and this is associated with lupus nephritis, lymphadenopathy or nephrotic syndrome[295-299]. Furthermore, IFN-γ-producing Th1 cells are detected in the blood and kidneys of patients with diffuse proliferative nephritis[300]. SLE patients also show increased circulating CXCL10, an IFN-γ-inducible serum chemokine, as well as higher expression of IFN-γ in urinary sediment, which correlates well with the SLE Disease Activity Index (SLEDAI)[301, 302]. Expression of IFN-γ, T-bet and STAT1 was increased in peripheral blood mononuclear cells (PBMCs) from SLE patients and also correlated with disease activity[303-305]. In addition, NK cells from active SLE patients showed an activated phenotype and abundant IFN-γ production[306]. The combination of Val14Met and

Gln64Arg polymorphisms within the IFN-γR1 and IFN-γR2 has also been identified in some SLE patients and is associated with the susceptibility to SLE[307]. All together, IFN-γ and genes related to IFN-γ signaling pathway may play an important role in SLE pathogenesis and can be used as biomarkers for disease activity.

IFN-γ is of particular interest to us since in our murine lupus model, *sanroque*, T cells exhibit increased production of this cytokine[227]. Similarly, high levels of IFN-γ are documented at the mRNA and protein levels in T cells of several different lupus-prone mice including MRL^{lpr}, NZB/W F1 and BXSB.Yaa[308-314]. There is also increased IL-12 expression in kidney-infiltrating mononuclear cells and tubular epithelial cells of MRL^{lpr} mice[315-317]. In other lupus-prone mouse models, IFN-γ or IFN-γR deficiencies in MRL^{lpr}, NZB/W F1 and pristine-induced lupus mice have been shown to reduce autoantibodies, glomerulonephritis severity or prolong survival[318-322]. The injections of IFN-γ in NZB/W F1 mice accelerated disease progression, whilst neutralising IFN-γ antibodies or soluble IFN-γR ameliorated disease and resulted in significant remission, prolonged survival and amelioration of glomerulonephritis[323, 324]. Consistent with this, depletion of IFN-γ using cDNA encoding IFN-γ/Fc receptor in MRL^{lpr} mice reduced autoantibody production, lymphoid hyperplasia, glomerulonephritis and mortality[325]. Even more striking is the fact that transgenic mice expressing IFN-γ in their epidermis, under the control of the involucrin promoter, developed inflammatory skin disease and a lupus-like syndrome charaterized by production of IgG anti-dsDNA, anti-histone autoantibodies and antinuclear autoantibodies (ANAs), and glomerulonephritis mediated by immune complex deposits in the kidney[326]. Further analysis of these mice suggests that antigen-specific αβ T cells targeted apoptotic epidermal keratinocytes as a source of

self-antigens led to the formation of pathogenic autoantibodies[327]. However, one study showed that recombinant IFN-γ (rIFN-γ) treatment to MRL^{lpr} mice has dichotomic effects, beneficial at the early stage of disease yet accelerating symptoms once SLE has manifested[328]. These data from several different lupus models (reviewed in[329]) emphasise the importance of IFN-γ/IFN-γR signaling for autoantibodies production and immune complex glomerulonephritis, and provide evidence that impeding IFN-γ signaling through IFN-γ blockade may be beneficial in the treatment of SLE in humans. Indeed, Amgen has recently conducted a phase Ib clinical trial (safety test) on a fully human monoclonal antibody that binds to IFN-γ (AMG 811) for the treatment of SLE.

The interferon signaling pathway, mainly the Type I IFNs (IFNα/IFNβ) secreted by innate cells such as pDC and mDC in response to proficient TLR receptor signaling[330], is considered the major signaling pathway involved in the pathogenesis of SLE. Indeed, most studies place IFN-γ downstream of this pathway. Further, excess type I IFNs are able to break peripheral tolerance through the unabated activation of myeloid DCs which expand rather than delete autoreactive T cells (including Th1 cells with subsequent excess production of IFN-γ) and B cells, resulting exacerbation of SLE through enhancement of the inflammatory cellular response, autoantibody secretion and immune complex formation[331]. Notably, low levels of type I IFNs can induce at least 10-fold enhancement of IFN-γ in DCs, NK cells and T cells via STAT1 and STAT4[332, 333].

The mechanism by which IFN-γ promotes SLE

The exact mechanism underlying the action of IFN-γ in SLE development is uncertain because of the highly pleiotropic properties of IFN-γ. There is the possibility that IFN-γ increases expression of MHC I and MHC II on APC and T cells with subsequent excessive self-peptide presentation culminating in excessive B cell help and autoantibody production. Additional mechanisms may include its indirect effect on B cell activation and maturation, whereby peripheral blood T cells from SLE patients that express significantly higher amounts of IFN-γ have been shown to induce soluble BAFF (B lymphocyte stimulator; Blys) via monocytes or macrophages. This subsequently results in excessive B cell activation and pathogenic autoantibody production[334]. In mice, IFN-γ induces Ig switching to pathogenic IgG2a (IgG2c in C57BL/6) and IgG3 autoantibodies that efficiently activate Ig FcγRIII and FcγRIV receptor and complement, leading to the initiation of the inflammatory response in lupus nephritis[335]. However, this may be different in humans: a recent study showed that blood $CXCR5^+$ or $CXCR5^-$ IFN-γ-producing Th1 cells failed to provide help to B cells[158]. Interestingly, there is also evidence that IFN-γ may participate in the early stage of the disease, specifically in the initiation of ANA production. Some rheumatoid arthritis patients receiving systemic treatment with rIFN-γ showed emergence of SLE with *de novo* anti-dsDNA antibody, glomerulonephritis and a butterfly rash, suggesting that the excessive amounts of IFN-γ in susceptible individuals can trigger an SLE-like autoimmune response[336].

An essential role of IFN-γ/IFN-γR signaling in the pathogenesis of immune complex-mediated renal disease in MRL^{lpr} mice has been well established. Kidney infiltrated T cells and mesangial cells secrete IFN-γ to induce colony stimulating factor (CSF)-1

and tumor necrosis factor (TNF)-α, which in turn, recruit and foster the expansion of macrophages and T cells. It also has been shown that IFN-γ produced by T cells could induce apoptosis of renal parenchymal cells in MRL^{lpr} mice. Thus, the influx of additional T cells into the kidney provides a positive amplification loop that results in apoptotic renal parenchymal cells and culminates in kidney destruction[319, 337]. In addition, infiltrating macrophages also produce IFN-γ, which in turn upregulates adhesion molecules, induces Mac accumulation and results in inflammation in the kidney even in the absence of autoantibody deposits. IFN-γ controls macrophage migration into the kidney, and the levels of IFN-γ secretion by Mac correlate with glomerulonephritis development[338]. All together, these studies show that IFN-γ is involved in the induction and progression of lupus nephritis by promoting inflammation and initiating a positive feedback loop that is responsible for amplifying autoimmune kidney damage. A direct effect of IFN-γ on promoting Tfh cell accumulation as a key early factor in SLE pathogenesis, which is the focus of this thesis, is not yet documented.

Thesis aims

This thesis aims to address several topics revolving around the regulation and function of physiological and pathogenic Tfh cell formation and their impact on both follicular and extrafollicular antibody responses.

The first results chapter of of this thesis (Chapter 3) focuses on aiding the understanding of the nature of T cells that drive extrafollicular B cell responses. Since our lab has previously shown that Bcl-6 is the transcription factor for GC Tfh and Bcl-6 expression in T cells is essential for initiation of GC reactions, we asked whether T cell expression this transcription factor is also required in the extrafollicular pathway.

The second and third results chapters of this thesis (Chapters 4 and 5) investigate a role for IFN-γ, a cytokine abundantly produced by *sanroque* T cells, in the accumulation of Tfh, spontaneous GC formation, and SLE pathogenesis of *sanroque* mice. Our results reveal that IFN-γ not only contributes to SLE pathogenesis via induction of istoype switching or downstream of the antibody response, but it itself alters the quality of the antibody response by influencing the differentiation and homeostasis of B cell helper T cells (Tfh cells). Targeting IFN-γ emerges as a tantalising concept in the development of therapeutics for Tfh and-mediated disease.

Chapter 2

Materials and Methods

Chapter 2 – Materials and Methods

Mice and Immunizations

C57BL/6, *Cd28*$^{-/-}$ mice, *Bcl6*$^{-/-}$, C57BL/6.*Fas*$^{-/-}$ [339], VavP-*Bcl2*^{340}, *Rag1*$^{-/-}$, *Roquin*$^{san/san}$ mice; and *Roquin*$^{san/san}$ mice crossed to *Ifngr*$^{-/-}$, *Ifng*$^{-/-}$, *Tbx21*$^{Du/Du}$ [341], *Icos*$^{-/-}$, *Tcr*$\alpha^{-/-}$, *Ig*$\alpha^{ken/ken}$ mice were maintained by the Australian National University Bioscience Services in specific pathogen-free conditions and had access to food and water ad libitium. SW_{HEL} CD45.1 mice, which carry a Vκ10κ light chain transgene and a knocked-in VH10 Ig heavy chain in place of the JH segments of the endogenous IgH gene that encode a high-affinity antibody for HEL, were obtained from the laboratory of R. Brink (Garvan Institute, Sydney, New South Wales, Australia) and maintained by Australian National University Bioscience Services. SW_{HEL} *Il21r*$^{+/+}$ CD45.2, SW_{HEL}.*Il21r*$^{-/-}$ CD45.2, and recipient C57BL/6 CD45.1 mice were maintained in the animal facility at the Garvan Institute. All procedures were performed with appropriate ethical and legal approval by the Australian National University's Animal Experimentation Ethics Committee.

Generation of fetal liver and bone marrow chimeras

Recipient *Rag1*$^{-/-}$ mice were irradiated with 500 Rad and reconstituted via i.v. injection with 2 × 10^6 donor fetal-liver or bone marrow–derived hematopoietic stem cells. Mice were reconstituted with the following combinations of fetal liver cells: for *S. enterica* infection and hapten (4-hydroxy-3-nitrophenyl) acetyl (NP) coupled to keyhole limpet haemocyanin (KLH) immunization experiments: 80% *Bcl6*$^{-/-}$

CD45.2:20% *Cd28*$^{-/-}$ CD45.1 and control 80% *Bcl6*$^{+/+}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 fetal liver cells; and for recipients of CD45.1 SW_{HEL} B cells, 80% *Bcl6*$^{-/-}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.2 and control 80% *Bcl6*$^{+/+}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.2 fetal liver cells. Bone marrow chimeras were reconstituted with the following combinations of bone marrow cells: 70% *Roquin*$^{san/san}$ *Tcr*$\alpha^{-/-}$:30% *Roquin*$^{san/san}$, 70% *Roquin*$^{san/san}$ *Tcr*$\alpha^{-/-}$:30% *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$, 70% *Roquin*$^{san/san}$ *Ig*$\alpha^{ken/ken}$:30% *Roquin*$^{san/san}$, 70% *Roquin*$^{san/san}$ *Ig*$\alpha^{ken/ken}$:30% *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$, and controls 100% *Roquin*$^{san/san}$, *Roquin*$^{+/+}$ and *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ bone marrow cells. All groups were maintained on antibiotics for 6 weeks, and experiments were performed 8 to 10 wk after reconstitution to allow for full reconstitution of the immune system.

S. enterica inoculations and liver bacterial counts

S. enterica serovar Dublin strain SL5631[342] was grown in Luria-Bertani medium overnight. Mice were inoculated with 10^6 colony-forming units (CFUs) from a log-phase culture administered i.p. in PBS, with control mice receiving PBS only. Liver bacterial load was measured at day 12 after infection by homogenizing organs, plating serial dilutions in PBS onto Luria-Bertani agar, and incubating at 37°C overnight. For experiments with *S. enterica* serovar Typhimurium strain SL3261, mice were immunized i.p. with 5×10^5 bacteria in PBS.

Antibodies detection by ELISA

ELISAs were used to quantify serum titers of anti-HEL, anti-NP and anti-*S. enterica* antibodies. To quantify anti-HEL and anti-*S. enterica* antibodies, 96-well plates were coated with either 1 μg/ml HEL protein or 250 μg/ml *S. enterica* lysate prepared as previously described in 0.05 M carbonate buffer (Na_2CO_3 + $NaHCO_3$, pH 9.6) overnight and subsequently blocked with PBS/1% BSA[6]. Serum was serially diluted onto the 96-well plates in PBS/1% BSA/0.05% Tween 20 buffer and incubated for 2 h at 37ºC. After washing, antigen-specific antibodies were detected with anti-mouse IgM, anti-mouse IgG1 or anti-mouse IgG2c antibodies conjugated to alkaline phosphatase (Vector Laboratories). Bound antibody was detected with para-nitrophenylphosphatete glycine buffer. Plates were read at 405 nm with a microplate reader (Thermomax; Molecular Devices), and titers for serum samples were calculated as the log serum concentration required to achieve 50% maximum optical density. To detect anti-NP antibodies 96-well plates were coated with 20 μg/ml NP_{13}-BSA in PBS overnight. Serum samples were serially diluted on plates in block solution (PBS/1% FCS/0.6% skim milk powder/0.05% Tween 20) and incubated for at least 20 h at room temperature. Anti-NP was shown with goat anti-mouse IgG1 conjugated to horseradish peroxidase (HRP; SouthernBiotech) and visualized with ABTS substrate (2'2-Azinobis [3-ethylbenzthiazoline sulfonic acid] diammonium salt; Sigma-Aldrich). Plates were read at 405 nm, with reference wavelength 490 nm (Molecular Devices).

ANA assessment

Serum was obtained from 8 to 10-wk-old mice by eye or cardiac bleed and used for immunoflourescence staining on fixed Hep-2 slides (Antibodies, Inc.) as previously described[6]. Briefly, slides were incubated with 20 µl serum for 45 min followed by 20 µl goat anti-mouse IgG-A488 (Invitrogen) incubation for 20 min. Slides were viewed using an Olympus microscope (model TH4-200; Olympus Optical) at 20X magnification and fluorescent intensity of the autoantibodies was measured by Image J software (National Institutes of Health, USA) on 20 randomly selected 2,400 μm^2 regions of clustered Hep-2 cells. ANA Ig autoantibodies levels in serum were also measured using Mouse ANAs Total Ig ELISA kit (Alpha Diagnostic) according to the manufacturer's instructions. Briefly, 100 µl serum and standards were incubated in 96-wells pre-coated with purified extractable nuclear antigens for 60 min at room temperature. Then, 100 µl of goat anti-mouse Ig coupled with HRP was added and incubated for 30 min. This is followed by 100 µl 3,3',5,5'-tetramethylbenzidine (TMB) substrate incubation for 15 min in the dark and 100 µl stop solution. Plates were then read at 405 nm using a microplate reader (Infinite 200® Pro; Tecan). The ANA Ig titers were calculated relative to mouse ANA standards.

Serum cytokine detection

IFN-γ levels in serum were evaluated by cytometric bead array (Mouse Th1/Th2 cytokine CBA kit, BD) according to the manufacturer's instructions. 50 µl serum and standards were added into 50 µl of cytokine capture bead suspension together with 50 µl PE-detection reaction and incubated for 2 h at room temperature in the dark.

Data were acquired with a LSRII flow cytometer. Detection of serum IFN-γ levels in mice at different stages of disease was performed using the Mouse IFN-γ ELISA kit II (BD) according to the manufacturer's instructions. Briefly, 50 µl serum and standards were added into 96-wells pre-coated with anti-mouse IFN-γ monoclonal antibody (mAb) and incubated for 2 h at room temperature. Then, 100 µl biotinylated anti-mouse IFN-γ antibody was added and incubated for 1 h. This is followed by 100 µl TMB substrate incubation for 30 min in the dark. Finally, 50 µl stop solution was added and plate was read at 450 nm using a microplate reader (Infinite 200® Pro; Tecan). The IFN-γ titers in serum were calculated according to the standard curve generated.

Immunization

For experiments involving SW_{HEL} mice, 10^5 SW_{HEL} B cells were transferred into recipients, which were simultaneously immunized i.v. with 2×10^8 sheep red blood cells (SRBCs) conjugated to (hen egg lysozyme) HEL or HEL^{2x} protein[41]. For experiments involving NP-KLH, immunizations with NP-KLH at a molar ratio of ~16:1 were performed. 100µg of NP-KLH was precipitated in alum and injected i.p.

Cell sorting, culture and stimulation

Single cell suspensions were prepared and resuspended in ice-cold sorting buffer (1% FCS, 0.1% NaN_3 in PBS) for sorting using a FASC Aria II (BD). 5 x 10^5 naïve ($CD44^{lo}$ $CD25^-$ $CD4^+$) sorted T cells were activated in Th1 polarizing conditions (10

ng/ml recombinant mouse IL-12p70 (eBioscience) and 10 ng/ml anti-IL-4 (Biolegend) in RPMI medium for 0, 1, 2, 4, 6, 12 and 24 h at 37°C in triplicate in 24-wells plate (Corning) pre-coated with 5 µg/ml anti-CD3 (BD) and 2.5 µg/ml anti-CD28 (BD). For some experiments, naïve cells were cultured in 10 ng/ml anti-IL-4 and 10 ng/ml anti-TGFβ (BioXcell) in the presence or absence of 2.5 µg/ml anti-CD28, 5 ng/ml IFN-γ (PeproTech) or 30 ng/ml rIL-6 (R&D Systems) for 24 and 72 h at 37°C. Cell culture supernatants were then aspirated and cell pellets were stored in TRIzol (Invitrogen) for real-time RT-PCR analysis.

Actinomycin D treatment

For actinomycin D treatment, 5 x 10^5 naive cells were cultured and activated in the presence of 5 µg/ml anti-CD3, 2.5 µg/ml anti-CD28 and 10 ng/ml IL-12p70 for 16 h in a 24-wells plate followed by treatment with 10µg/ml actinomycin D (transcriptional inhibition reagent, Sigma-Aldrich) in triplicate for 0, 0.5, 1 and 3 h.

cDNA preparation and real time RT-PCR

RNA was extracted using the TRIzol extraction method, and cDNA was prepared using M-MLV reverse-transcription with oligo(dT) (Invitrogen). Quantitative PCR was performed with a ABI Prism detection system (model 7900; Applied Biosystems) using Power SYBR® Green PCR Master Mix (Applied Biosystem). Forward and reverse primers used are: *Ifng* 5'-ACAGCAAGGCGAAAAAGGAT-3' and 5'-TGAGCTCATTGAATGCTTGG-3'; *Il2* 5'-CCACAGTTGCTGACTCATCATC-3' and 5'-

AAGGGCTCTGACAACACATTTG-3'; *bcl6* 5'-TGTCCTCACGGTGCCTTTTT-3' and 5'-CACACCCGTCCATCATTGAA-3'; *gapdh* 5'-TGACGTGCCTGGAGAAA-3' and 5'-AGTGTAGCCCAAGATGCCCTTCAG-3'. Quantification for *Ifng*-, *Il2*- and *bcl6*-specific fold change, normalized to *gapdh*, was performed using the $2^{-\Delta\Delta ct}$ method.

OT-II and SW_{HEL} adoptive cell transfer experiments and HEL-Ovalbumin (OVA) immunization

For collaborative responses of SW_{HEL} B cells and OT-II T cells, 10^5 sorted CD45.2 SW_{HEL} B cells and 20 μg HEL-$OVA_{323–339}$ peptide[43] were injected i.v. into *Cd28*$^{-/-}$ CD45.1 mice. 4 h later, 10^5 sorted naive $CD44^{lo}$ $CD25^-$ $CD4^+$ CD45.2 OT-II T cells were injected i.v. into same recipient mice, and mice were immunized i.p. with 100 μg of OVA (Sigma-Aldrich) in alum (Thermo Fisher Scientific) immediately after adoptive cell transfer.

T cell passive transfer experiments

For experiment involving T cell transfer, $CD4^+$ T cells from spleens and lymph nodes of *Roquin*$^{+/+}$, *Roquin*$^{san/san}$ and *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice and $B220^+$ from spleens and lymph nodes of *Roquin*$^{+/+}$ mice were sorted as described in the previous section. 5 x 10^6 $CD4^+$ T cells of genotypes indicated in the text and 7 x 10^6 *Roquin*$^{+/+}$ $B220^+$ sorted cells were injected i.v. into *Rag1*$^{-/-}$ recipients. Experiments were performed 8 weeks after transfer.

Immunohistochemistry

NP-binding cells were visualized by freezing spleen samples in OCT (Tissue-Tek; Sakura). Spleen sections were prepared and stained as described[343]. Antibodies and conjugates used were B220-biotin (RA3-6B2), goat anti-mouse IgG1-HRP (SouthernBiotech) and streptavidin alkaline phosphatase (SouthernBiotech). Antibodies were visualized with substrates AEC (Vector Laboratories) and FastBlue (Vector Laboratories).

Immunofluorescence

To visualize the responses to *S. enterica* and SRBC immunization, spleen samples were fixed for 20 min in ice-cold acetone (for *S. enterica*) or 4% paraformaldehyde for 2 h on ice, incubated in six changes of sucrose buffer overnight (for SRBCs), and embedded in Tissue-Tek OCT compound. Sections were blocked with streptavidin and biotin Blocking Kit (Vector Laboratories) before staining. Sections were stained for CD3 using anti-CD3 (BD) followed by anti-Armenian hamster Cy5 (Jackson ImmunoResearch Laboratories, Inc.). For Bcl-6, anti-mouse Bcl-6 (Santa Cruz Biotechnology, Inc.) was followed by donkey anti-rabbit FITC (Jackson ImmunoResearch Laboratories, Inc.) and then Alexa Fluor 488- conjugated goat anti-FITC (Invitrogen). PD-1 was stained with purified anti-PD-1 (BioLegend) followed by anti-rat Cy3 (712-166-153, Jackson ImmunResearch Laboratories, Inc.). For AID, anti-mouse AID (eBioscience) was followed by biotinylated anti-rat IgG, then streptavidin-HRP conjugates (Zymed) were followed by TSATM tetramethylrhodamine (PerkinElmer). Stained sections were mounted in

Fluoromount-G (SouthernBiotech) and analyzed with a confocal laser-scanning microscope (DMRXA2; Leica; SRBC experiments) or a laser-scanning confocal microscope (LSM510; Carl Zeiss) a microscope (AxioVert 100M; Carl Zeiss) using a 40X objective. The latter images were subsequently analyzed on the LSM Image Browser (Carl Zeiss).

Quantification of cells in tissue sections

Quantification of $Bcl6^+$ $CD3^+$ $PD\text{-}1^{hi/lo}$ cells was carried out as follows. 10 T zone areas were randomly selected. For each image, the number of $CD3^+$ $Bcl6^+$ cells and their PD-1 status (bright/intermediate or negative) was quantified, and the total number of cells in each spleen was calculated as described previously[344]. Cell numbers and densities were estimated using point counting, and counts were adjusted for the different sizes of the spleens seen throughout the study by multiplying the cells/mm^3 by the mass of the spleen.

Anti-IFN-γ treatment

5-week-old female *Roquin*$^{san/san}$ mice were bled prior to treatment and then injected i.v. with 500 µg anti-IFN-γ mAb (XMG 1.2; BioXcell) or 500 µg rat IgG1 Isotype control mAb (BioXcell) every 3 days for 3 weeks.

Assessment of renal pathology

Kidneys slides were prepared and stained with hematoxylin and eosin (H & E), and 2% aqueous uranyl acetate for electron microscopy as described previously[6]. Nephritis severity was assessed by histological analysis and scored blindly according to the criteria in **Appendix 2.1**.

Flow cytometry

To identify specific cell subsets, single cell suspension of splenocytes in ice-cold FACS buffer (2% FCS and 0.1% NaN_3 in PBS) were stained with the antibodies detailed below, all from BD unless specified, with thorough washing between stain layers. Flow cytometry data was acquired on flow cytometers (LSRII; BD) running FACSDiva and analyzed with Flowjo version 8 (Tree Star).

NP-binding cells were identified by staining with IgM Alexa Fluor 580 (conjugated in house, Invitrogen), NP-APC (conjugated in house[345]), CD138-PE, PNA-FITC (Vector Laboratories), B220-PE Cy7 and CD45.1-Pacific Blue. HEL-binding GC B cells were identified by first staining cells with 200 ng/ml HEL solution, followed by GL7-FITC, CD95-PE, CD45.2-PerCP Cy5.5, anti-HEL HyHEL9-Alexa Fluor 647 (conjugated in-house with an Alexa Fluor 647 antibody labeling kit; Invitrogen), B220-APC Cy7, and CD45.1-Pacific Blue (BioLegend). HEL^+ EFPBs were identified by treating spleens with collagenase and DNase; spleens were cut into ~1 mm^3 pieces and incubated at 37ºC for 30 min with 1 ml of 1 mg/ml type II collagenase and 0.1% DNase in RPMI/10% FCS. After washing in FACS buffer as above, cells were stained with

CD45.2-PerCP Cy5.5, B220-APC Cy7 and CD45.1-Pacific Blue, permeabilized using BD Cytofix/Cytoperm kit (BD) according to the manufacturer's instructions, and stained for intracellular HEL binding and IgG1 with HEL protein conjugated to Alexa Flour 647 as above and anti-mouse IgG1-FITC, respectively.

The presence of GC B cells in *S. enterica*-infected and uninfected mice was identified by staining cells with GL7-FITC, CD95-PE, CD19-Alexa Flour 700 (eBioscience), IgG2c-biotin, IgG1-APC, and B220-APC Cy7. NK cells were identified using NK1.1-APC, CD49b-PE, CD11b-APC and CD11c-PerCP Cy5.5 (Biolegend). T cell phenotypes were investigated using combinations of the following antibodies: CXCR5-biotin + streptavidin-PE Cy7 (eBioscience), PD-1-PE (eBioscience), IL21-APC (R&D Systems), CD4-APC Cy7, B220-FITC, CD44-Pacific Blue (BioLegend), CD25-PerCP Cy5.5, CD45.2-PerCP Cy5.5, CD4-PerCP, FoxP3-Alexa Flour 700 (eBioscience), T-bet-PerCP Cy5.5 (eBioscience), Ki-67-Alexa Flour 647, Bcl-6-Alexa Flour 647, IFN-γ-FITC, IL-4-PE Cy7 (eBiosicence), IL-17-Alexa Flour 700 and mouse IgG1 isotype control-Alexa Flour 647. For the detection of cytokine-producing Tfh cells, cells were stained with 1 h CXCR5-biotin and 30 min SA-PE Cy7 prior to PMA, Ionomycin and Golgi stop stimulation. For Annexin-V/7-aminoactinomycin D (7AAD; Invitrogen) staining, cells were incubated at 37°C for 2 h before staining for surface molecules and then Annexin-V-Pacific Blue (BioLegend) was added for 15 min in the dark at room temperature in Annexin-V binding buffer (BioLegend). Cells were stained with 7AAD immediately before data acquisition.

Statistical analysis

Two-way analysis of variance was used to interrogate whether IL-21R signaling was a significant contributor to variance over a time-course (**Figure 3.8 and 3.9**). For other experiments, nonparametric Mann-Whitney test (U-test) was used except mixed bone marrow chimeras experiments, in which paired Student's t-test was used; and *in vitro* culture experiments, in which unpaired *t* test was used. All statistical analysis was performed with Prism software (GraphPad Software). Statistically significant differences are indicated as * = $P \leq 0.05$, ** = $P \leq 0.01$; *** = $P \leq 0.001$; and ns = not significant. Unless otherwise specified, each dot in graphs corresponds to one biological replicate or an individual mouse and bars represent median values in each group.

Chapter 3 - 5

Results

Chapter 3 – B Cell Priming for Extrafollicular Antibody Responses Requires Bcl-6 Expression by T Cells

Chapter introduction

During thymus-dependent responses, B cells that make cognate interactions with T-cells in the outer T zone of secondary lymphoid tissues can differentiate along either follicular or extrafollicular pathways. In the follicular pathway, activated B cells form the GC, where they undergo somatic hypermutation, selection, and eventually exit as high affinity long-lived plasma cells or memory B cells[39]. In the extrafollicular pathway, B blasts migrate to the splenic bridging channels or junction zones at the border between the T zones and red pulp or the lymph node extramedullary cords, where they form foci of short-lived plasmablasts[49]. These plasmablasts provide a wave of early antibody that, although unmutated and generally of modest affinity, can be critical for protection against infection[51]. Bcl-6 expression in T cells is required for the formation of Tfh cells, which are essential to support GC reactions[136, 152, 153]. Whether Bcl-6 expression in T cells is required for B cell differentiation along the extrafollicular pathway is still unknown.

Bcl-6 and Blimp-1 are important transcriptional regulators of terminal differentiation of T and B cells; they are mutually repressive, and their reciprocal abundance appears to specify one or other cell fate when two differentiation pathways are possible. In B cells, Bcl-6 is essential for the development of GC B cells[174-176], whereas Blimp-1 is required for extrafollicular plasma cell formation[346]. T cells require Bcl-6 expression for upregulation of CXCR5, the receptor for the chemokine

CXCL13, which is produced by follicular DCs in B cell follicles and GC T cells themselves[159, 347]. Coordinated down-regulation of CCR7 and up-regulation of CXCR5 by T cells is required for the interactions at the T-B border that precede follicular migration[180, 347]. During this initial T-B cognate interaction, T cells provide signals that initiate Ig isotype switching [30, 343].

There is evidence to suggest that the nature of T cell help required to promote extrafollicular antibody responses may differ from that required to drive GC development. First, extrafollicular responses occur in response to pure polysaccharide antigens in the absence of T cell help, whereas T-independent GCs are only found in exceptional circumstances[348]. Second, while SAP expression in T cells is required for differentiation of Tfh cells and GC B cells[45, 172], extrafollicular antibody responses are less dependent on this adaptor molecule[6].

Although it is thought that Th1 and Th2 cells, which form in a Bcl-6-independent manner, can drive extrafollicular switched antibody responses, there is indirect evidence to suggest that Bcl-6 may play a role in these responses. Early antibody production is diminished in LCMV-infected mice expressing low levels of Bcl-6 in T cells as a consequence of Blimp-1 overexpression[152]. Also, in autoimmune lupus-prone MRL^{lpr} mice, T cells that share some but not all phenotypic markers of Tfh cells and are Bcl-6-dependent have been found in extrafollicular foci, and they appear to promote autoantibody production[76, 170]. In contrast, the early antibody response to protein antigen was reported to be intact in irradiated *Rag1*$^{-/-}$ mice reconstituted with Bcl-6-deficient bone marrow[175]. However, it is difficult to distinguish follicular from extrafollicular origin of antibody because most

immunization strategies do not result in development of only one or the other pathway of B cell differentiation. Furthermore, isotype switching is comparable in both pathways, and up to 25% of the plasma cells found in splenic extrafollicular foci after standard TD immunization protocols are of GC origin[349].

In this study, we report the results of experiments from several different models in which we can distinguish antigen-specific plasmablasts and/or antibodies of either extrafollicular or follicular origin. We show that Bcl-6-expressing T cells are essential for extrafollicular production of IgG1 in responses to the model protein antigens HEL and NP-KLH and for the production of *S. enterica*-specific IgG2c. Bcl-6-expressing helper T cells are initially situated at the interface between the B cell follicle and the T zone and, unlike Tfh cells located in the GC, do not express high levels of PD-1.

Results

Bcl-6-expressing PD-1^{lo} CD3^{+} T cells are seen at the T-B border early in the course of an antibody response

We began by investigating whether Bcl-6 expression is confined to GC T cells during T-dependent immune responses by assessing the distribution and kinetics of Bcl-6-expressing cells during a T-dependent response to SRBCs. In unimmunized mice, there were virtually no Bcl-6^{+} T cells detected outside rare background GCs (**Figure 3.1A**). Between days 2 and 3, CD3^{+} Bcl-6^{+} PD-1^{lo} cells were seen at the T-B border (**Figure 3.1B**). By day 7, CD3^{+} cells expressing higher amounts of Bcl-6 and PD-1 appeared within newly formed GCs (**Figure 3.1C**). As has been reported before[180, 213], most Bcl-6^{+} CD3^{+} PD-1^{hi} T cells were rarely seen at sites other than GC. We conclude that high expression of PD-1 identifies GC Tfh cells. Together, these results suggest that the T cells that interact with B cells at the outer T zone have up-regulated Bcl-6, raising the possibility that expression of Bcl-6 influences B cell commitment to both the follicular and the extrafollicular pathways.

Bcl-6 expression in T cells boosts T-dependent extrafollicular antibody responses to a haptenated protein

To evaluate the influence of T cell-expressed Bcl-6 on the early antibody response to NP-KLH, we constructed mixed chimeras in which sublethally-irradiated *Rag1*$^{-/-}$ mice were reconstituted with 80% *Bcl6*$^{-/-}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 fetal liver. *Cd28*$^{-/-}$ fetal liver cells help maintain the chimeras' health and provide a source of Bcl-6^{+} B cells and T cells that cannot differentiate into effector cells, including Tfh cells. A

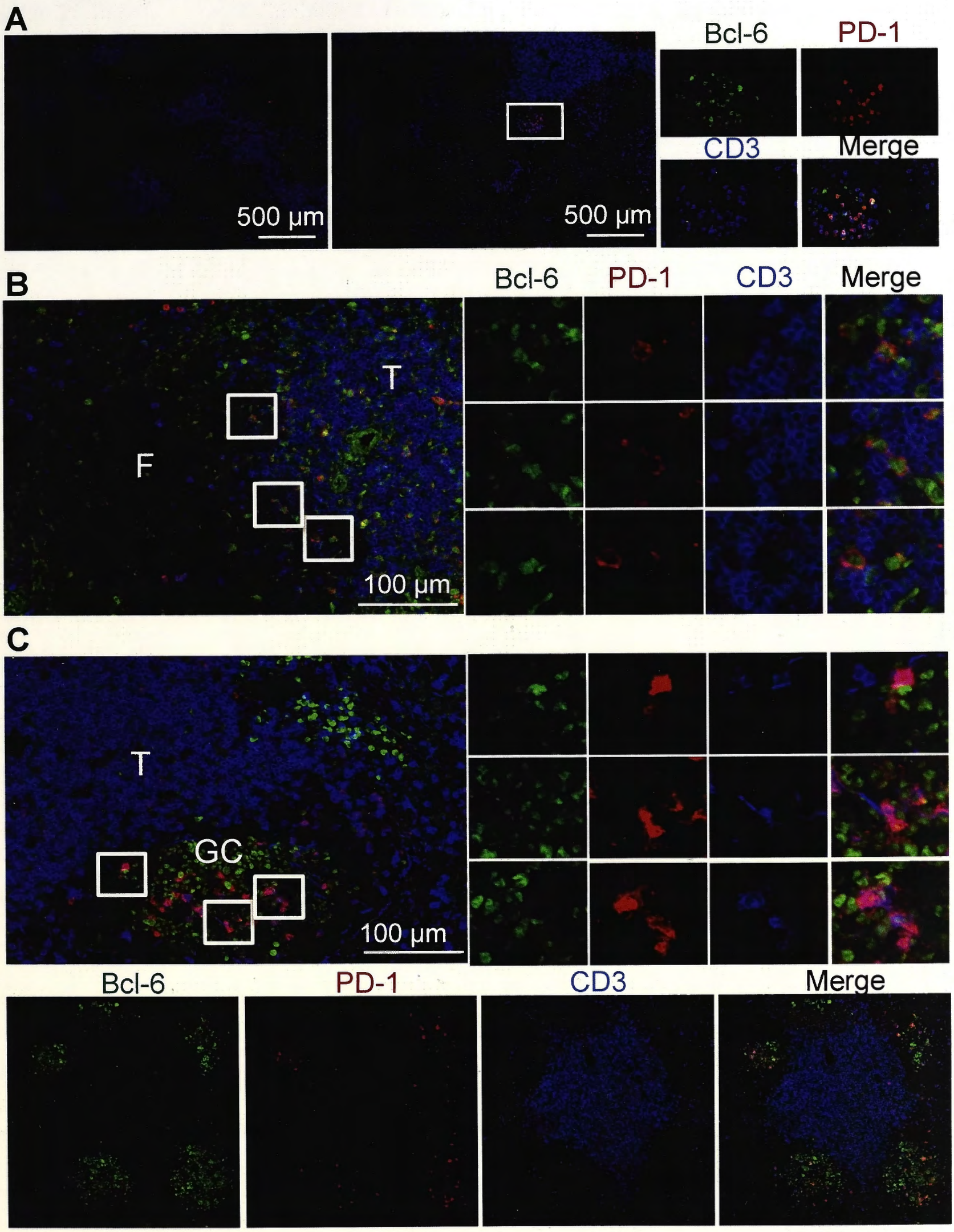


Figure 3.1. Bcl-6^{+} PD-1^{lo} CD3^{+} cells are seen at the T-B border after SRBC immunization.

Immunofluorescence stains of spleen sections from C57BL/6 mice at 0 (**A**), 3 (**B**) and 7 d (**C**) after SRBC immunization. Boxes areas indicate the location of the zoomed-in images on the right, in the same order (from top to bottom). These data are representative of two independent experiments with four mice per experiment. F, follicle; T, T zone.

control group was reconstituted with 80% *Bcl6$^{+/+}$* CD45.2:20% *Cd28$^{-/-}$* CD45.1 fetal liver.

Chimeric mice were immunized i.p. with NP-KLH in alum. 7 d after immunization, mice lacking functional Bcl-6-expressing T cells did not form GC (**Figure 3.2A and 3.2B**) or Tfh cells (**Figure 3.2C and 3.2D**). This concurs with previously published data showing that Bcl-6-expressing Tfh cells are required for the formation of GCs[136, 152, 153]. Mice lacking functional Bcl-6-expressing T cells had an ~50% reduction in switched Syndecan-1^{+} (CD138^{+}) plasma cells in the spleen (**Figure 3.3A and 3.3B**). NP-specific IgG1 titers were reduced by >40-fold, and a more modest reduction in NP-specific IgM titers was also observed (**Figure 3.4A**). By day 7, few extrafollicular foci containing IgG1^{+} plasma cells were visible (**Figure 3.4B**) in the spleens of chimeras lacking Bcl-6 expression in T cells. Together, these results suggest Bcl-6-expressing T cells are important for optimal extrafollicular antibody responses to protein antigens.

Bcl-6-expressing T cells are required for the development of antigen-specific extrafollicular plasmablasts (EFPBs)

Although the aforementioned results suggest a role for Bcl-6 in extrafollicular antibody responses, the origin of the antibody-producing cells cannot be precisely identified in this model. For example, an early wave of memory B cells that develops in parallel to the extrafollicular antibody response independently of GC, Bcl-6, ICOS, and IL-21 has been now described by several groups[43, 177, 192, 211, 350]. Another potential caveat of these experiments is that the absence of Bcl-6-expression in 80%

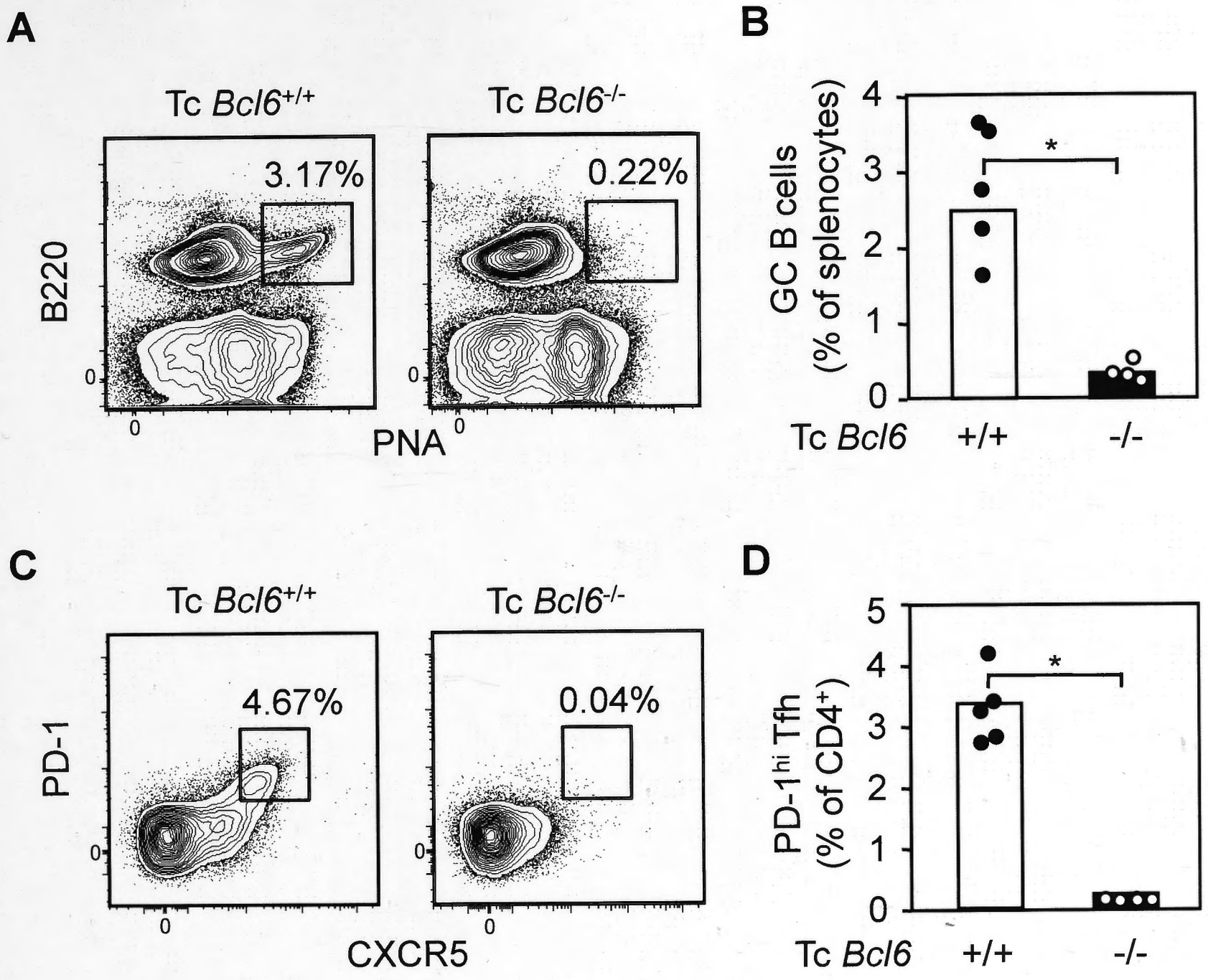


Figure 3.2. Reduced formation of GC B cell and Tfh cell to NP-KLH in the absence of T cell-expressed Bcl-6.

Representative flow cytometric contour plots (**A and C**) and quantification (**B and D**) of GC B cells identified as $B220^{+}$ PNA^{hi} splenocytes (**A and B**) and Tfh cells identified as $CXCR5^{hi}$ PD-1^{hi} (gated on $CD4^{+}$ cells; **C and D**) from 80% *Bcl6*$^{-/-}$ CD45.2:20% *Cd28*$^{-/-}$CD45.1 (i.e., effector T cells lack Bcl-6; Tc *Bcl6*$^{-/-}$) or control 80% *Bcl6*$^{+/+}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{+/+}$) fetal liver chimeric mice immunized 7 d earlier with NP-KLH. These data are representative of two independent experiments with five mice per group in each cohort. The experiment was performed by Dimitra Zotos.

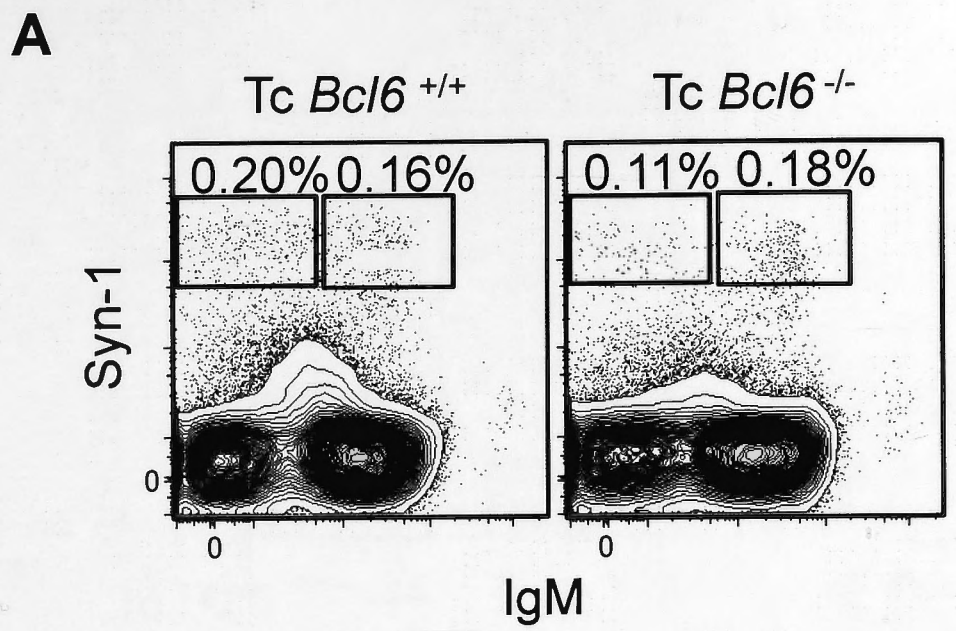


Figure 3.3. Reduced splenic switched plasma cells in mice lacking Bcl-6 expression in T cells.

Representative flow cytometric contour plots (**A**) and quantification (**B**) of switched (IgM^-) $CD138^+$ plasma cells in the 80% *Bcl6*$^{-/-}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{-/-}$) or control 80% *Bcl6*$^{+/+}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{+/+}$) fetal liver chimeric mice immunized 7 d earlier with NP-KLH. These data are representative of two independent experiments with five mice per group in each cohort. The experiment was performed by Dimitra Zotos.

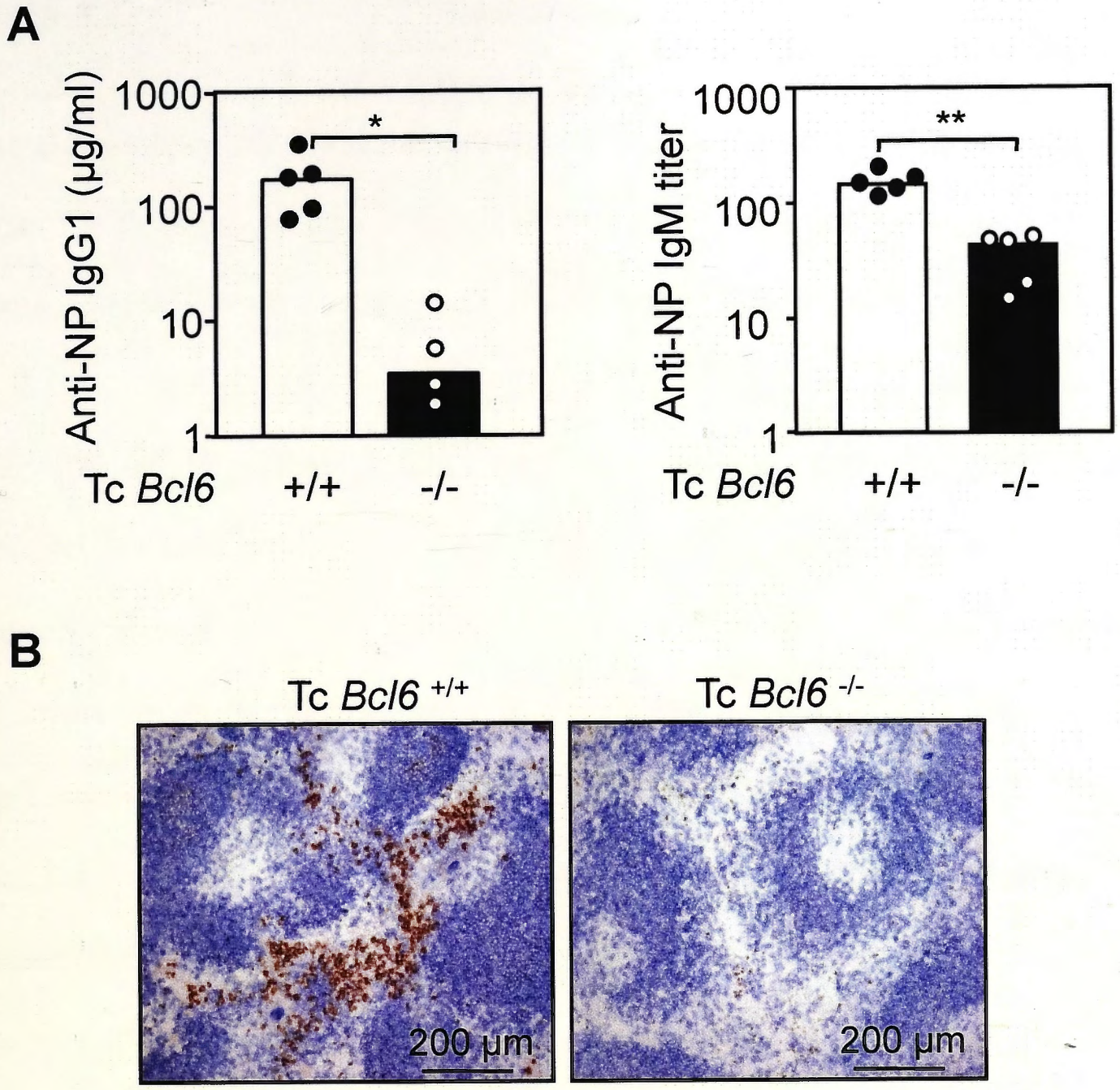


Figure 3.4. Reduced plasma cell responses to NP-KLH in the absence of T cell-expressed Bcl-6.

A) NP-specific IgG1 and NP-specific IgM antibodies from 80% *Bcl6*$^{-/-}$ CD45.2:20% *Cd28*$^{-/-}$CD45.1 (i.e., effector T cells lack Bcl-6; Tc *Bcl6*$^{-/-}$) or control 80% *Bcl6*$^{+/+}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{+/+}$) fetal liver chimeric mice immunized 7 d earlier with NP-KLH.
B) Photomicrographs of spleen sections stained with B220 (follicles; blue) and IgG1 (plasma cells; brown). These data are representative of two independent experiments with five mice per group in each cohort. The experiment was performed by Dimitra Zotos.

of the B cells may influence the extrafollicular plasma cells response, although Bcl-6-deficient B cells would be expected to give rise to enhanced rather than decreased plasma cell responses as the result of unopposed Blimp-1 expression.

To control for these possibilities and accurately quantify the number of EFPBs produced, we took advantage of the SW_{HEL} adoptive transfer system. CD45.1 SW_{HEL} transgenic B cells bearing a rearranged HEL-specific VDJ_H element targeted into the IgH chain locus combined with an HEL-specific κ L-chain transgene[351] were transferred i.v. into mixed *Bcl6*$^{-/-}$ CD45.2:*Cd28*$^{-/-}$ CD45.2 or *Bcl6*$^{+/+}$ CD45.2:*Cd28*$^{-/-}$ CD45.2 chimeric mice in combination with HEL protein conjugated to SRBC. Thus, transferred SW_{HEL} B cells have an intact *bcl6* gene, can undergo class switching and develop into both GC B cell and EFPB populations in response to HEL[41, 351]. In this model, GC B cells and EFPBs can be identified by flow cytometry[41, 351].

The development of HEL-binding, CD45.1 EFPBs and GC B cells was quantified 4.5 d after transfer. The gating strategies are shown in **Figure 3.5A**; GC B cells derived from transferred SW_{HEL} cells are CD45.1 B220^{+} GL-7^{hi} Fas^{hi} and HEL-binding. In the absence of Bcl-6-expressing T cells, the development of antigen-specific GC B cells was reduced to ~10% of the numbers found in the presence of Bcl-6-expressing T cells (**Figure 3.5B**), which is consistent with the previously described lack of functional Tfh cells in these chimeric mice[136].

The EFPB response derived from donor SW_{HEL} B cells can be measured by enumerating CD45.1^{+} B220^{lo} intracellular HEL-binding^{hi} cells[41, 351] (**Figure 3.6A**). In the absence of Bcl-6-expressing T cells, the percentage of EFPBs was reduced to

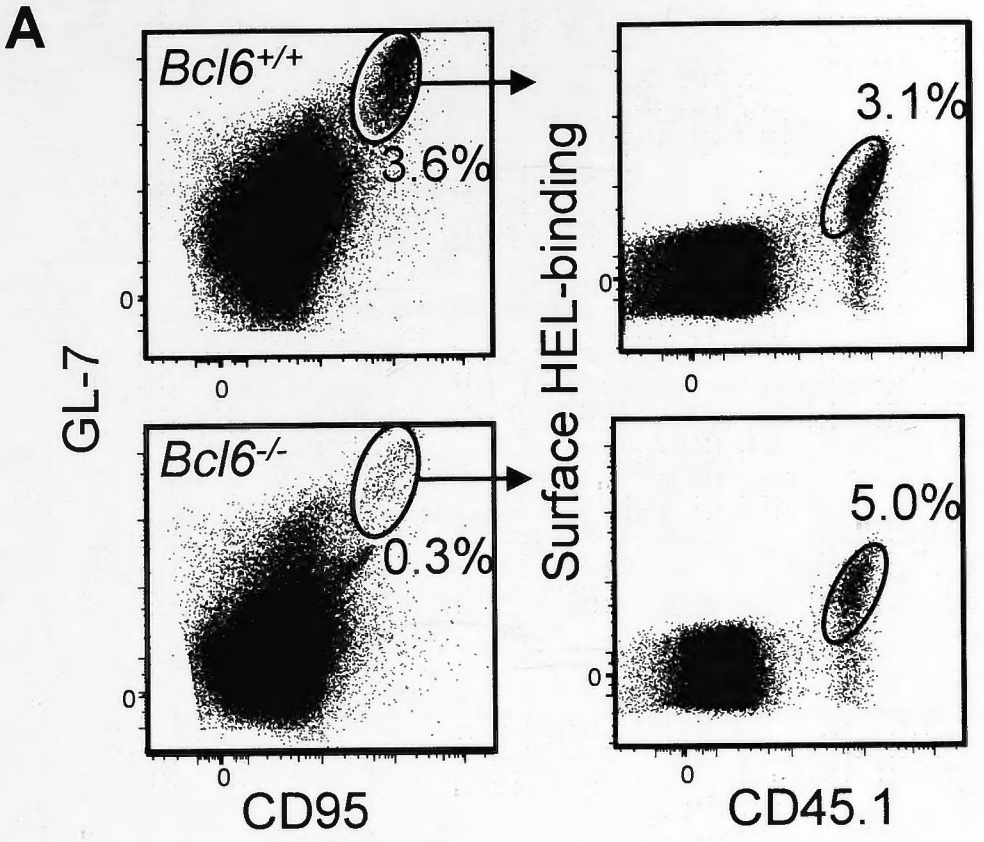


Figure 3.5. Development of HEL-specific GC B cells is greatly impaired in the absence of T cell-expressed Bcl-6.

Representative flow cytometric dot plots (**A**) and quantification (**B**) of SW_{HEL} GC B cells identified as $B220^+$ $GL\text{-}7^+$ Fas^+ HEL-binding CD45.1 cells in CD45.2 chimeric mice sufficient (Tc *Bcl-6*$^{+/+}$) or deficient (Tc *Bcl-6*$^{-/-}$) in Bcl-6-expressing T cells 4.5 d after adoptive transfer of SW_{HEL} B cells and HEL-SRBC immunization. Control (HEL^-) mice received SW_{HEL} B cells and were immunized with SRBCs that had not been conjugated to HEL. These data are representative of two independent experiments with five mice per group immunized with HEL-SRBC and two mice per group in the HEL-only control group for each independent experiment. The experiment was done with Robert Rigby.

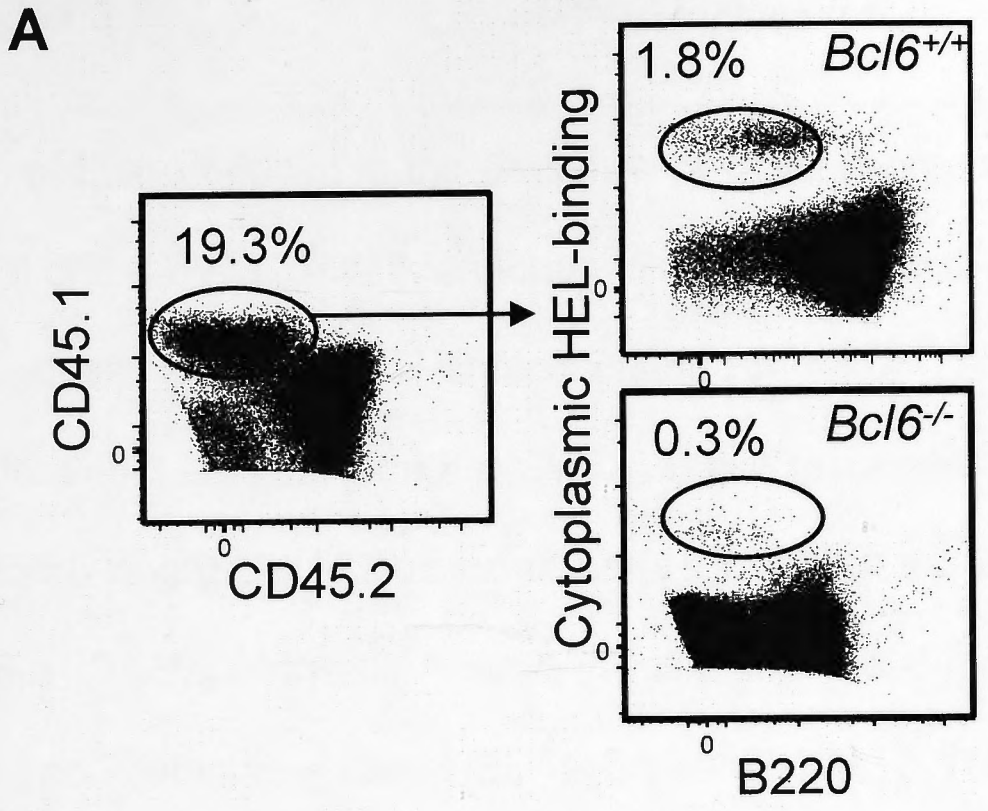


Figure 3.6. Development of HEL-specific EFPBs is greatly impaired in the absence of T cell-expressed Bcl-6.

Representative flow cytometric dot plots (**A**) and quantification (**B**) of EFPBs identified as CD45.1$^+$ B220^{lo} intracellular HEL-binding cells in CD45.2 chimeric mice sufficient (Tc *Bcl-6*$^{+/+}$) or deficient (Tc *Bcl-6*$^{-/-}$) in Bcl-6-expressing T cells 4.5 d after adoptive transfer of SW_{HEL} B cells and HEL-SRBC immunization. Control (HEL$^-$) mice received SW_{HEL} B cells and were immunized with SRBCs that had not been conjugated to HEL. These data are representative of two independent experiments with five mice per group immunized with HEL-SRBC and two mice per group in the HEL-only control group for each independent experiment. The experiment was done with Robert Rigby.

~5% of that produced in the presence of Bcl-6-expressing T cells (**Figure 3.6B**). On day 5 of the SW_{HEL} B cell response to HEL-SRBC, anti-HEL antibodies are produced exclusively by EFPBs[41]. Quantification of serum anti-HEL IgM and IgG1 (the predominant isotype found in SW_{HEL} B-cell responses to HEL-SRBC[351]) antibodies revealed negligible production of anti-HEL IgM in the mice lacking Bcl-6-expressing T cells (0.18% of the titer found in mice with Bcl-6-expressing T cells; **Figure 3.7**) with no detectable anti-HEL IgG1 antibodies. These results demonstrate a requirement of Bcl-6-expressing T cells for the production of switched and unswitched EFPBs in response to a TD antigen.

IL-21 acts early to promote follicular and extrafollicular antibody responses

IL-21 is produced by Tfh cells and acts directly on B cells to maximize Bcl-6 expression and promote GC B cell growth and survival[192, 211]. IL-21 also promotes Blimp-1 expression and plasma cell formation[216]. We compared side by side the effects of IL-21 in the course of a SW_{HEL} B cell-derived GC and EFPB response.

SW_{HEL} B cells sufficient or deficient for the IL-21R (*Il21r*$^{-/-}$) were transferred to CD45.1 congenic C57BL/6 mice, which were immunized with HEL^{2X}-SRBC. HEL^{2x} is a mutant HEL protein that binds the SW_{HEL} BCR with lower affinity. In the absence of IL-21R signaling, the production of GC B cells was reduced to ~12% of the levels seen with IL-21R-sufficient donor cells at day 4.5 (**Figure 3.8 and 3.9A**), which is consistent with the described dependency of GC B cells on IL-21. The development of EFPBs was also impaired in the absence of IL-21R signaling, with only around

10% of the cell numbers seen with IL-21R-sufficient donor cells at day 4.5 (**Figure 3.10A**). In the absence of IL-21-mediated signaling, the extrafollicular response peaked 12 hours later, on day 5. At this time point, there was a 4-fold reduction in the number of EFPBs in the absence of IL-21R (**Figure 3.8 and 3.9B**). The GC and EFPB responses to SRBCs elicited by the host CD45.1 *Il21r*$^{+/+}$ B cells were comparable in both groups of adoptively transferred mice[352] (**Figure 3.10B**).

The effects of IL-21 deficiency were apparent after 4 d in the course of both follicular and extrafollicular antibody responses (**Figure 3.9A and 3.9B**), suggesting that IL-21 produced by T cells acts at the stage of T-B interaction to enhance and accelerate B cell activation prior to their differentiation into either GC cells or extrafollicular plasma cells. IL-21 has recently been shown to promote GC B cell proliferation and survival, at least in part through maximizing Bcl-6 expression in B cells[192, 211]. Our findings extend this effect of IL-21 to extrafollicular antibody responses and are consistent both with reports that IL-21 also promotes Blimp-1 expression and plasma cells accumulate when overexpressed[216] and with the paucity of extrafollicular plasma cells 7 d after NP-KLH immunization in IL-21R and IL-21 knockout mice[211]. Although Bcl-6 and Blimp-1 are mutual antagonists by inhibiting each other's expression[427], IL-21 induced both Blimp-1 and Bcl-6[216]. This maybe explained by its ability to enhance B cell activation and differentiation at different stages of B cell maturation; IL-21 exerts effects on Ig production, isotype switching and plasma cell production in a context-regulated manner.

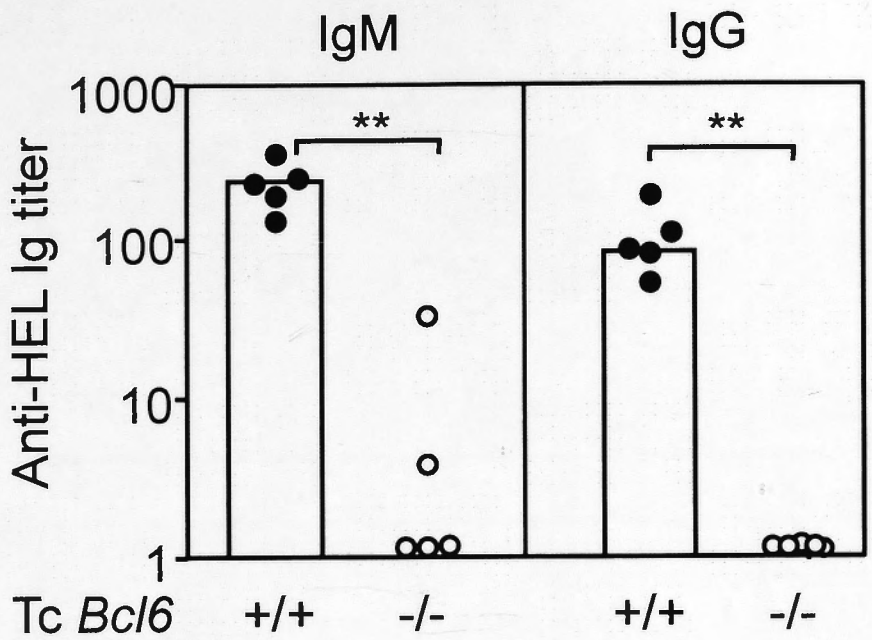


Figure 3.7. Reduced splenic plasma cells in mice lacking Bcl-6 expression in T cells.

HEL-specific antibody titers in the same HEL-SRBC immunized chimeric mice as in Figure 3.5 and 3.6. These data are representative of two independent experiments with five mice per group immunized with HEL-SRBC and two mice per group in the HEL-only control group for each independent experiment.

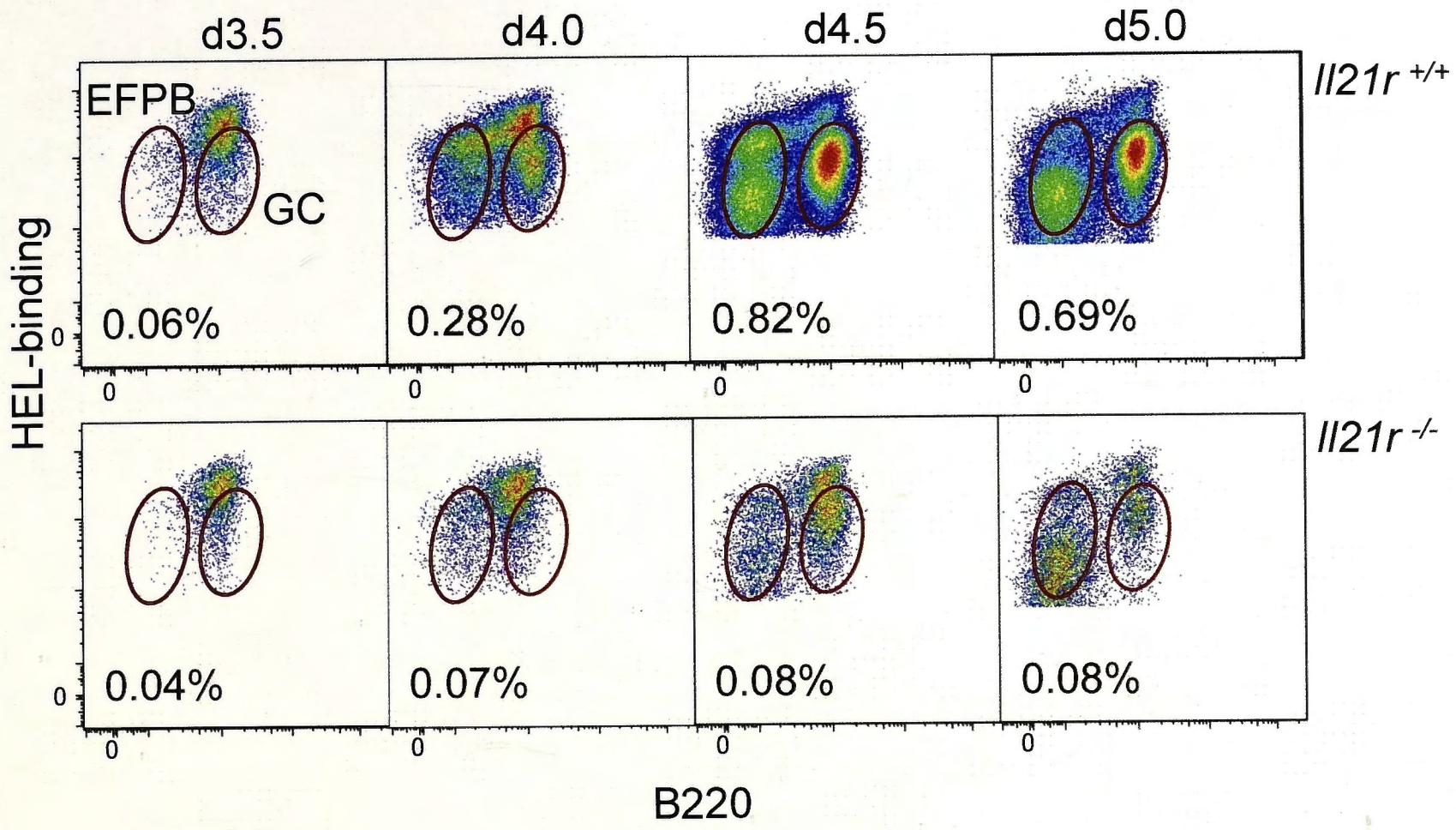


Figure 3.8. IL-21 influences GC and EFPB responses.

Representative flow cytometric dot plots showing HEL-binding versus B220 staining of *Il21r*$^{+/+}$ or *Il21r*$^{-/-}$ CD45.2 SW_{HEL} B cells at the indicated days after transfer into congenic (CD45.1) C57BL/6 recipients immunized with HEL^{2X}-SRBC at the time of transfer. The gates in each plot are drawn around GC B cells ($B220^{hi}$; right) and EFPBs ($B220^{lo}$; left). Numbers in plots indicate the percentage of HEL-binding CD45.2 SW_{HEL} cells out of total splenocytes. These data are representative of two independent experiments with three mice per group per time point. The experiment was performed by Tyani Chan.

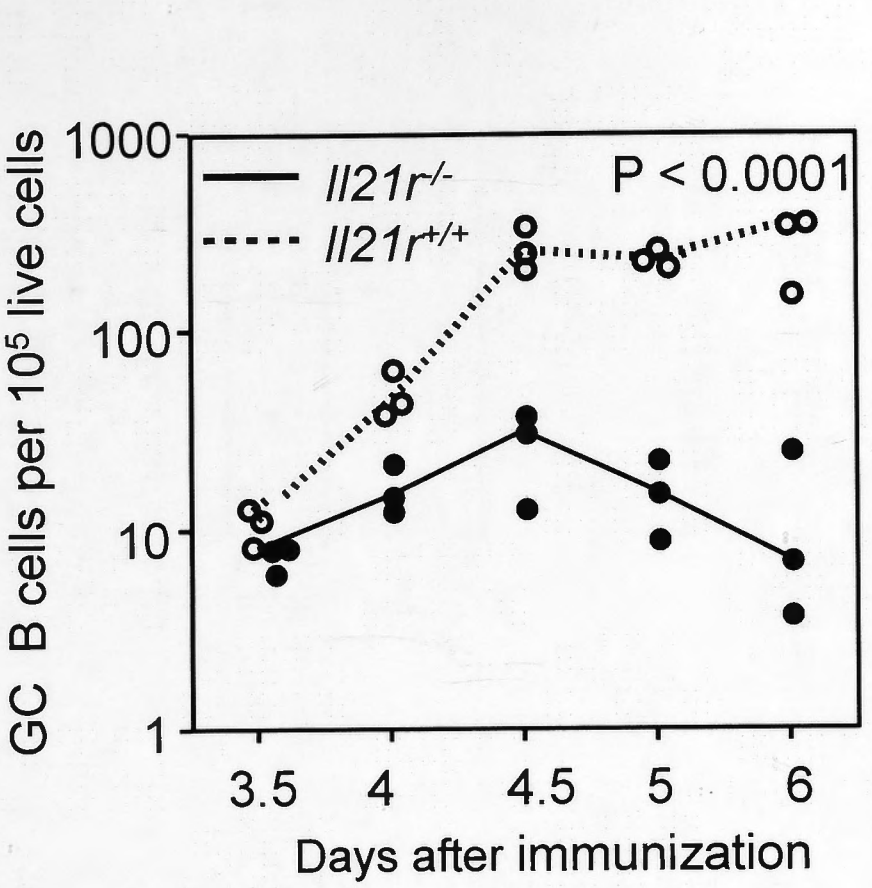
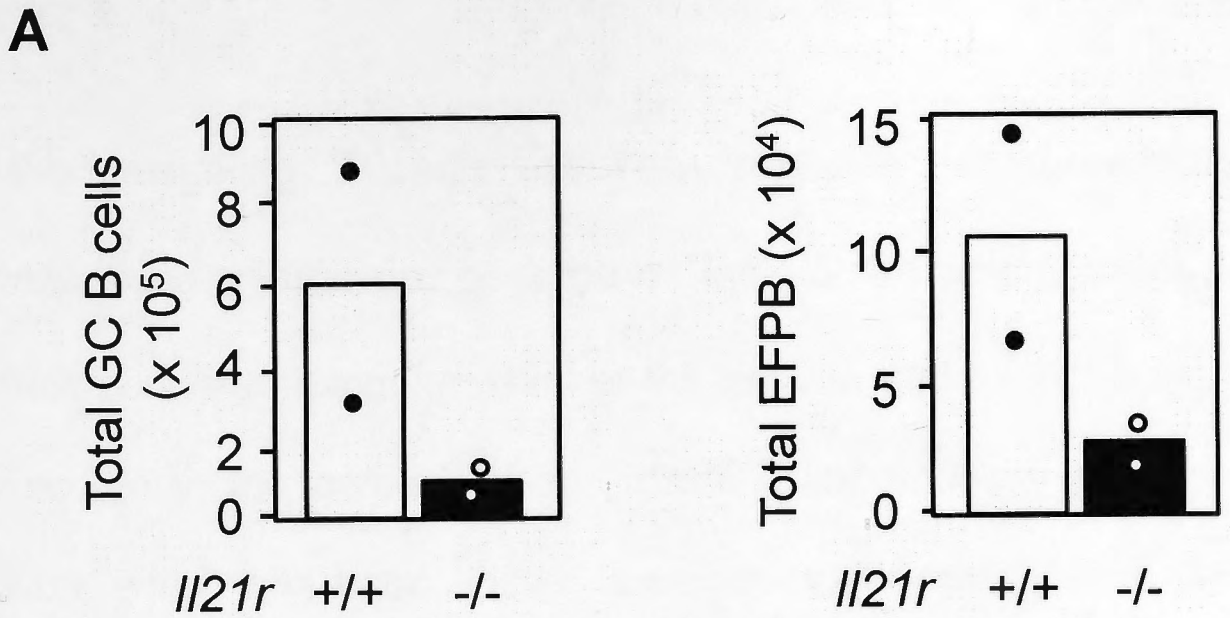


Figure 3.9. IL-21 influences GC and EFPB responses from the early stages of B cell activation.

Number of SW_{HEL} GC B cells (**A**) and EFPBs (**B**) in the same mice using the gates shown in Figure 3.8. These data are representative of two independent experiments with 3 mice per group per time point. The P-values were calculated using a two-way analysis of variance that interrogates the variance over the entire time course between the two groups of mice receiving either *Il21r*$^{+/+}$ or *Il21r*$^{-/-}$ SW_{HEL} B cells. The experiment was performed by Tyani Chan.



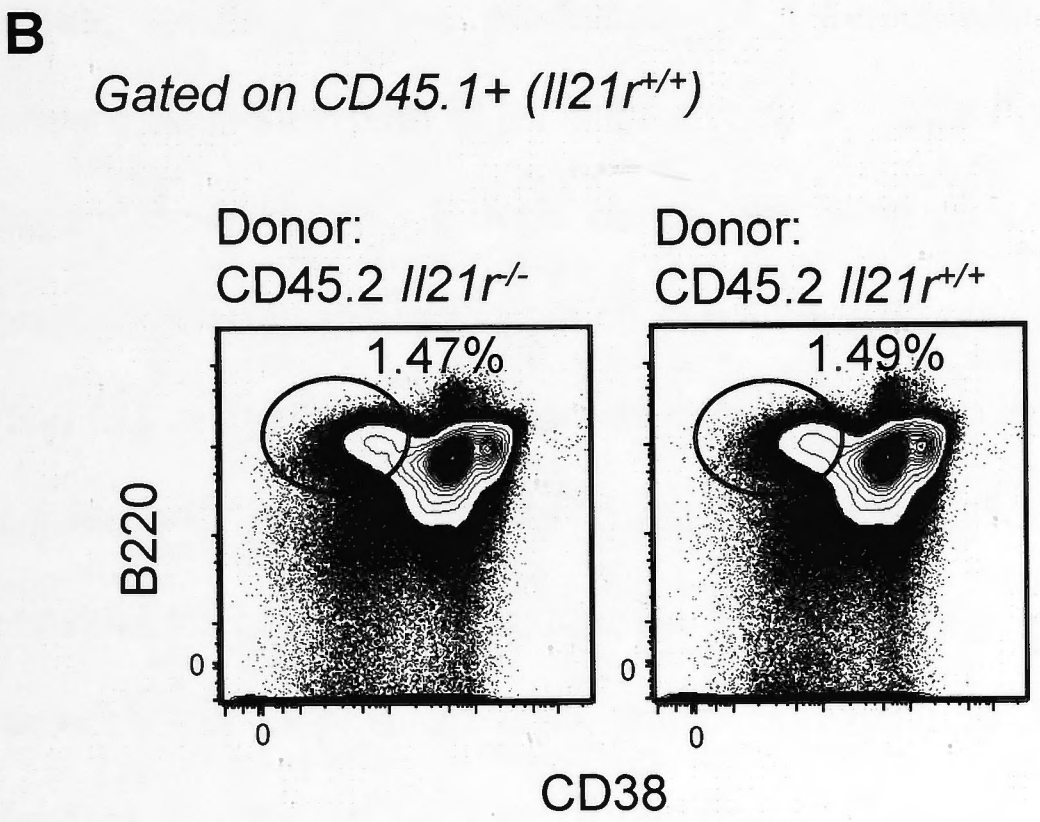


Figure 3.10. Host-derived GC formation in mice receiving *Il21r*$^{+/+}$ or *Il21r*$^{-/-}$ SW_{HEL} B cells after SRBC immunization.

A) Total numbers of donor-origin *Il21r*$^{+/+}$ or *Il21r*$^{-/-}$ SW_{HEL} GC B cells and EFPBs at day 5 after immunization.
B) Representative flow cytometric plots showing endogenous GC B cells identified as B220^{+} CD38^{lo} in CD45.1 recipient mice. These data are representative of two independent experiments with two to three mice per group per time point. The experiment was performed by Tyani Chan.

Bcl-6-expressing T cells are required for the development of class-switched extrafollicular plasma cells in *S. enterica* infection

We next sought to examine whether the requirement for Bcl-6 expression in T cells also occurs in the context of an extrafollicular antibody response to *S. enterica* infection, which has been shown to occur independently of IL-21[192]. The immune response to infection with *S. enterica* induces a potent extrafollicular plasma cell response, resulting in the production of T-independent IgM antibodies and T-dependent class switching of B cells to produce IgG2a (IgG2c in C57BL/6 mice) and IgG2b antibodies [213, 353]. T dependency has been previously demonstrated by the absence of *S. enerica*-specific switched antibody responses in mice lacking CD28[213, 353]. The GC response is considerably delayed, developing between days 20 and 35 after infection[344]. It has also been established that IFN-γ-producing Th1 cells are responsible for bacterial clearance, starting on week 2 after immunization[354]. Thus, *S. enterica* infection is an ideal model in which to investigate whether Bcl-6-expressing T cells rather than conventional Th1 cells are required to drive the extrafollicular plasma cell responses without the potential interference of GC B cell-derived antibodies or GC Tfh cells.

Bcl6$^{+/+}$ CD45.2:*Cd28*$^{-/-}$ CD45.1 or *Bcl6*$^{-/-}$ CD45.2:*Cd28*$^{-/-}$ CD45.1 mixed fetal liver chimeric mice were infected i.p. with *S. enterica.* As shown previously[344], this immunization did not increase GC cells above background 7 d after infection (**Figure 3.11A and 3.11B**). At this same time point, anti-*S. enterica* IgM and IgG2c titers were measured by ELISA. Bcl-6 deficiency in T cells did not reduce the production of specific IgM (**Figure 3.12A**) but significantly reduced the titer of anti-*S. enterica* IgG2c antibodies (**Figure 3.12B**). This mirrored the requirement of CD28 for specific

IgG2c but not IgM production in a cohort of C57BL/6 *Cd28*$^{-/-}$ mice (**Figure 3.12A and 3.12B**). Together, these results suggest that switched antibody responses to *S. enterica* require help from T cells in a Bcl-6-dependent manner.

Our data so far suggest that Bcl-6-expressing T cells rather than conventional Th1 cells are required for extrafollicular antibody responses. To confirm the presence of functional *S. enterica*-specific Th1 cells in mice lacking Bcl-6 expression in T cells, we investigated bacterial clearance, which has been shown to be critically dependent on the presence of functional Th1 cells[354]. Although bacterial counts were increased by nearly 2 logs in nonchimeric *Cd28*$^{-/-}$ mice that lack Th1 cells (**Figure 3.13A**), lack of Bcl-6 expression in T cells did not alter bacterial load in the livers of chimeric mice 12 d after infection (**Figure 3.13A**).

Next, we investigated when and where Bcl-6^{+} T cells appear during the response to *S. enterica.* A population of CD3^{+} Bcl-6^{+} PD-1$^{lo/-}$ cells was detected at outer T zone on day 4 after infection (**Figure 3.15A and 3.16**), with total numbers increasing ~10-fold above background. This is consistent with our previous finding of a 20-fold increase in numbers of IgG2c switched plasma cells as early as day 4 after *S. enterica* infection[344]. At this time point (day 4), CD3^{+} Bcl-6^{+} PD-1^{hi} cells were absent (**Figure 3.15A and 3.16**), but they became visible 35 d after immunization, when *S. enterica*-induced GCs appear, and they were located within GC (**Figure 3.15B and 3.16**). Together, these results highlight a necessary role for T cell-expressed Bcl-6 soon after infection to promote switching. Given that the IgG2c response occurs normally in the absence of IL-21 but requires IFN-γ, it is likely that early Th1 cells

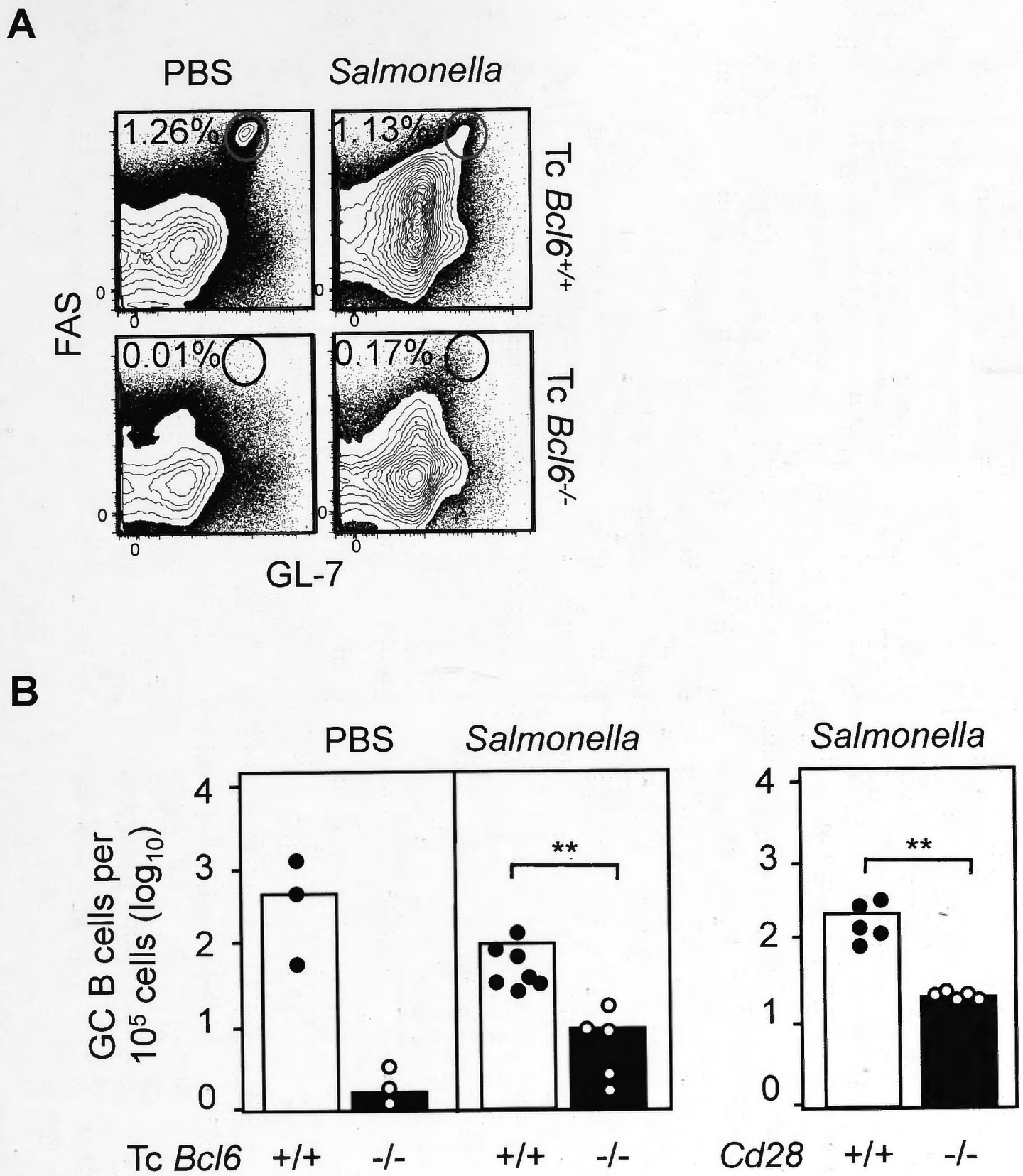


Figure 3.11. GC responses to *S. enterica* are impaired in the absence of T cell-expressed Bcl-6.

A) Representative flow cytometric plots showing Fas versus GL-7 stains from spleens of 80% *Bcl6*$^{-/-}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{-/-}$) chimeras or control 80% *Bcl6*$^{+/+}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{+/+}$) fetal liver chimeric mice injected 7 d previously with *S. enterica*; oval gates identify GC B cells.
B) Bar graphs show the number of splenic GC B cells in the same mice (left) and a group of *Cd28*$^{-/-}$ and control *Cd28*$^{+/+}$ mice infected with *S. enterica* at the same time (right). These data are representative of three independent experiments with three mice per PBS-only control groups and five to seven mice per *S. enterica*-infected groups.

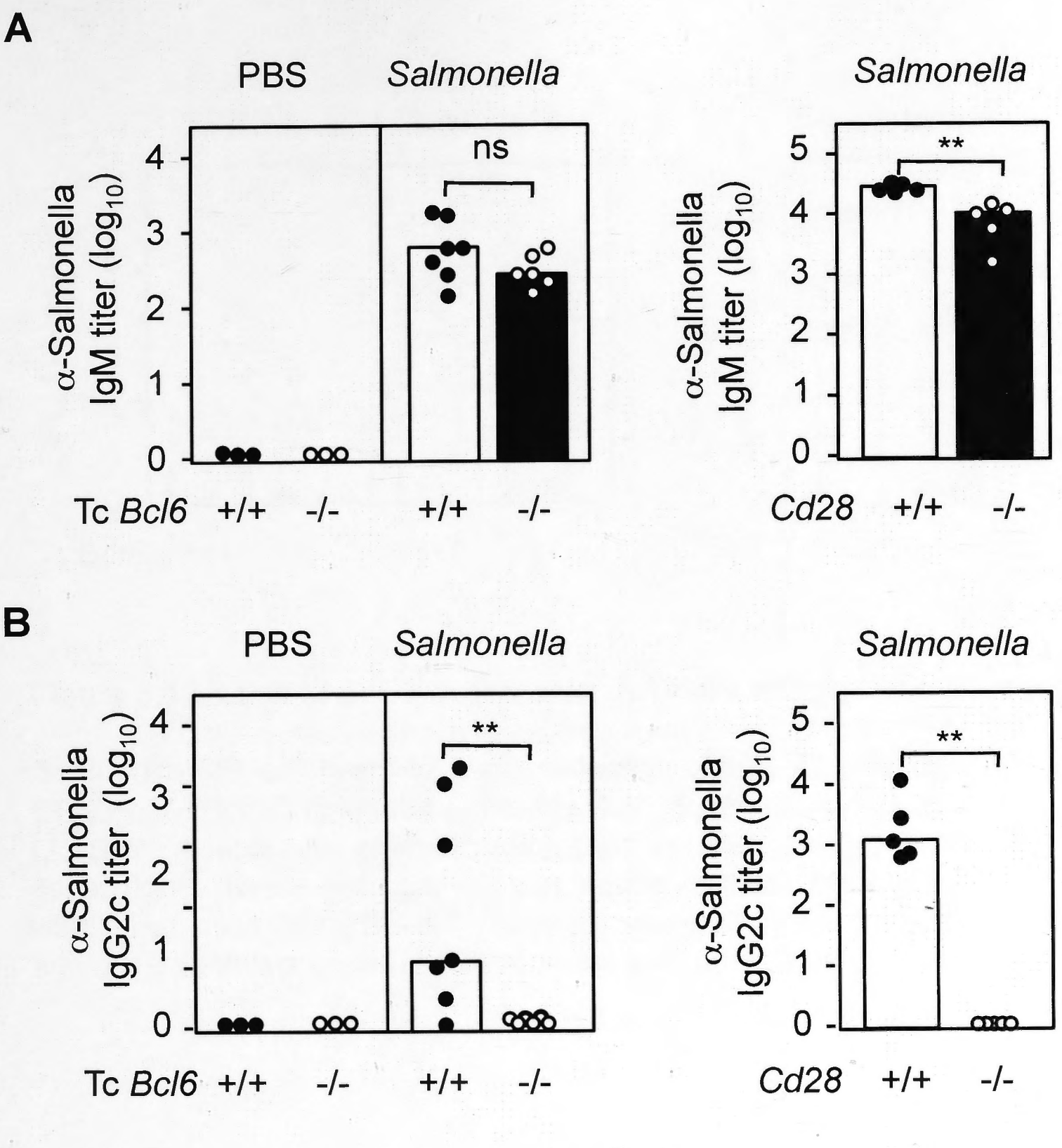


Figure 3.12. IgG2c extrafollicular antibody responses to *S. enterica* are impaired in the absence of T cell-expressed Bcl-6.

S. enterica-specific IgM (**A**) and IgG2c (**B**) antibody titers 7 d after *S. enterica* infection in 80% *Bcl6*$^{-/-}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{-/-}$) chimeras or control 80% *Bcl6*$^{+/+}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{+/+}$) fetal liver chimeric mice, or nonchimeric *Cd28*$^{-/-}$ and *Cd28*$^{+/+}$ mice. These data are representative of three independent experiments with three mice per PBS-only control groups and five to seven mice per *S. enterica*-infected groups. The experiment was performed by Roybel Ramiscal.

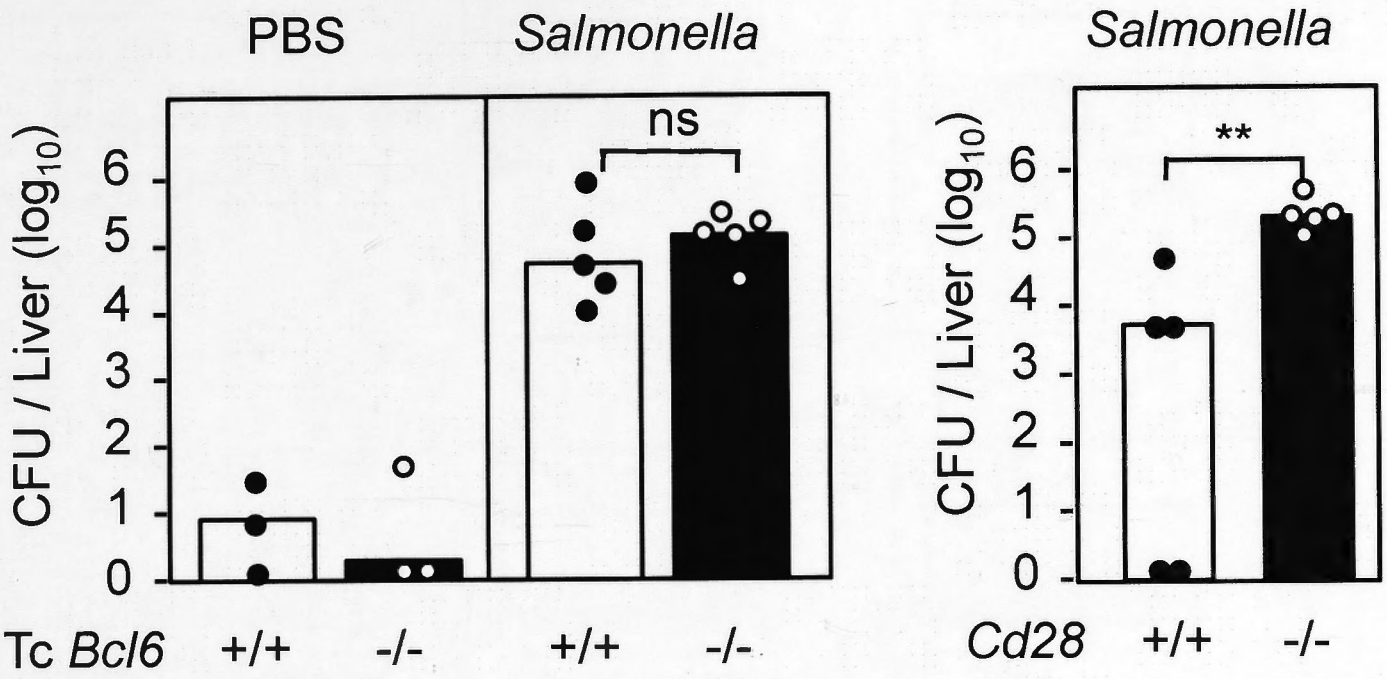


Figure 3.13. Lack of Bcl-6 expression in T cells did not alter bacterial load.

S. enterica CFUs in livers 12 d after infection of 80% *Bcl6*$^{-/-}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{-/-}$) chimeras or control 80% *Bcl6*$^{+/+}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{+/+}$) fetal liver chimeric mice (left) or nonchimeric *Cd28*$^{-/-}$ and *Cd28*$^{+/+}$ mice (right). These data are representative of three independent experiments with three mice per PBS-only control groups and five to seven mice per *S. enterica*-infected groups. The experiment was performed by Roybel Ramiscal.

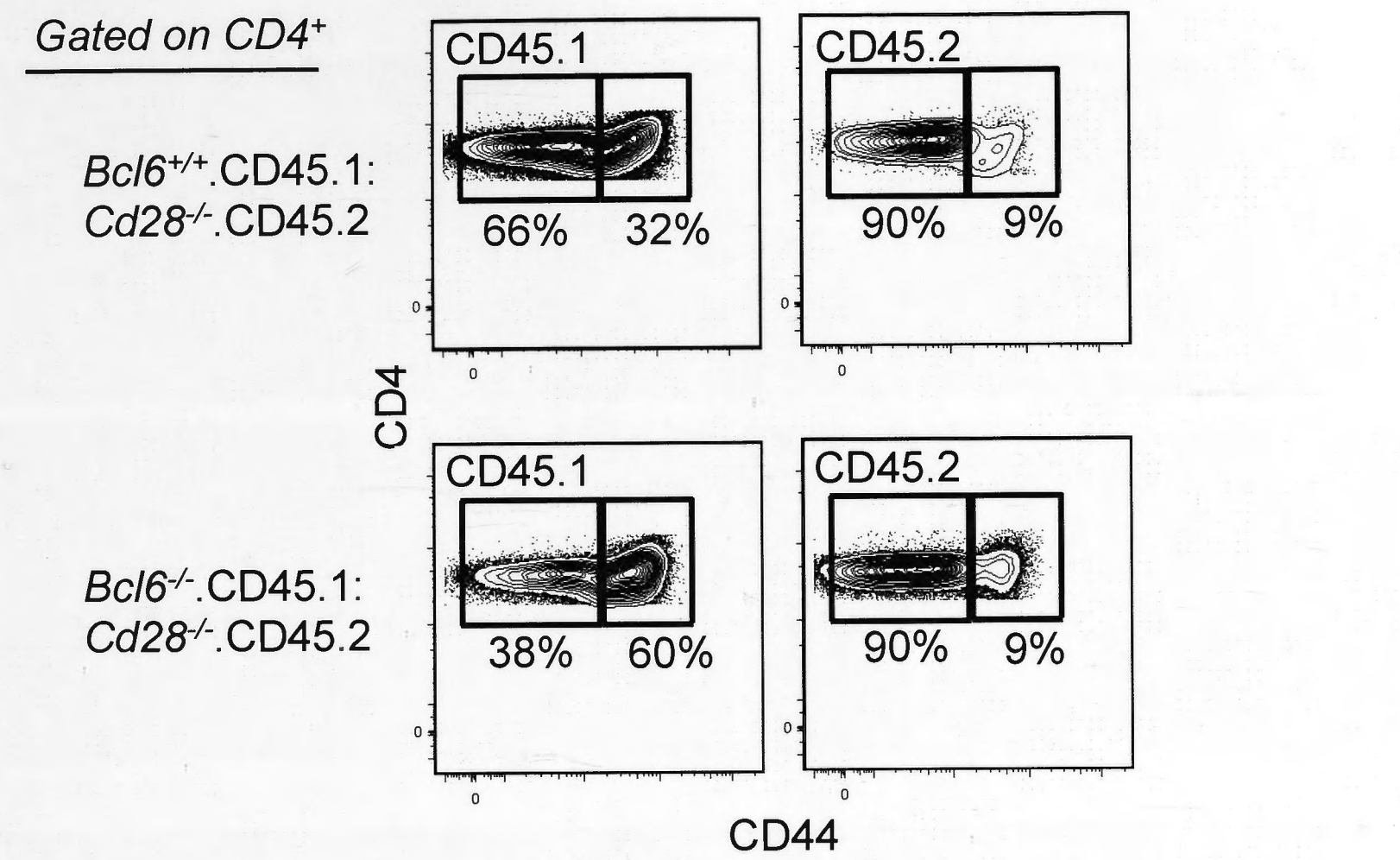


Figure 3.14. Increased proportion of effector cells amongst Bcl-6$^{-/-}$ T cells from mixed chimeric mice.

A) Representative flow cytometric plots showing CD4 versus CD44 staining of spleens from unimmunized 80% *Bcl6*$^{-/-}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{-/-}$) or control 80% *Bcl6*$^{+/+}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{+/+}$) fetal liver chimeric mice.
B and C) Proportion of CD44^{lo} (left) and CD44^{hi} (right) cells gated on CD45.1 (B) and CD45.2 (C). These data are representative of three independent experiments with three mice per PBS-only control groups and five to seven mice per *S. enterica*-infected groups.

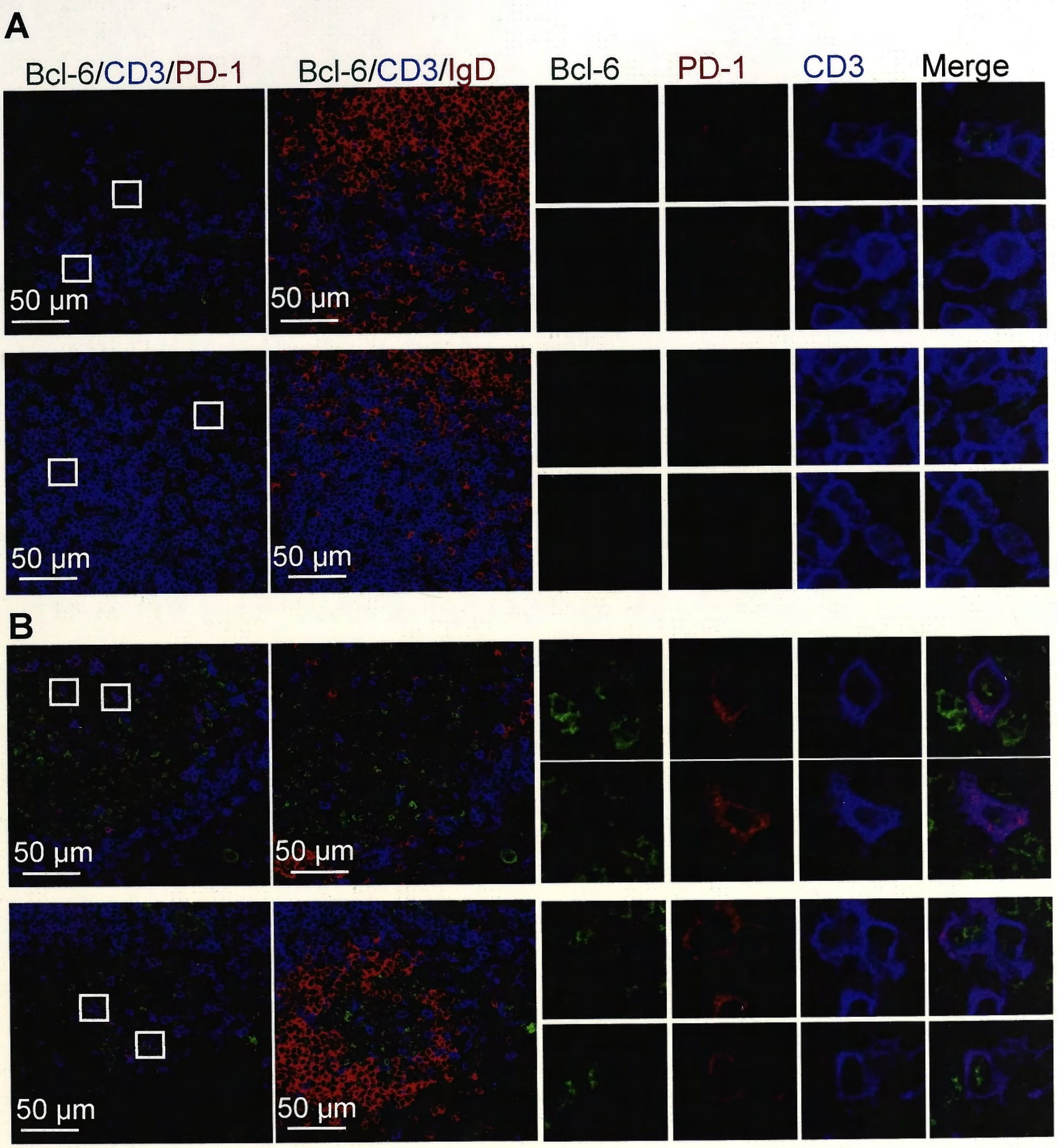


Figure 3.15. Bcl-6^{+} T cells induced by *S. enterica* infection are PD-1^{lo} and locate to the T-B border.

Immunofluorescence stains of spleen sections from C57BL/6 immunized 4 (**A**) or 35 d (**B**) previously with *S. enterica*. Boxes areas indicate the location of the zoomed-in images on the right, in the same order (from top to bottom). These data are representative of two independent experiments with four mice per group in each cohort. The experiment was performed by Jennifer Marshall.

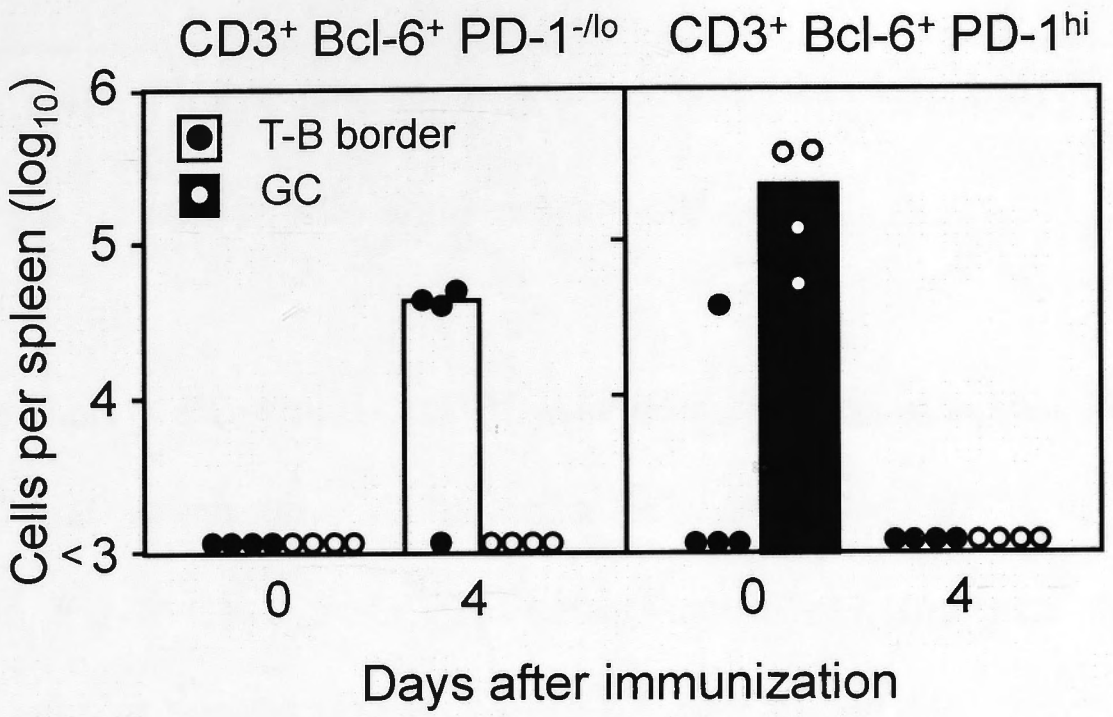


Figure 3.16. Bcl-6$^+$ T cells induced by *S. enterica* infection are PD-1^{lo}.

Quantification of PD-1^{lo} Bcl-6$^+$ CD3$^+$ (left) and PD-1^{hi} Bcl-6$^+$ CD3$^+$ (right) cells in the T-B border and GCs at the indicated times after immunizations. These data are representative of two independent experiments with four mice per group in each cohort. The experiment was performed by Jennifer Marshall.

producing IFN-γ up-regulate Bcl-6 that facilitates migration to the T-B border and thus cognate interaction with antigen-primed B cells.

The expression of PD-1 and CXCR5 was evaluated on effector (CD4^{+} CD44^{hi}) cells. On day 7, at which time *S. enterica* fails to induce GCs regardless of Bcl-6 expression, PD-1^{hi} CXCR5^{hi} GC Tfh cells (**Figure 3.17**, red gate; and **Figure 3.18C**) that were seen at background levels in PBS-immunized chimeras were low or absent after *S. enterica* immunization in all chimeras. Immunized chimeras lacking Bcl-6 expression in T cells selectively lacked a PD-1^{lo} CXCR5^{+} population (**Figure 3.17**, blue gate; and **Figure 3.18B**), which is likely to correlate with those cells shown in **Figure 3.1B** that localize to T-B border and prime B cells. Non-Tfh effectors identified as CD4^{+} CD44^{hi} CXCR5^{-} PD-1^{-} (**Figure 3.17**, green gate; and **Figure 3.18A**) were equally represented in both sets of chimeras, irrespective of T cell expression of Bcl-6. Together, these data suggest that CD4^{+} CD44^{hi} CXCR5^{+} Bcl-6^{+} PD-1^{lo} cells may be important for the initiation of an extrafollicular antibody response.

Bcl-6 is required for a T and B cell antigen-specific extrafollicular antibody response

A potential confounding factor in the aforementioned experiments is the increased proportion of activated *Bcl6*$^{-/-}$ CD44^{hi} CD4^{+} cells in mixed chimeras reconstituted with *Bcl6*$^{-/-}$ fetal liver (**Figure 3.14A-C**). To overcome this issue, we performed adoptive transfers of 10^5 naïve CD45.2 OT-II cells that express a transgenic TCR specific for OVA peptide 323-339 and were either deficient or sufficient in Bcl-6 into CD45.1 *Cd28*$^{-/-}$ mice. Recipient mice also received 10^5 SW_{HEL} B cells and were immunized

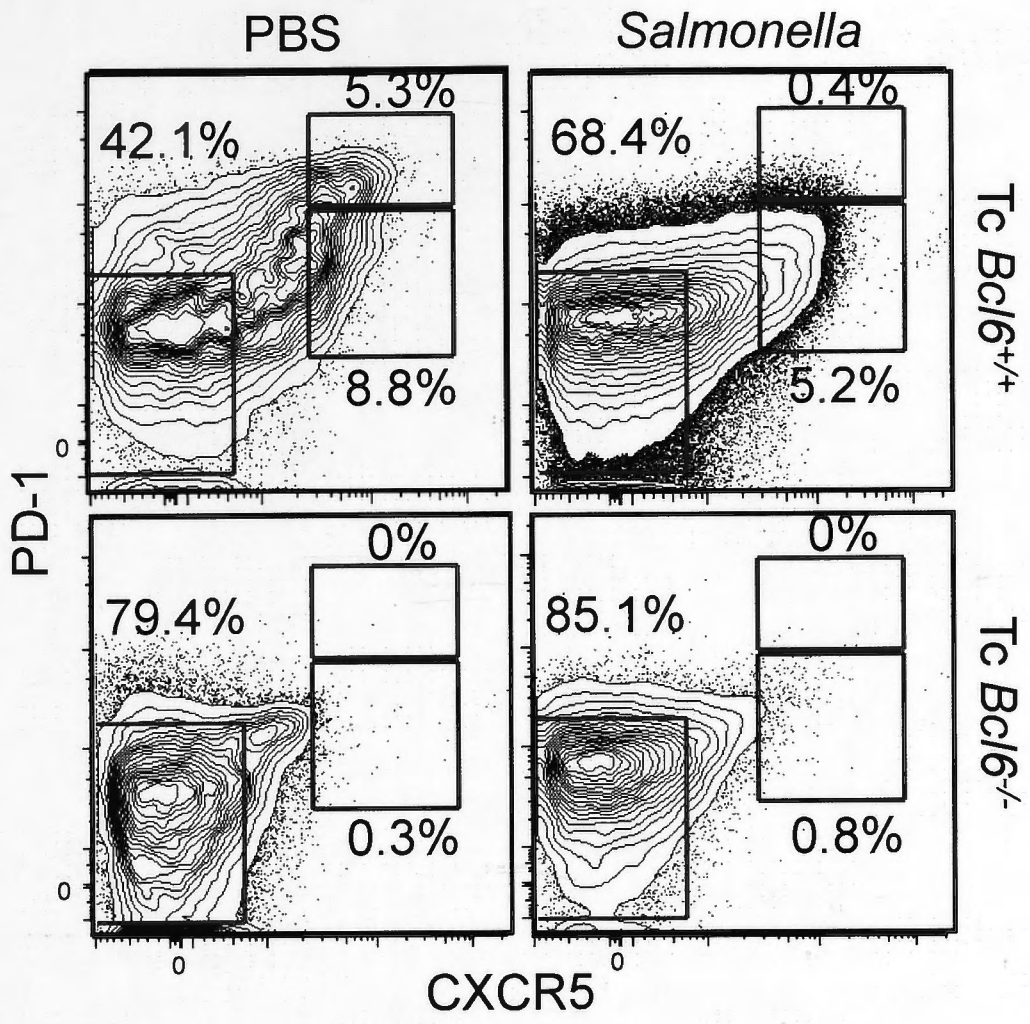


Figure 3.17. PD-1^{lo} CXCR5^{+} cells are reduced in the absence of T cell-expressed Bcl-6 7 d after *S. enterica* infection.

Representative flow cytometic plots showing PD-1 versus CXCR5 expression on effector/memory CD4^{+} CD44^{hi} cells from 80% *Bcl6*$^{-/-}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{-/-}$) or control 80% *Bcl6*$^{+/+}$ CD45.2:20% *Cd28*$^{-/-}$ CD45.1 (Tc *Bcl6*$^{+/+}$) fetal liver chimeric mice injected 7 d previously with *S. enterica* (green gate, non-Tfh effectors; blue gate, PD-1^{lo} Tfh cells; and red gate, PD-1^{hi} Tfh cells). These data are representative of two independent experiments with four mice per group in each cohort.

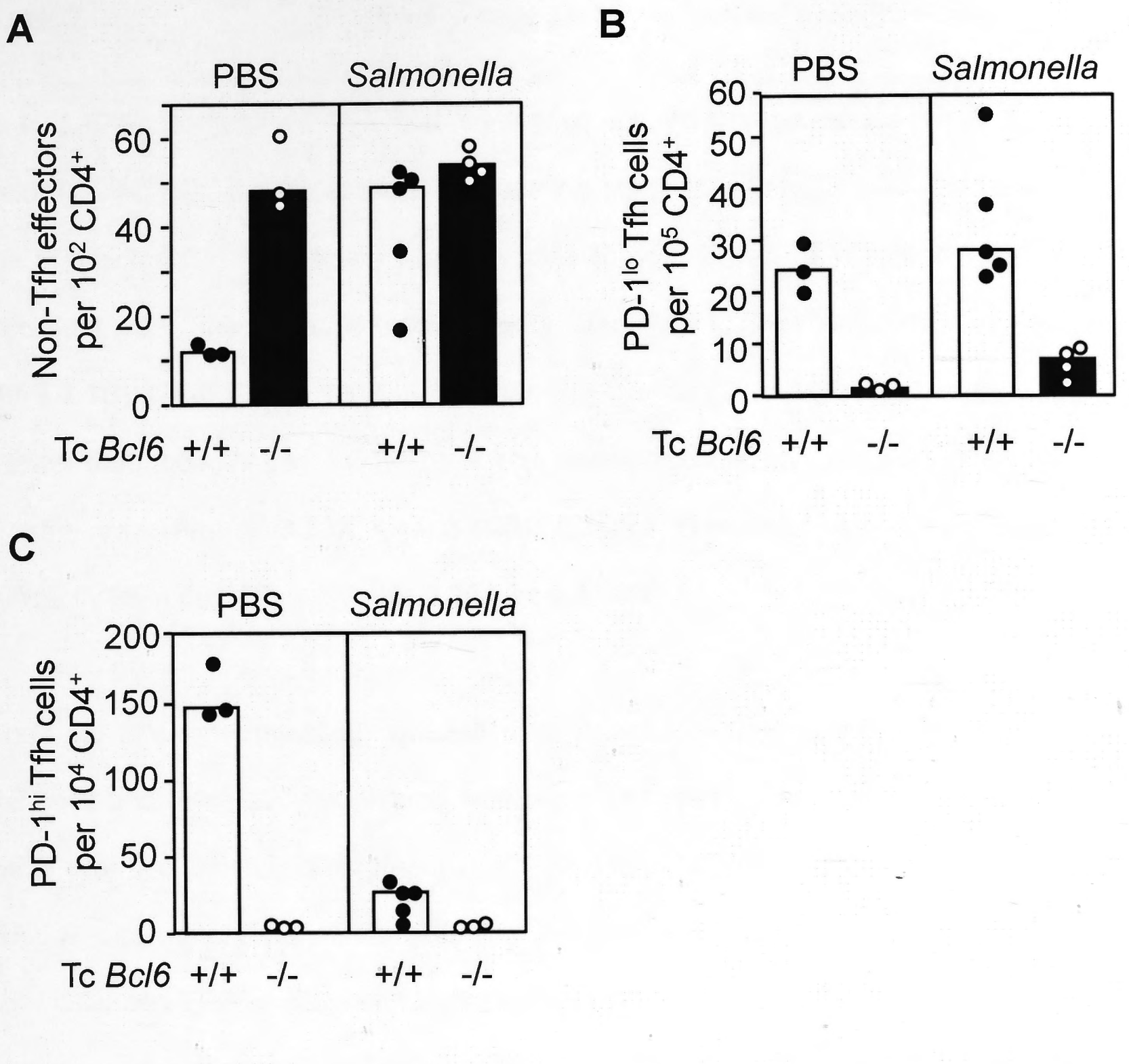


Figure 3.18. PD-1^{lo} $CXCR5^+$ cells are reduced in the absence of T cell-expressed Bcl-6.

Frequencies of non-Tfh effectors (**A**), PD-1^{lo} Tfh cells (**B**) and PD-1^{hi} Tfh cells (**C**) 7 d after *S. enterica* infection. Gating strategy is described in Figure 3.17. These data are representative of three independent experiments with three mice per PBS-only control groups and five to seven mice per *S. enterica*-infected groups.

with HEL-OVA conjugates. We first compared the phenotype of Bcl-6-sufficient effector (CD44^{hi}) OT-II cells at the B cell priming stage (pre-GC Tfh, day 2) and the stage in which GC Tfh cells are readily visible (day 4; **Figure 3.19A and 3.19B**). Concordant with the immunohistochemistry data from SRBC-immunized mice (**Figure 3.1B**), OT-II T cells had up-regulated PD-1 on day 2, but the levels were ~3-fold lower than those of day 4 OT-II cells from immunized recipient mice analyzed on the same day (**Figure 3.20A and 3.20B**). CXCR5 expression did not increase significantly from day 2 to 4 (**Figure 3.20A and 3.20B**).

On day 5.5 after immunization, spleens were harvested, and distinct HEL-specific GC B cells and extrafollicular plasma cells were identified by flow cytometry on the basis of size and differential expression of B220, CXCR5, and HEL-binding (**Figure 3.21A**). Recipients of *Bcl6*$^{-/-}$ OT-II cells showed a parallel and comparable reduction in both GCs and EFPBs (**Figure 3.21B and 3.21C**). Staining with PD-1 and CXCR5 demonstrated the absence of PD-1^{hi} CXCR5^{hi} Tfh cells among OT-II *Bcl6*$^{-/-}$ T cells on day 5.5 (**Figure 3.22A and 3.22B**). These results confirm that T cell-expressed Bcl-6 plays a role in B cells differentiating along the extrafollicular pathway. GL-7 has been recently described as a marker of GC Tfh cells[355]. The appearance of GL-7^{hi} CD4^{+} cells among OT-II cells lacking Bcl-6 expression (these cells expressed low amounts of PD-1 and CXCR5; **Figure 3.23**) suggests GL-7^{hi} cells are a heterogeneous population comprised of activated CD4^{+} cells other than GC Tfh cells.

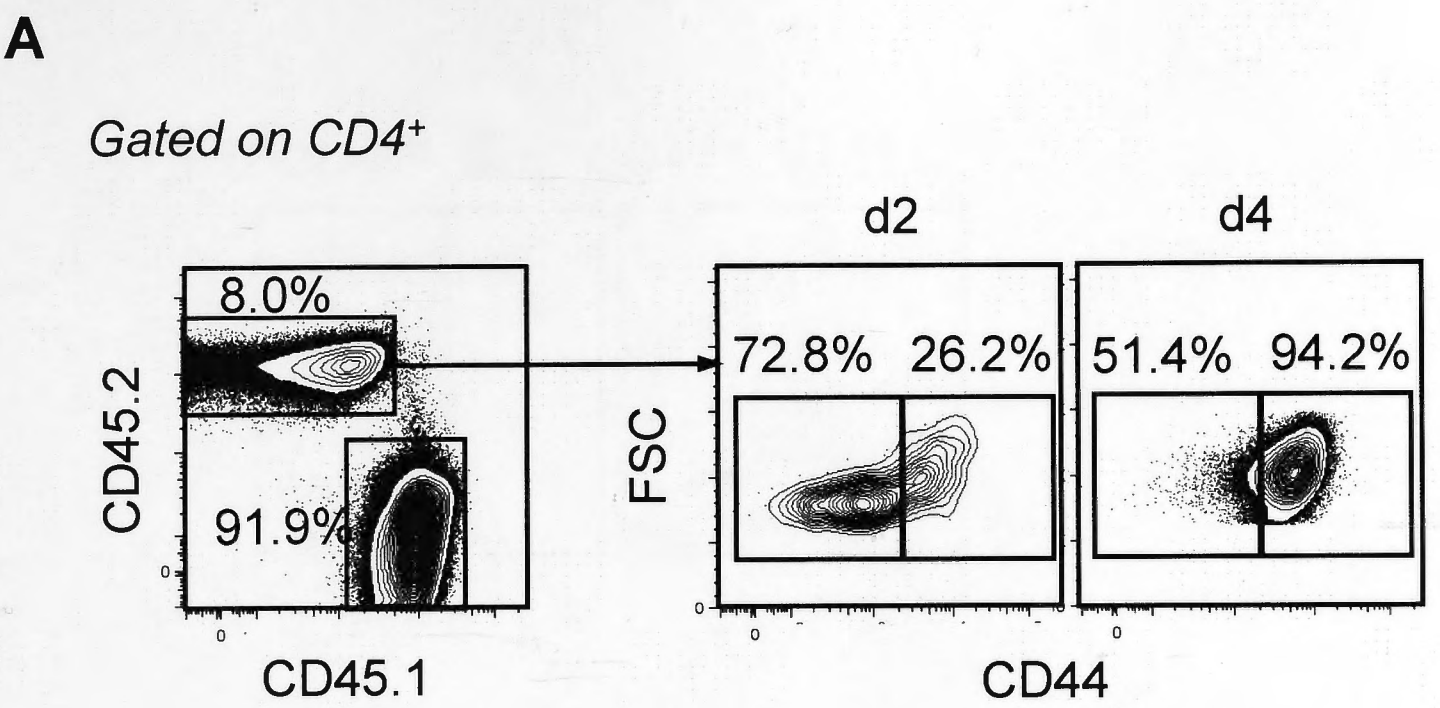


Figure 3.19. OVA-specific T cells expressing Bcl-6 are PD-1^{lo} at the B cell priming stage.

Naive OT-II CD45.2 cells were transferred i.v. into *Cd28*$^{-/-}$ CD45.1 recipient mice, which were then immunized with OVA in alum i.p. Spleens were harvested 2 and 4 days after adoptive transfer.

A) Gating strategy to identify naïve versus activated donor OT-II cells on day 2 or 4 after transfer.

B) Representative flow cytometric plots showing CXCR5 versus PD-1 expression of naïve (CD44^{lo}) and activated (CD44^{hi}) OT-II *Bcl6*$^{+/+}$ cells on either day 2 or 4 after adoptive cell transfer and immunization, analyzed on the same day. These data are representative of two independent experiments with three to six mice per group.

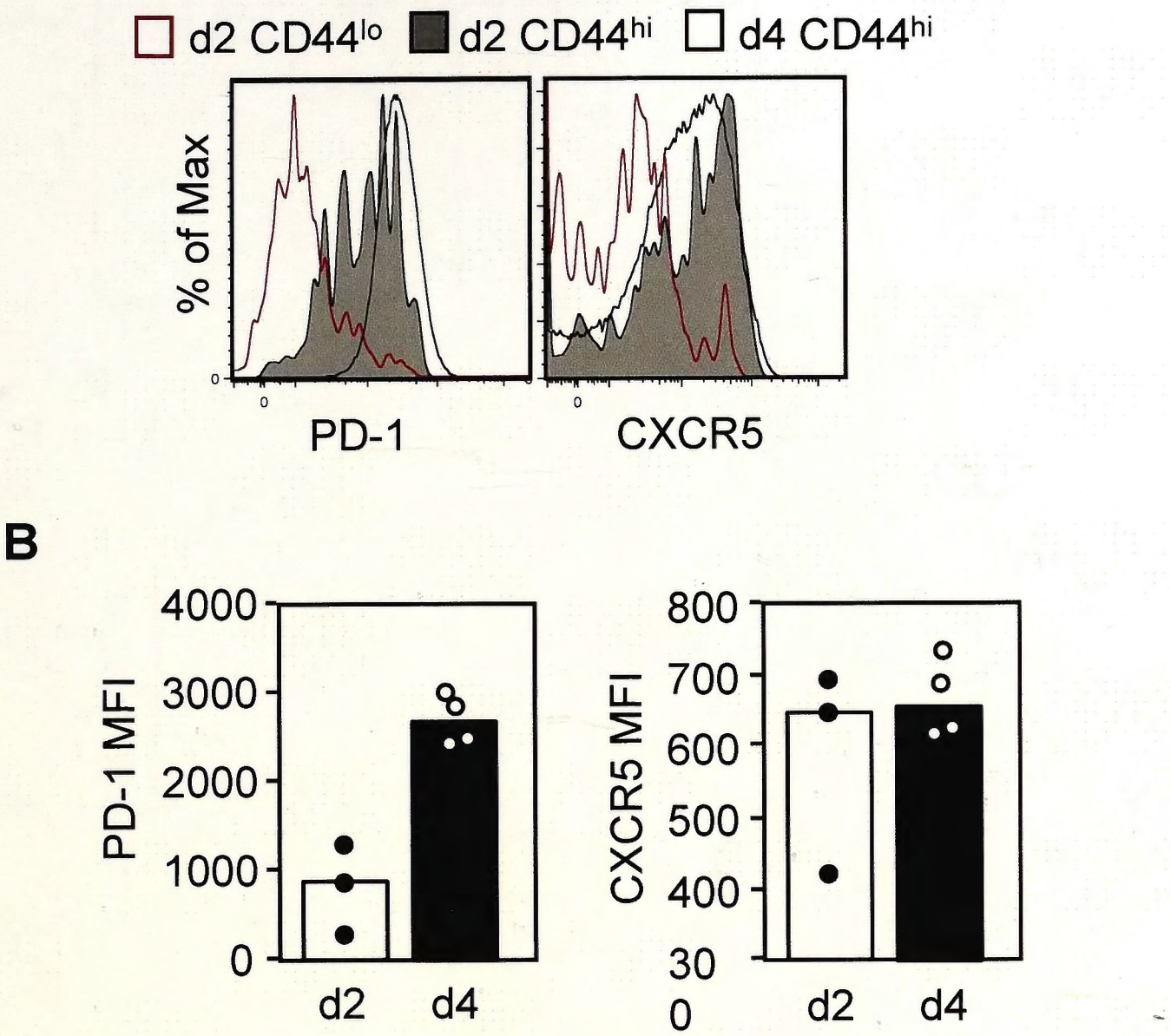


Figure 3.20. OVA-specific T cells expressing Bcl-6 are PD-1^{lo} at day 2 after adoptive transfer and immunization.

Histograms (**A**) and bar graphs (**B**) showing PD-1 and CXCR5 expression of naïve (CD44^{lo}) and activated (CD44^{hi}) OT-II *Bcl6*$^{+/+}$ cells on either day 2 or 4 after adoptive cell transfer and immunization, analyzed on the same day. These data are representative of two independent experiments with three to six mice per group.

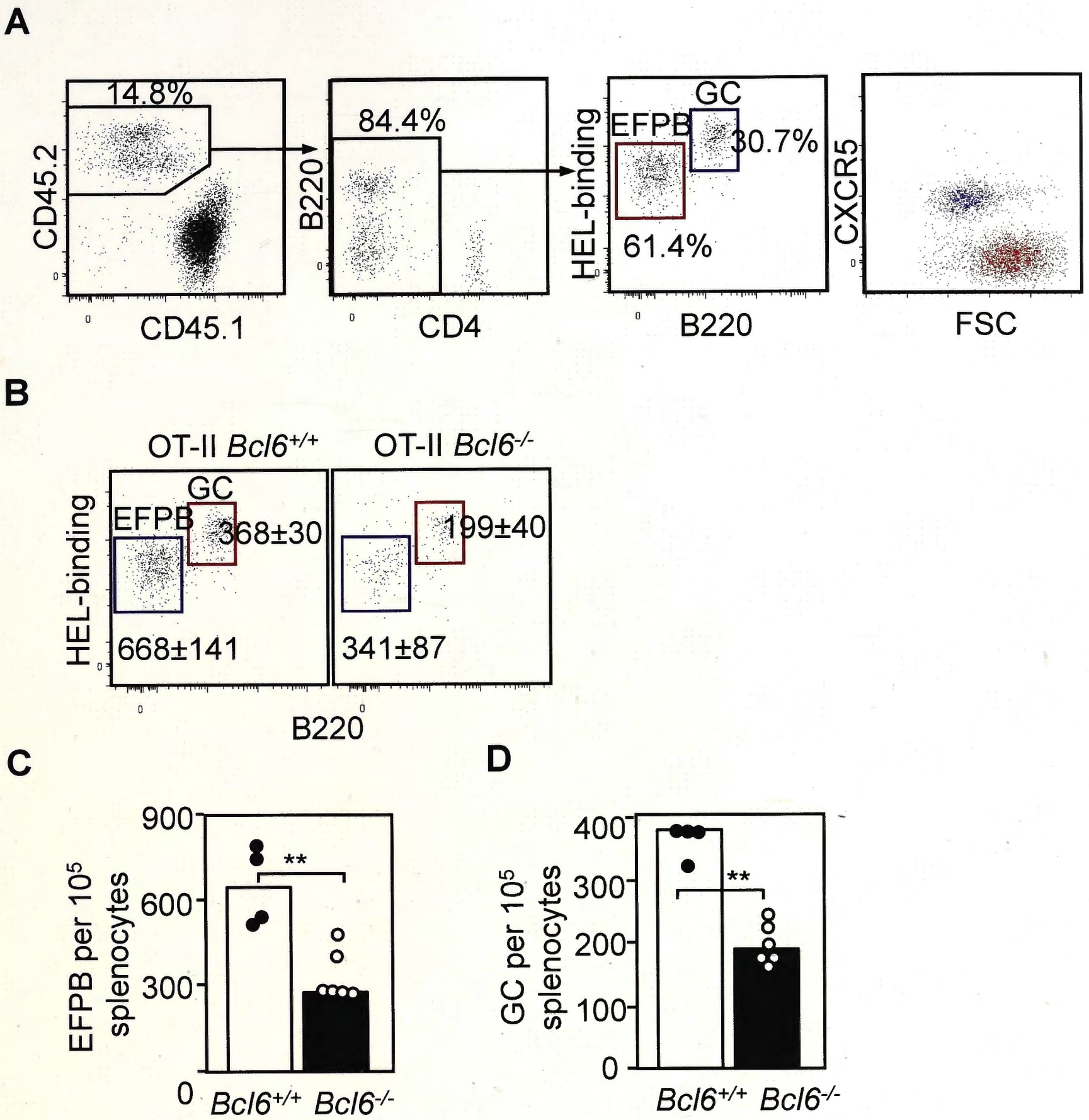


Figure 3.21. Development of HEL-specific GC and EFPBs is impaired in the absence of OVA-specific T cells expressing Bcl-6.

Naive OT-II *Bcl6*$^{+/+}$ or OT-II *Bcl6*$^{-/-}$ with or without SW_{HEL} B cells were transferred i.v. 4 h apart into *Cd28*$^{-/-}$ CD45.1 recipient mice, which were then immunized with HEL-OVA in alum i.p. Spleens were harvested 5.5 days after adoptive transfer.

A) Representative flow cytometric plots showing the gating strategy to identify donor-origin SW_{HEL} GC B cells (CD45.2 B220^{hi} HEL-binding^{hi} CXCR5^{hi} small forward scatter [FSC]) and EFPBs (CD45.2 B220^{lo} HEL-binding^{lo} CXCR5^{lo} large FSC) in the spleen of *Cd28*$^{-/-}$ CD45.1 recipient mice.

B-D) Flow cytometric profiles with frequencies ± standard deviation. (B) and quantification of SW_{HEL} EFPBs (C) and GC B cells (D) in the same recipient mice. These data are representative of two independent experiments with three to six mice per group. This experiment was performed by Louis Tsai.

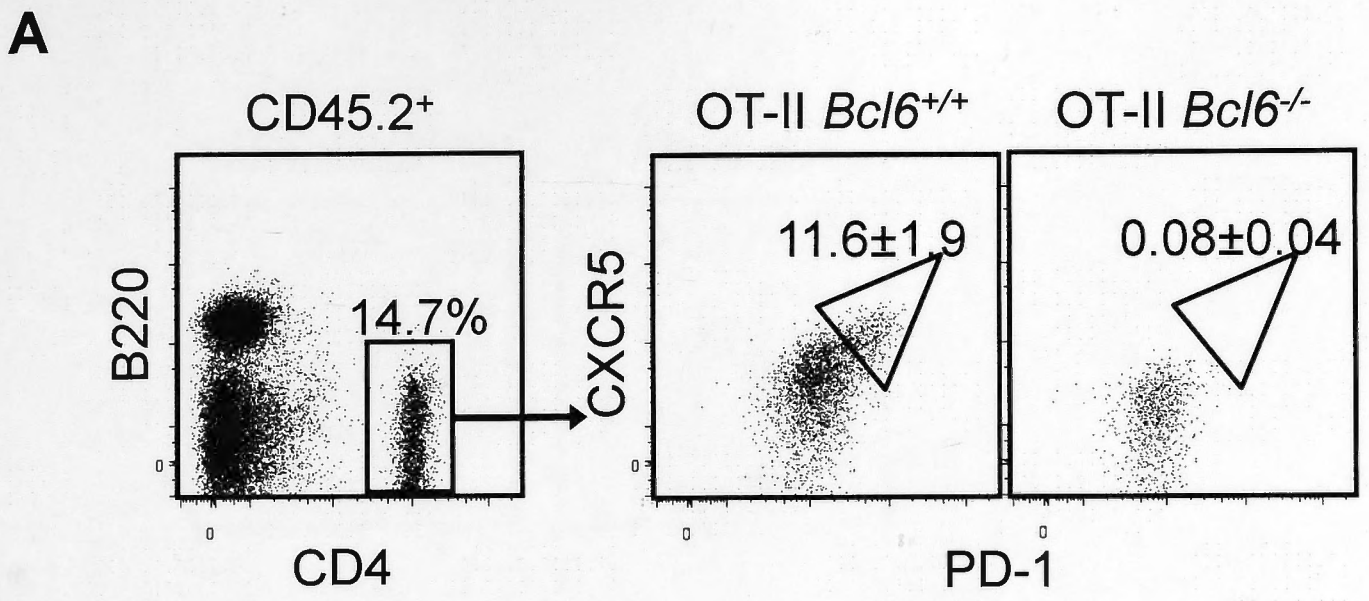


Figure 3.22. Formation of Tfh cell is impaired in the absence of OVA-specific T cells expressing Bcl-6.

Representative flow cytometric plot showing gating strategy (**A**) and quantification (**B**) of donor-origin Tfh cells identified as CXCR5^{hi} PD-1^{hi} CD45.2 (gated on CD4^{+} cells) after transfer of OT-II *Bcl6*$^{+/+}$ or OT-II *Bcl6*$^{-/-}$ T cells. These data are representative of two independent experiments with three to six mice per group. This experiment was performed by Louis Tsai.

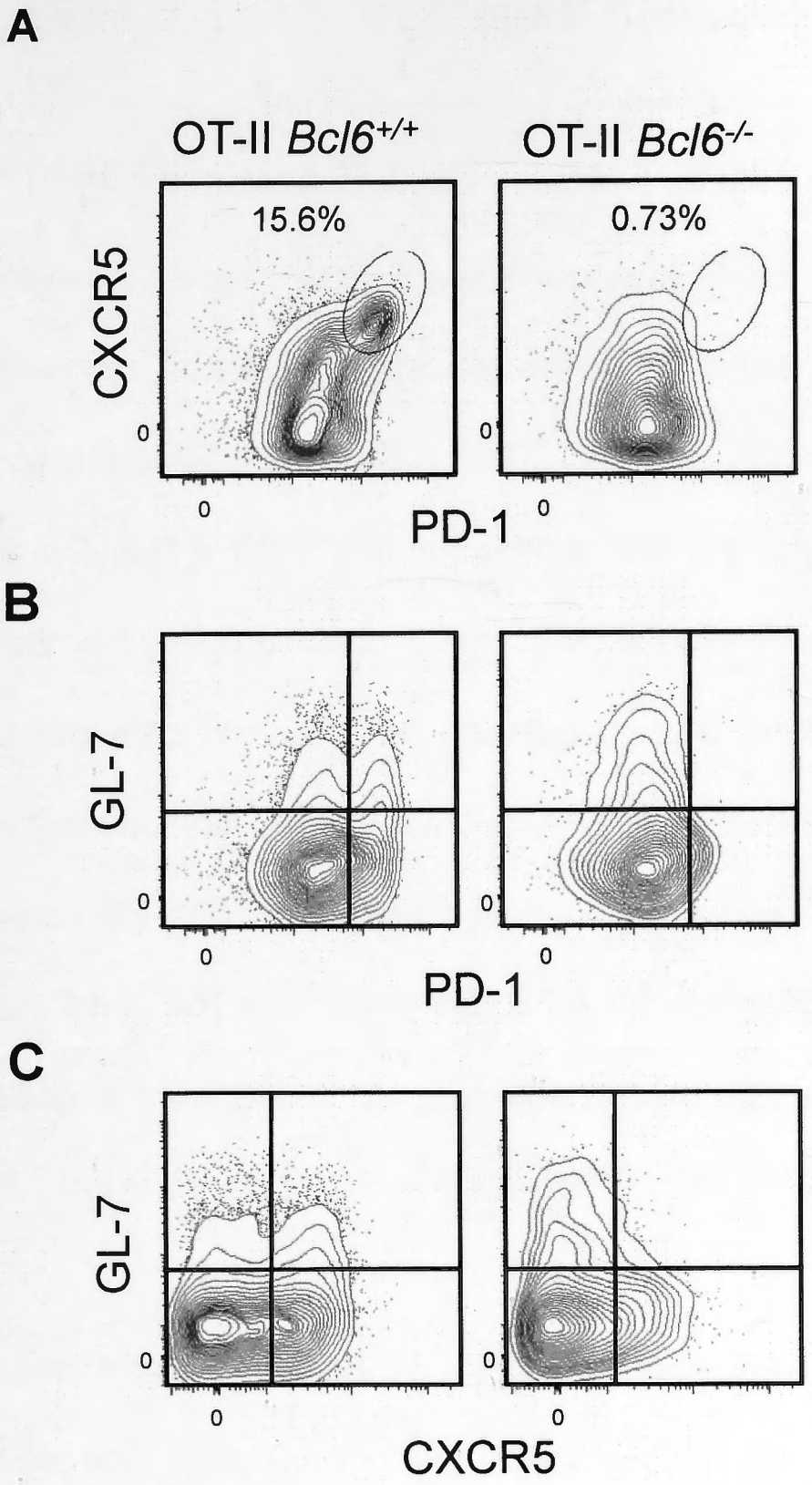


Figure 3.23. PD-1^{hi} but not GL-7^{hi} cells fail to form in the absence of Bcl-6.

Representative flow cytometric plots showing CXCR5 versus PD-1 (**A**), GL-7 versus PD-1. (**B**), and GL-7 versus CXCR5 (**C**) staining of donor OT-II cells sufficient or deficient in Bcl-6, 5.5 days after transfer into *Cd28*$^{-/-}$ CD45.1 mice and OVA immunization. Cells have been gated on CD4^{+} CD45.2. Numbers represent the percentages in the gates. These data are representative of two independent experiments with four to six mice per group. This experiment was performed by Louis Tsai.

Chapter Discussion

We observed significantly fewer switched EFPBs in the absence of T cell-expressed Bcl-6 across all experimental systems used in this study. A previous report described normal antibody titers in the first 2 wk after immunization of chimeric mice lacking Bcl-6 in T and B cells[177]. It is possible that the requirement for Bcl-6$^+$ T cells may be overcome by signals from other cells; B cells differentiating along the extrafollicular route are also known to be boosted by IL-6 and/or APRIL (a proliferating-inducing ligand)-producing cells that colocalize with EFPBs[356]. It is also conceivable that the plasma cells and antibodies detected on day 14 in mice lacking Bcl-6$^+$ T cells have arisen from an alternative route of differentiation. Indeed, several groups have reported a third B cell differentiation route into early memory B cells that is GC$^-$, ICOS$^-$, Bcl-6$^-$, and IL-21$^-$ independent[43, 177, 192, 211, 350].

Our data establish that Bcl-6 expression enables T cells to provide effective help to B cells for both follicular and extrafollicular antibody responses to protein antigens. PD-1 expression distinguishes outer T zone PD-1$^{lo/int}$ Bcl-6$^+$ pre-GC Tfh cells from the PD-1^{hi} Bcl-6$^+$ Tfh cells that appear within GC (GC Tfh cells). Bcl-6 has been shown to be critical for up-regulation of CXCR5 and down-regulation of CCR7[136, 152, 153], which are required for T and B cell localization to the T-B border[180, 347]. Thus, expression of Bcl-6 appears important to facilitate the initial T and B cell encounter at this location that results in the provision of T cell help required to initiate T-dependent B cell differentiation and Ig isotype switching. Indeed and contrary to common belief, there is evidence that production of >99% of switch transcripts occurs at this time and therefore precedes GC formation[30, 343]. In agreement with this, we have shown here that in the absence of Bcl-6 expression by CD4$^+$ T cells, the switched antibody

responses to all antigens are completely abrogated, including the extrafollicular IgG2c response to *S. enterica*, against which only antigen-specific IgM, of probable T-independent origin, is maintained. We also have observed that PD1-1^{lo} cells were not induced by *S. enterica* infection in wild-type mice. It is possible that there are inhibitors in *S. enterica* that selectively limit the development of PD-1^{lo} cells or Tfh cell precursors.

Our finding that T cell-expressed Bcl-6 acts at the time of T-B interaction does not exclude the possibility that Bcl-6-expression in T cells may also be required at the stage of plasmablast growth in extrafollicular foci. CXCR5^{lo} CXCR4^{hi} CD4^{+} T cells have been found in splenic extrafollicular foci of autoimmune MRL/*Fas*^{lpr} mice[76], and these are also Bcl-6 dependent[170]. This works suggests that Tfh are even more heterogeneous than we thought. Bcl-6^{+} T cells in the outer T zones appear competent to help B cells differentiate along either follicular or extrafollicular pathway. This highlights the need for refining the nomenclature of B follicular helper T cells. As has been previously proposed[79], pre-GC Tfh cells can be used to refer to T cells that prime B cells at the T-B border, which are shown here to be PD-1$^{lo/int}$ Bcl-6^{+}, whereas GC-Tfh can be used to refer to those CD4^{+} T cells located within GCs, which are generally, albeit not universally, PD-1^{hi}.

Chapter 4 - Interferon-gamma Excess Leads to Pathogenic Accumulation of Follicular Helper T cells and Germinal Centers

Chapter Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease caused by autoantibodies targeting nuclear components that affect multiple organs[357]. Clinical manifestations range from skin rashes and arthritis to life-threatening kidney or cardiovascular involvement. The relative paucity of effective drugs for lupus indicates that we need a better understanding of its pathogenesis. Only one new drug has been approved for lupus by the FDA in the past 50 years (anti-BAFF mAb, Belimumab), and this treatment has a relatively modest effect on disease activity in a small subset of patients[244]. Part of the problem is heterogeneity of causes, with several different pathways contributing to disease in different patients: dysregulation in Th1, Th17, Tfh and Treg cells, or excessive production of pro-inflammatory cytokines and growth factors such as type I interferon, TNF, or BAFF, can promote activation of self-DNA-reactive B cells triggering lupus[244, 357-359]. Recent evidence of crosstalk between some of these pathways suggests lupus pathogenesis may be less heterogeneous than previously thought: A link between dysregulated Th17 activity and Tfh cells has recently been described[229] as has been a feed back loop between excessive IFN-γ and BAFF production[360]. To date, a pathogenic pathway that links excessive IFN-γ production and Tfh cell dysregulation has not been described.

Follicular helper T cells have recently emerged as a specialized helper T cell subset specialized in providing help to B cells for both GC and extrafollicular antibody responses[3, 4, 79, 178, 361]. Aberrant formation and/or accumulation of Tfh cells which mediate positive selection of B cells that have acquired somatic mutations in the Ig V region genes has recently emerged as a key driver of autoantibody-mediated diseases[5]. In mice, dysregulated Tfh cell formation is found in lupus- and type 1 diabetes-prone *Roquin*$^{san/san}$ mice, lupus-prone BXSB.Yaa mice, autoimmune hepatitis-prone *Pdcd1*$^{-/-}$ mice and in IL-27-dependent pristane-induced lupus[193, 228, 230, 258]. Dysregulated IL-17-producing GC Tfh cells have also been proposed to explain lupus in BXD2 mice[229] and autoimmune arthritis of K/BxN mice[362]. Furthermore, Tfh cells found in extrafollicular foci have been shown to promote autoantibody formation in MRL^{lpr} mice[76, 170]. In humans, associations have been described between increased circulating Tfh-like cells and SLE[232], and between an increased proportion of IL-17-producing circulating Tfh-like cells and juvenile dermatomyositis[158].

Homozygosity for the *"san"* allele of *Roquin* that increases the binding affinity for mRNA has recently emerged as a powerful driver of Tfh cell accumulation[6, 227, 248]. *Roquin*$^{san/san}$ mice develop T cell-autonomous accumulation of Tfh cells and lupus. By contrast, mice expressing a null allele of *Roquin* have reduced GCs and do not develop autoimmunity[254]. The observation that abrogating Tfh cell formation prevented lupus in *Roquin*$^{san/san}$ mice together with induction of spontaneous GCs upon Tfh cell transfer demonstrated a pathogenic role for Tfh cells in lupus[6]. *Roquin* was shown to repress *Icos* mRNA posttranscriptionally[249, 253] through its ability to directly bind RNA[248, 253]. Although *Roquin*-mediated overexpression of ICOS[249]

promotes Tfh cell formation and resistance to Treg cell suppression[363], as we show here, it cannot alone explain Tfh cell accumulation and lupus development.

Th1 cells and their dominant effector cytokine, IFN-γ, have long since been associated with lupus development[329]. In mice, genetic disruption of *Ifngr* prevents the appearance of autoantibodies and kidney damage in MRL^{lpr} and NZB/W mice[319, 321] and blockade of IFN-γ signaling with antibodies against IFN-γ or IFN-γR prevents renal disease in NZB/W mice[323]. Furthermore, treatment with IFN-γR-Fc reverses disease in MRL^{lpr} mice[325]. In humans, associations between polymorphisms in *IFNG* gene and lupus have been described, including a risk allele leading to elevated IFN-γ expression[364]. In addition, increased IFN-γ in culture supernatants from PBMC of SLE patients strongly correlated with SLE Disease Activity Index scores[365].

The results from this chapter show that failure to repress *Ifng* posttranscriptionally by *Roquin* acts in a cell-intrinsic manner to produce excess IFN-γ. In turn, this excessive production of IFN-γ initiates Tfh-driven lupus in *Roquin*$^{san/san}$ mice; ablation of IFN-γR signaling in *Roquin*$^{san/san}$ mice reduced autoantibody production and renal disease significantly.

Results

Accumulation of IFN-γ-producing cells in lupus-prone *Roquin*$^{san/san}$ mice

We have previously reported T cell autonomous accumulation of effector/memory CD4$^+$ cells, including Tfh cells, in lymph nodes and spleen of *Roquin*$^{san/san}$ mice[6, 227]. Measurement of cytokines in culture supernatants had also revealed increased IFN-γ production by *Roquin*$^{san/san}$ T cells[227]. To evaluate the relative abundance of cytokine-producing CD4$^+$ T cells in *Roquin*$^{san/san}$ mice, we enumerated effector/memory (CD44^{hi}) cells producing IFN-γ, IL-4 and IL-17. IFN-γ-producing cells were the most abundant T cell effectors (13% of total CD44^{hi} effector/memory cells), followed by IL-4$^+$ cells (1%) and IL-17$^+$ cells (0.9%; **Figure 4.1A and 4.1B**). Total numbers of IFN-γ-producing effector cells were 7-fold higher than those of wild-type littermates (**Figure 4.2**). There was also a mild increase in the number of IL-17-producing cells in *Roquin*$^{san/san}$ mice (**Figure 4.2**). IFN-γ was detected (50-350 pg/ml) in the serum of *Roquin*$^{san/san}$ mice from 7 weeks of age in *Roquin*$^{san/san}$ mice coinciding with disease onset (**Figure 4.3A and 4.3B**); IFN-γ remained below the level of detection in wild-type littermates (**Figure 4.3A**).

Next, we evaluated whether the IFN-γ-producing cells also included Tfh cells and found 2- and 3-fold increases in the number of pre-Tfh and Tfh cells producing IFN-γ in *Roquin*$^{san/san}$ mice compared to wild-type counterparts (**Figure 4.4A and 4.4B**). IFN-γ-producing cells amongst non-Tfh CD44^{hi} effector/memory cells were 9-fold higher in *Roquin*$^{san/san}$ compared with *Roquin*$^{+/+}$ mice (**Figure 4.4A and 4.4B**). There

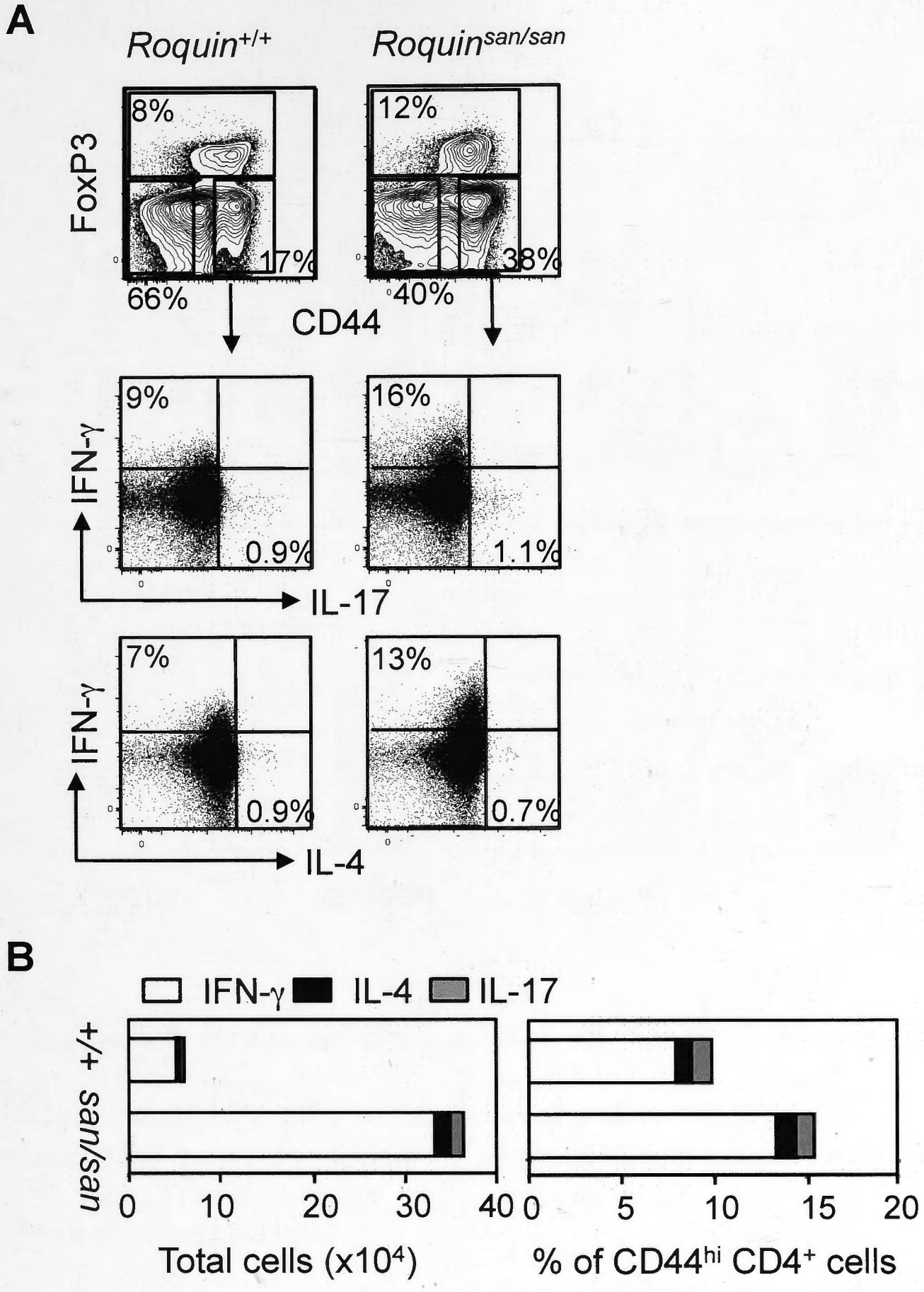


Figure 4.1. Enhanced production of IFN-γ-producing cells in *Roquin*$^{san/san}$ mice.

Representative flow cytometric plots showing intracellular stains of IFN-γ versus IL-17, and IFN-γ versus IL-4 gated on CD44^{hi} FoxP3^{-} CD4^{+} cells (**A**) and quantification of total numbers and percentages of IFN-γ, IL-4 and IL-17-producing CD4^{+} cells (**B**) from 8-wk-old *Roquin*$^{+/+}$ and *Roquin*$^{san/san}$ mice. These data are representative of two independent experiments with four mice per group.

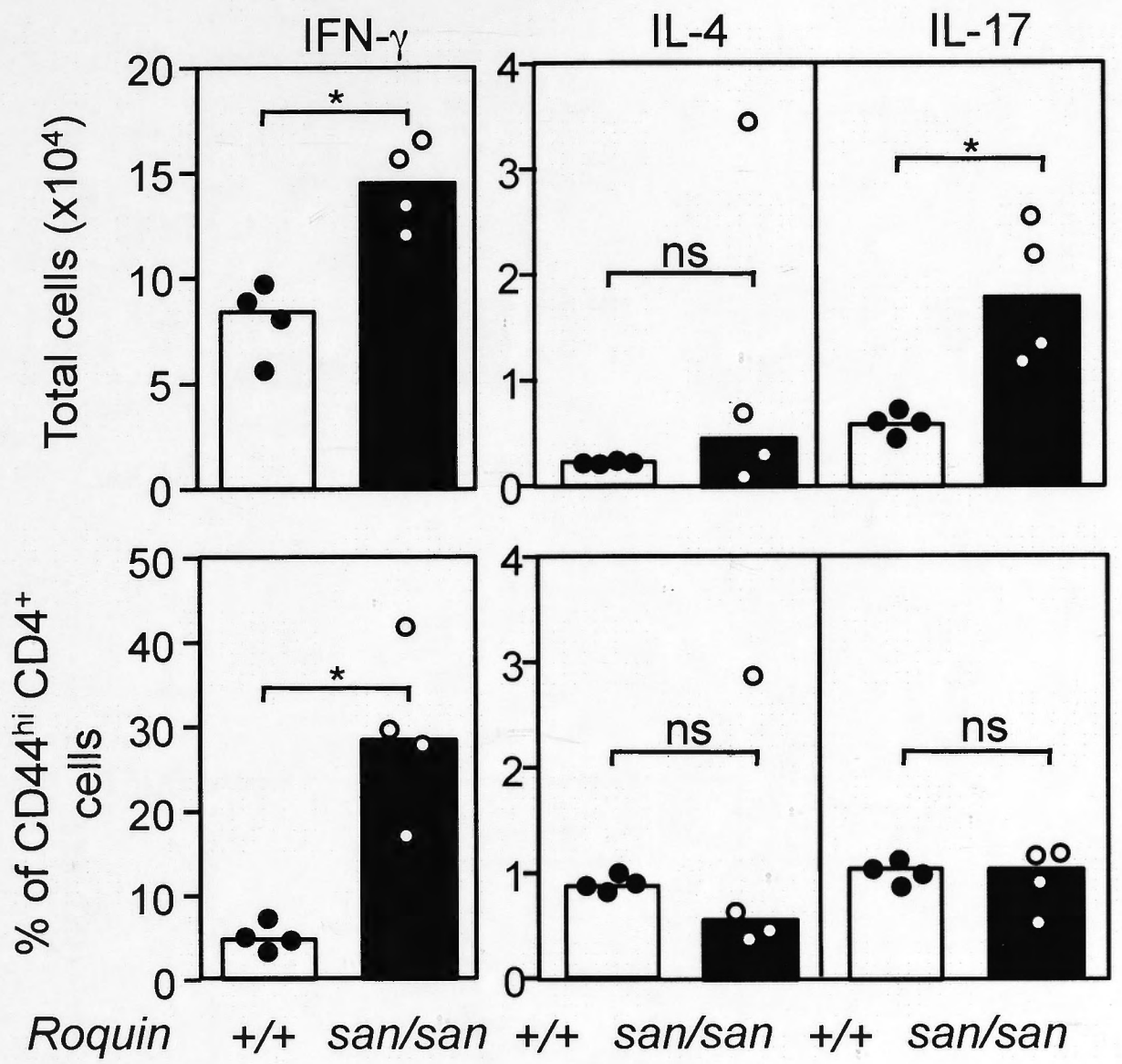
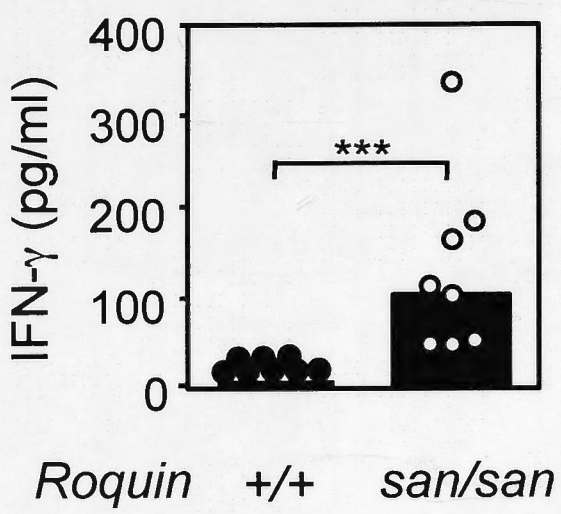


Figure 4.2. Increased IFN-γ-producing T cells in *Roquin*$^{san/san}$ mice.

Quantification of total numbers (top) and percentages (bottom) of IFN-γ, IL-4 and IL-17-producing CD44^{hi} CD4^{+} cells from 8-wk-old *Roquin*$^{san/san}$ and *Roquin* $^{+/+}$ mice. These data are representative of two independent experiments with four mice per group.



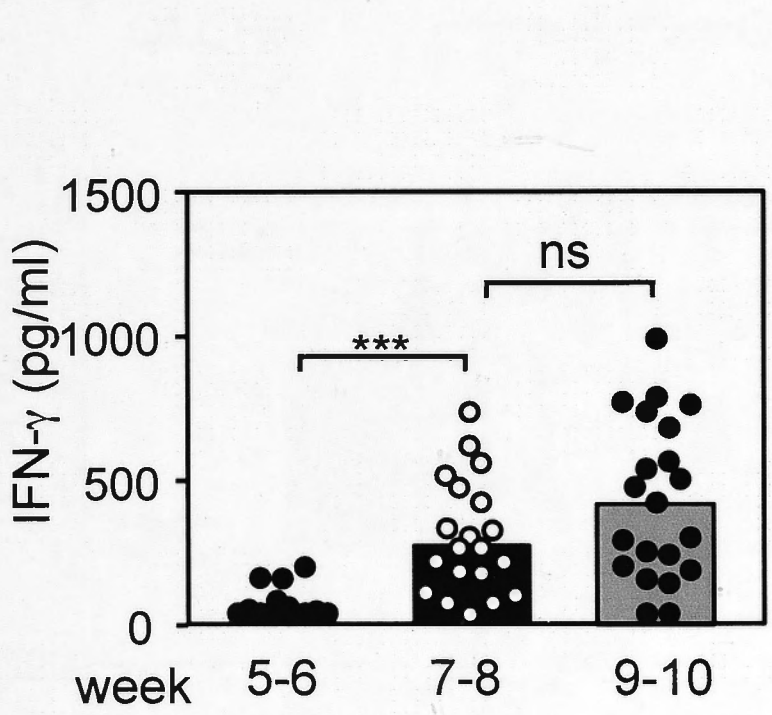


Figure 4.3. Increased IFN-γ concentration in serum of *Roquin*$^{san/san}$ mice.

A) IFN-γ concentration in serum of 8 to 10-wk-old *Roquin*$^{san/san}$ mice and *Roquin*$^{+/+}$ littermates analyzed by Cytometric Bead Array.
B) IFN-γ titers in serum of *Roquin*$^{san/san}$ mice of indicated ages measured by ELISA. These data are representative of two independent experiments with more than eight mice per group.

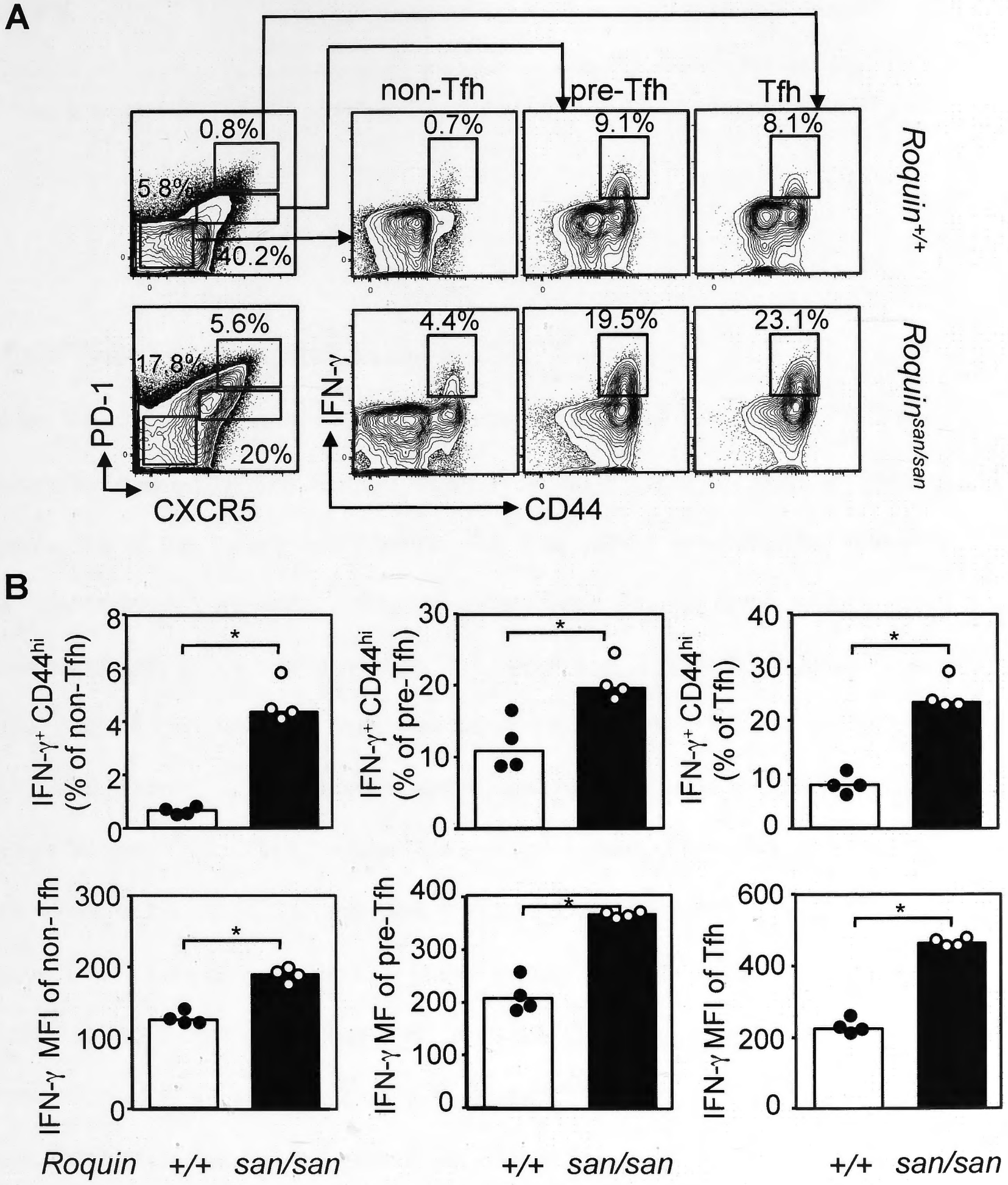


Figure 4.4. Enhanced production of IFN-γ-producing Tfh cells in *Roquin*$^{san/san}$ mice.

A) Representative flow cytometric plots showing IFN-γ versus CD44 gated on non-Tfh (CXCR5$^-$ PD-1$^-$ CD4$^+$; green), pre-Tfh (CXCR5$^+$ PD-1^{int} CD4$^+$, blue) and Tfh (CXCR5$^+$ PD-1$^+$ CD4$^+$; red) cells from 8-wk-old *Roquin*$^{+/+}$ and *Roquin*$^{san/san}$ mice.
B) Percentages of IFN-γ$^+$ cells and mean fluorescence intensity (MFI) of IFN-γ expression amongst non-Tfh, pre-Tfh and Tfh cells from 8-wk-old *Roquin*$^{+/+}$ and *Roquin*$^{san/san}$ mice. These data are representative of two independent experiments with four mice per group.

was also a higher proportion of NK cells producing IFN-γ in *sanroque* mice (**Figure 4.5**).

Roquin^{san} delays Ifng mRNA decay in CD4^{+} T cells

Next we investigated whether increased production of IFN-γ by *Roquin*$^{san/san}$ T cells was directly caused by the *Roquin* mutation in CD4^{+} cells, or was an indirect consequence of the inflammatory milieu. For this, mixed bone marrow chimeras were constructed. Recipient *Rag1*$^{-/-}$ *mice* were sublethally-irradiated and reconstituted with a 1:1 mix of *Roquin*$^{+/+}$ CD45.1 and *Roquin*$^{san/san}$ CD45.2 bone marrow cells. A control set of mice was reconstituted with a 1:1 mix of *Roquin*$^{+/+}$ CD45.1 and *Roquin*$^{+/+}$ CD45.2 bone marrow. The proportion of IFN-γ-producing cells amongst *Roquin*$^{san/san}$ CD45.2 cells was ~4-fold higher than amongst *Roquin*$^{+/+}$ CD45.1 cells in the same chimeric mice (**Figures 4.6A and 4.6B**). IFN-γ expression in naïve T cells was also significantly higher within *Roquin*$^{san/san}$ CD45.2 than within *Roquin*$^{+/+}$ CD45.1 cells (**Figures 4.6C and 4.6D**). These data indicate that the accumulation of IFN-γ-producing cells in *Roquin*$^{san/san}$ mice is T cell-intrinsic. We previously showed that the increase in Tfh cells in *Roquin*$^{san/san}$ mice is also T cell-intrinsic[6] (**Figure 4.7**).

To investigate if ROQUIN delays *Ifng* mRNA decay as described for *Icos* mRNA[249, 253], purified *Roquin*$^{san/san}$ and *Roquin*$^{+/+}$ naïve (CD44^{lo}) CD4^{+} T cells were activated with anti-CD3 and anti-CD28 for 24 h and *Ifng* mRNA was measured by quantitative RT-PCR. *Roquin*$^{san/san}$ CD4^{+} T cells expressed 40-fold higher amounts of *Ifng* mRNA than *Roquin*$^{+/+}$ CD4^{+} T cells at 4 h (**Figure 4.8A**). Next, we investigated whether

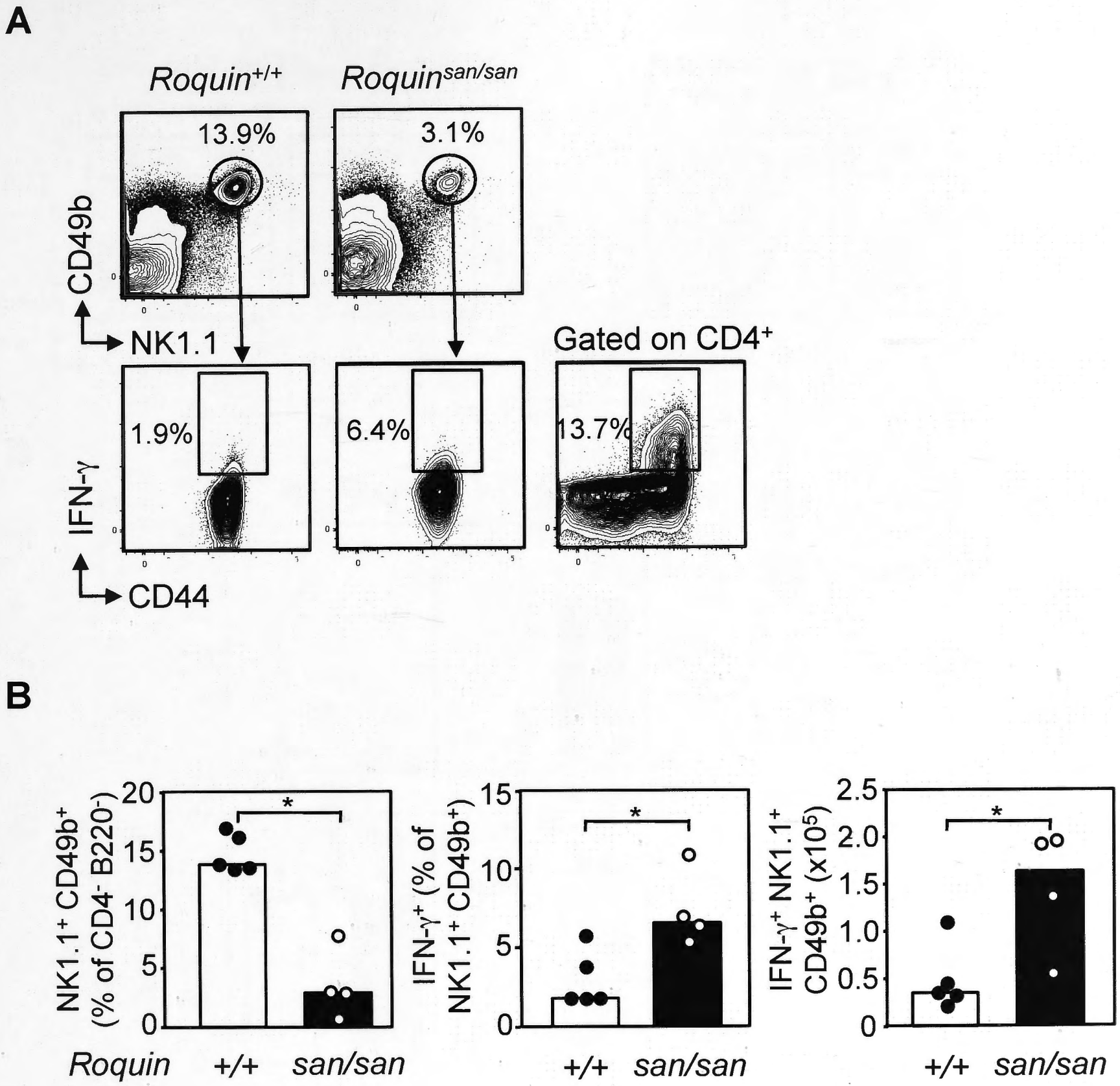


Figure 4.5. Increased IFN-γ-producing NK cells in *Roquin*$^{san/san}$ mice.

A) Representative flow cytometric plots showing gating strategies of NK cells (NK1.1$^+$ CD49b$^+$) and IFN-γ production by NK cells (a plot showing IFN-γ production by *Roquin*$^{san/san}$ CD4$^+$ cells from the same stain is included as a positive control) from 10-wk-old *Roquin*$^{san/san}$ and *Roquin*$^{+/+}$ mice.
B) Quantification of percentages of NK cells (NK1.1$^+$ CD49b$^+$; left) and IFN-γ-producing NK cells (middle); and total numbers IFN-γ-producing NK cells (right) from 10-wk-old *Roquin*$^{san/san}$ and *Roquin*$^{+/+}$ mice. These data are representative of two independent experiments with four to five mice per group.

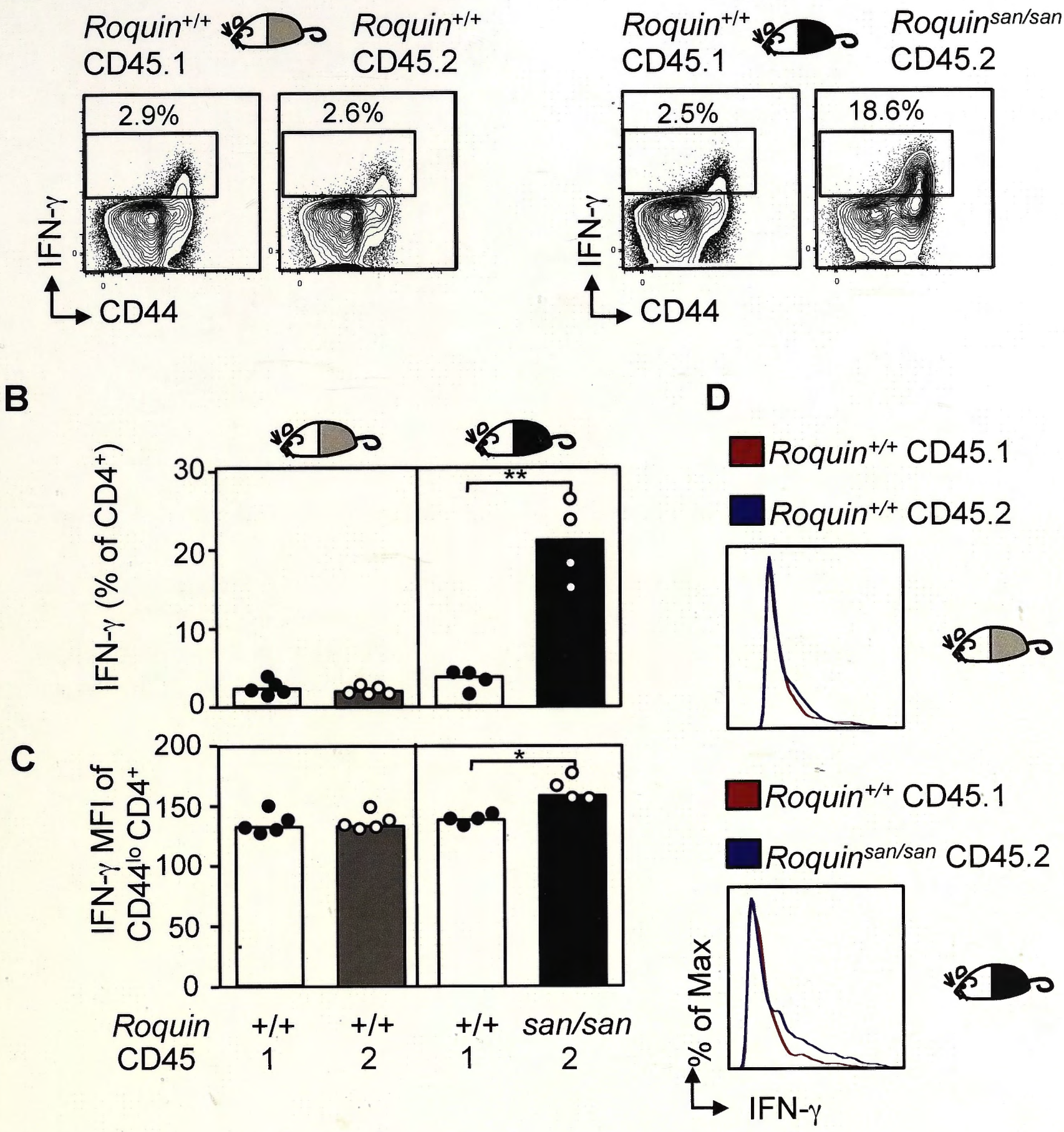


Figure 4.6. T cell-intrinsic overproduction of IFN-γ in *Roquin*$^{san/san}$ mice.

A and B) Representative flow cytometric plots (A) and percentages (B) of IFN-γ$^+$ CD4$^+$ cells from 50% *Roquin*$^{+/+}$ CD45.1:50%: *Roquin*$^{san/san}$ CD45.2 or control 50% *Roquin*$^{+/+}$ CD45.1:50% *Roquin*$^{+/+}$ CD45.2 bone marrow chimeras 10 weeks after cell reconstitution.
C and D) Bar graphs (C) and histograms (D) showing IFN-γ expression in naïve (CD44^{lo}) CD4$^+$ cells. These data are representative of four independent experiments with four to five mice per group.

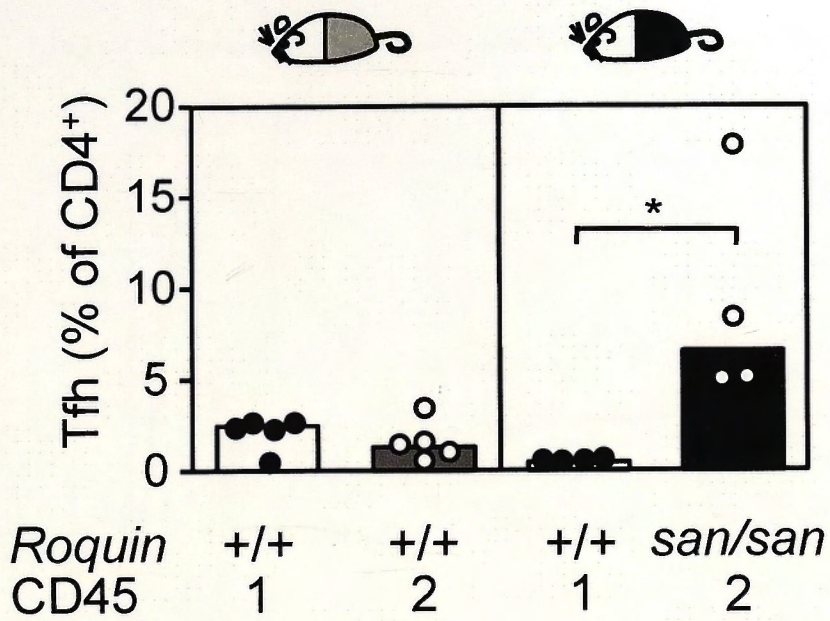


Figure 4.7. Tfh cell accumulation in *Roquin*$^{san/san}$ mice is T cell-intrinsic.

Bar graph showing percentages of Tfh cells from 50% *Roquin*$^{+/+}$ CD45.1:50%: *Roquin*$^{san/san}$ CD45.2 or control 50% *Roquin*$^{+/+}$ CD45.1:50% *Roquin*$^{+/+}$ CD45.2 bone marrow chimeras. These data are representative of four independent experiments with four to five mice per group.

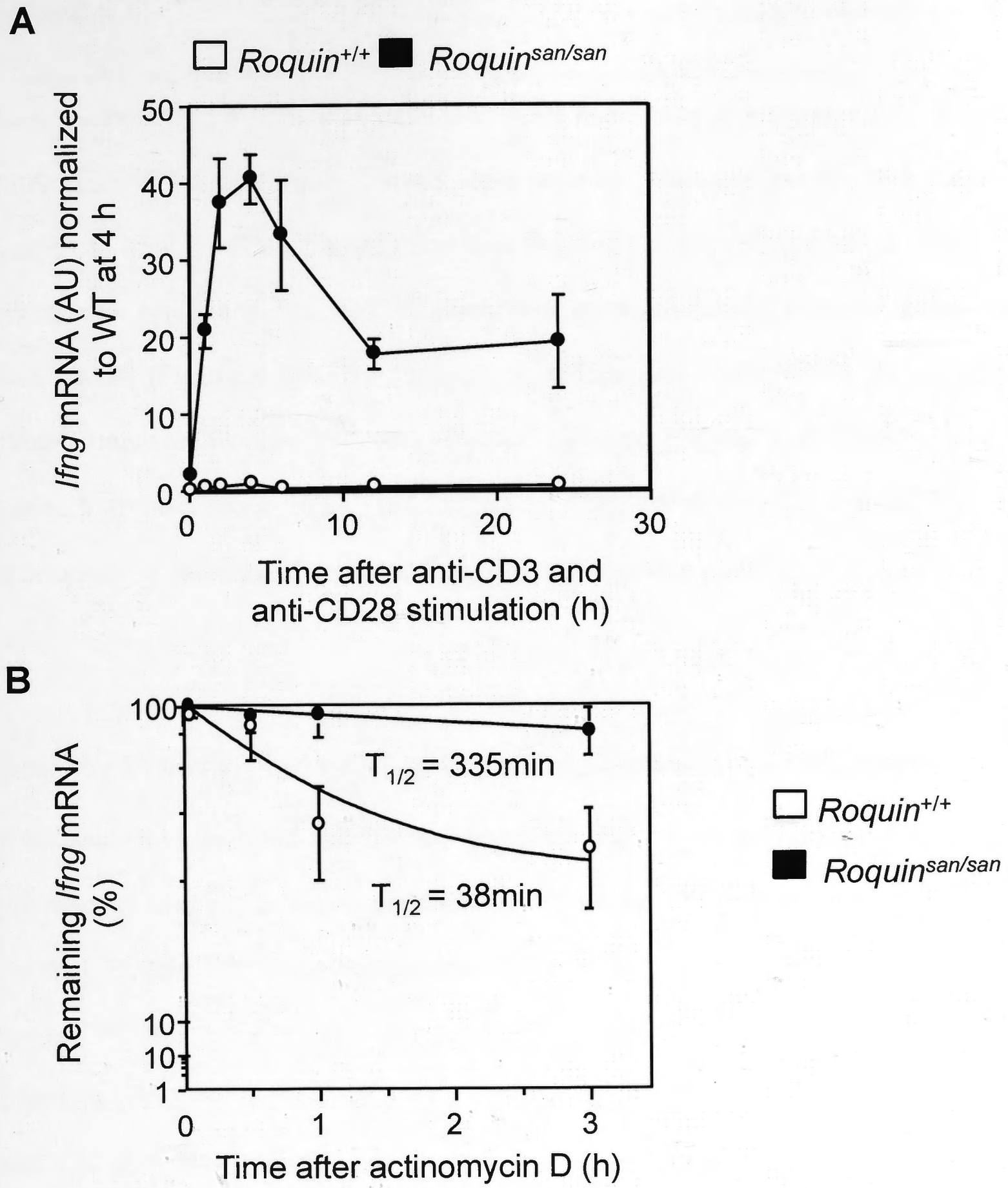


Figure 4.8. T cell-intrinsic overproduction of IFN-γ in *Roquin*$^{san/san}$ mice is caused by increased *Ifng* mRNA stability.

A) Quantitative RT-PCR analysis of *Ifng* mRNA expression in sorted CD44^{lo} CD4^{+} cells from 8-wk-old *Roquin*$^{san/san}$ and *Roquin*$^{+/+}$ mice cultured for indicated times with anti-CD3, anti-CD28 in the presence of IL-12p70 and anti-IL-4.
B) Remaining *Ifng* mRNA levels in activated *Roquin*$^{san/san}$ and *Roquin*$^{+/+}$ cells using same culture condition as in A for 18 h, then treated with actinomycin D and measured at indicated times using quantitative RT-PCR. *Ifng* mRNA levels at time 0 in each group were assigned as 100%. Trendlines were fitted to predict *Ifng* mRNA half-life ($T_{1/2}$). These data represent mean values ± standard deviation with three biological replicates in two independent experiments.

accumulation of *Ifng* mRNA was occurring posttranscriptionally. Naïve $CD4^+$ T cells from $Roquin^{san/san}$ and $Roquin^{+/+}$ mice were treated with actinomycin D 3 h after activation to inhibit mRNA transcription and *Ifng* mRNA was measured at different intervals after treatment. $Roquin^{san/san}$ delayed *Ifng* mRNA decay, prolonging its half-life by ~9-fold (**Figure 4.8B**). By contrast, no differences in *Il2* mRNA decay were observed between $Roquin^{san/san}$ and $Roquin^{+/+}$ T cells (**Figure 4.9**). These results indicate that excessive IFN-γ production in the presence of $Roquin^{san}$ is a consequence of failed posttranscriptional repression of *Ifng* mRNA.

Deficiency in IFN-γR but not T-bet or ICOS rescues hypercellularity

The results above suggest excessive IFN-γ production may contribute to the lupus-like syndrome and autoimmune susceptibility of $Roquin^{san/san}$ mice. To test this, we generated $Roquin^{san/san}$ mice deficient in either IFN-γR ($Roquin^{san/san}$ $Ifngr^{-/-}$), IFN-γ ($Roquin^{san/san}$ $Ifng^{-/-}$) or T-bet ($Roquin^{san/san}$ $Tbx21^{Du/Du}$)[341]. We also generated C57BL/6.$Roquin^{san/san}$ mice lacking ICOS; previously ICOS hemizygosity ($Icos^{+/-}$) in a mixed C57BL/6.CBA background was shown to decrease ICOS overexpression, albeit not to the level of wild-type T cells, reducing $Roquin^{san/san}$ splenomegaly and lymphadenopathy[249].

Lack of IFN-γR signaling in $Roquin^{san/san}$ $Ifngr^{-/-}$ mice prevented hypercellularity and splenomegaly (**Figures 4.10A and 4.10B**). $Roquin^{san/san}$ $Ifngr^{-/-}$ mice also showed a significant reduction in the percentage of effector/memory ($CD44^{hi}$) $CD4^+$ cells but maintained the elevated Treg cell numbers typical of $Roquin^{san/san}$ mice (**Figure 4.10B**). By contrast, complete deficiency of either T-bet or ICOS failed to rescue the

splenomegaly and effector/memory T cell expansion (**Figure 4.10A, 4.10B, 4.11A and 4.12**). Of note, *Roquin*$^{san/san}$ *Tbx21*$^{Du/Du}$ mice still overproduced IFN-γ compared to wild-type mice (**Figure 4.11B**), suggesting that mutant *Roquin* leads to IFN-γ overproduction independently of T-bet-mediated transcriptional control. In contrast to the effect of ICOS hemizygosity in *Roquin*$^{san/san}$ mice, complete lack of ICOS not only failed to reduce splenomegaly but further increased splenic cellularity (**Figures 4.10A, 4.10B and 4.12**) and led to the production of homogeneous nuclear antinuclear antibodies (ANAs; data not shown), different from the mixed nuclear/cytoplasmic pattern typical of *Roquin*$^{san/san}$ mice. This is likely to be explained by the observed reduction in FoxP3^{+} CD4^{+} Treg cell numbers in the absence of ICOS in *Roquin*$^{san/san}$ mice (**Figure 4.10B and 4.12**) plus defective Treg cell function that characterizes ICOS-deficient Treg cells[363].

Excessive IFN-γ signaling promotes accumulation of Tfh cells and germinal centers, and triggers lupus

Next we sought to investigate a possible link between IFN-γ overproduction and the aberrant Tfh accumulation and spontaneous GC formation in *Roquin*$^{san/san}$ mice. Tfh and GC B cells were enumerated using flow cytometric staining. Lack of IFN-γ signaling significantly reduced total CD4^{+} T cells, Tfh and GC B cell numbers in *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice (**Figure 4.13A and 4.13B**). Complete IFN-γ deficiency in *Roquin*$^{san/san}$ *Ifng*$^{-/-}$ mice also reduced the proportion of Tfh and GC (**Figure 4.14**). Consistent with the described roles for IFN-γ in switching to IgG2a (IgG2c in

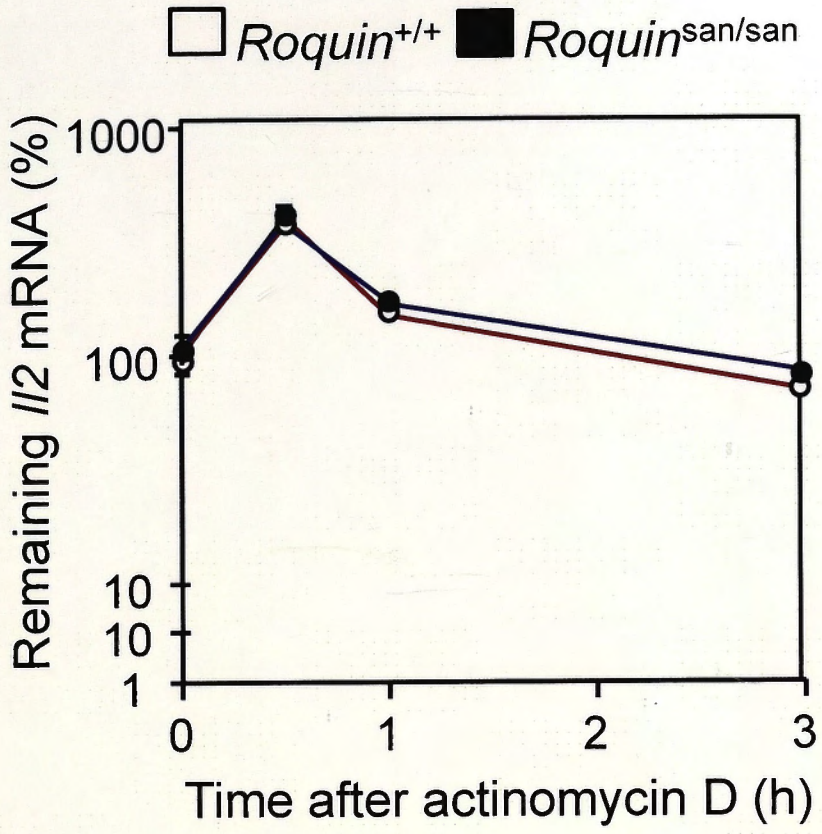


Figure 4.9. No difference in *Il2* mRNA stability between *Roquin*$^{san/san}$ and *Roquin*$^{+/+}$ T cells.

Remaining *Il2* mRNA levels in activated *Roquin*$^{san/san}$ and *Roquin*$^{+/+}$ T cells treated with actinomycin D and measured at the indicated times using quantitative RT-PCR. *Il2* mRNA levels at time 0 in each group were assigned as 100%. These data represent mean values ± standard deviation with three biological replicates in two independent experiments. This experiment was done by Jaime Martin.

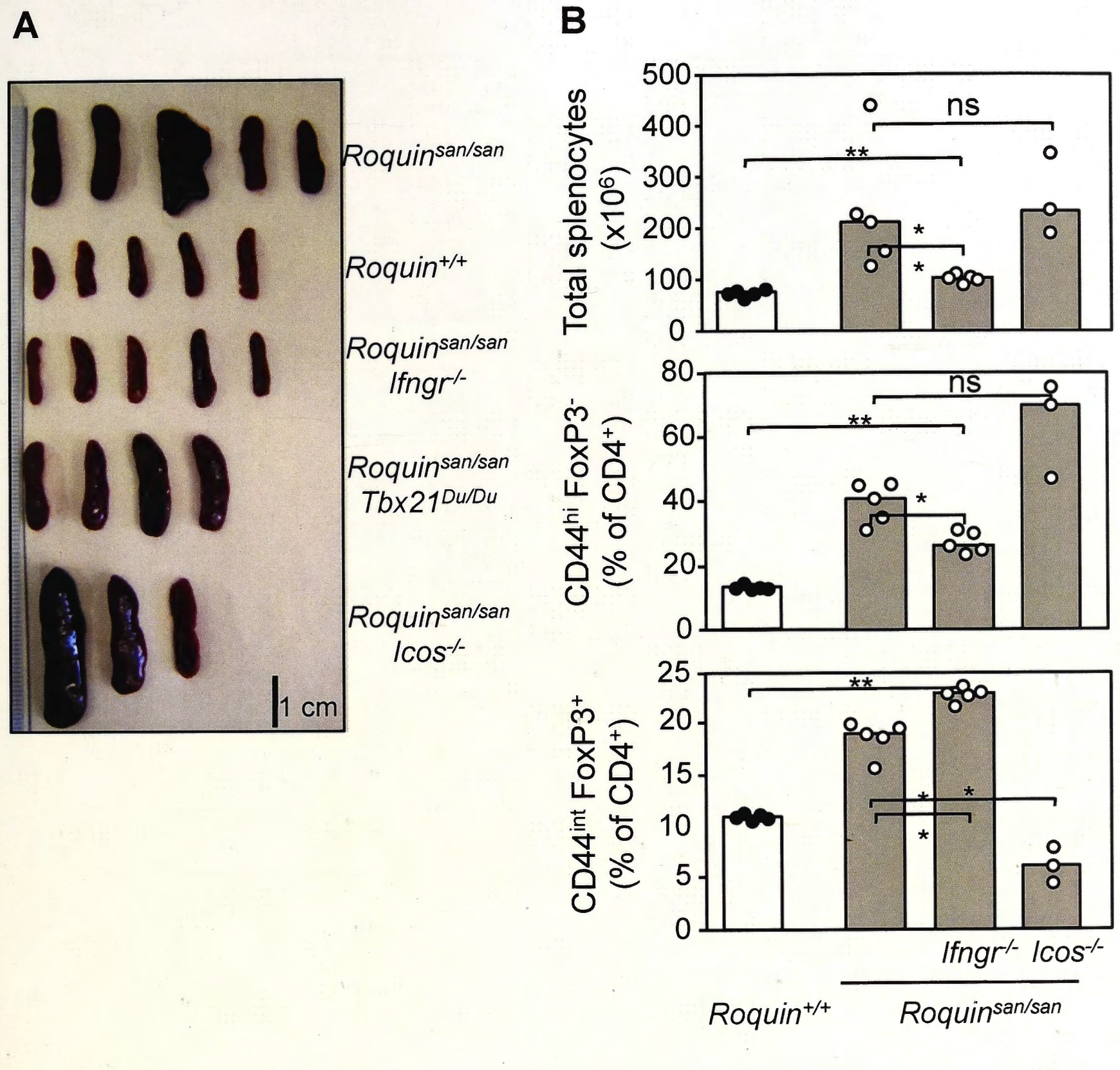


Figure 4.10. IFN-γR deficiency in *Roquin*$^{san/san}$ mice reduces hypercellularity.

A) Photograph indicates spleens from 8-wk-old *Roquin*$^{+/+}$, *Roquin*$^{san/san}$, *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$, *Roquin*$^{san/san}$ *Tbx21*$^{Du/Du}$ and *Roquin*$^{san/san}$ *Icos*$^{-/-}$.
B) Quantification of total numbers of splenocytes *(top)*, and frequencies of effector/memory (CD44^{hi} Foxp3^{-}; middle) and Treg (CD44^{int} FoxP3^{+}; bottom) CD4^{+} cells in mice with indicated genotypes (data for *Roquin*$^{san/san}$ *Tbx21*$^{Du/Du}$ is listed in Figure 4.11).

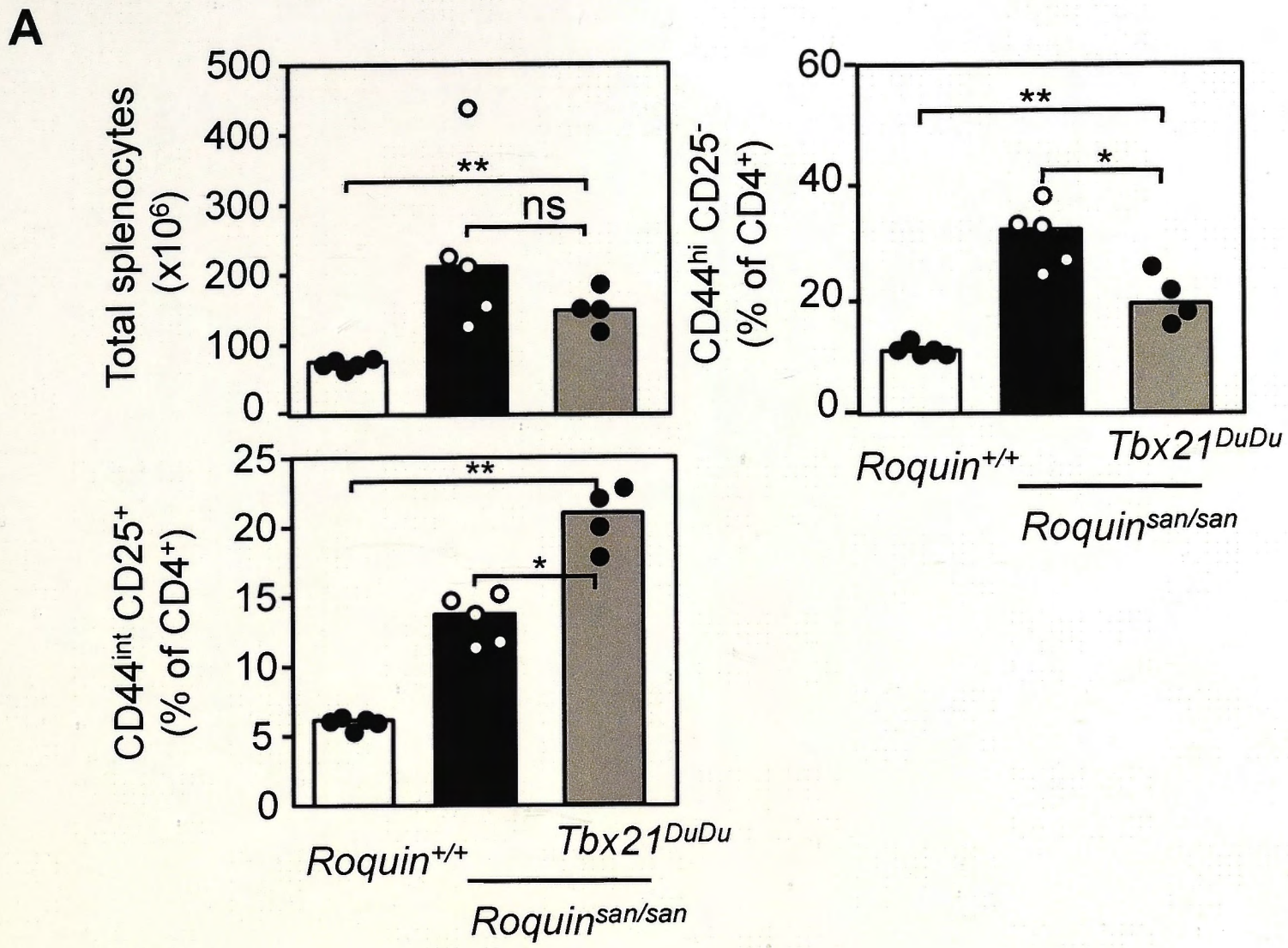


Figure 4.11. T-bet deficiency in *Roquin*$^{san/san}$ mice does not reduce hypercellularity.

A) Bar graphs enumerating total numbers of splenocytes (top left), the percentages of effector/memory ($CD44^{hi}$ $CD25^{-}$; top right) and $CD44^{int}$ $CD25^{+}$ (bottom) $CD4^{+}$ cells from 8-wk-old *Roquin*$^{+/+}$, *Roquin*$^{san/san}$ and *Roquin*$^{san/san}$ *Tbx21*$^{Du/Du}$ mice.
B) Representative flow cytometric plots of IFN-γ^{+} cells gated on $CD4^{+}$ T cells of *Roquin*$^{+/+}$, *Roquin*$^{san/san}$ and *Roquin*$^{san/san}$ *Tbx21*$^{Du/Du}$ mice. These data are representative of three independent experiments with four to five mice per group.

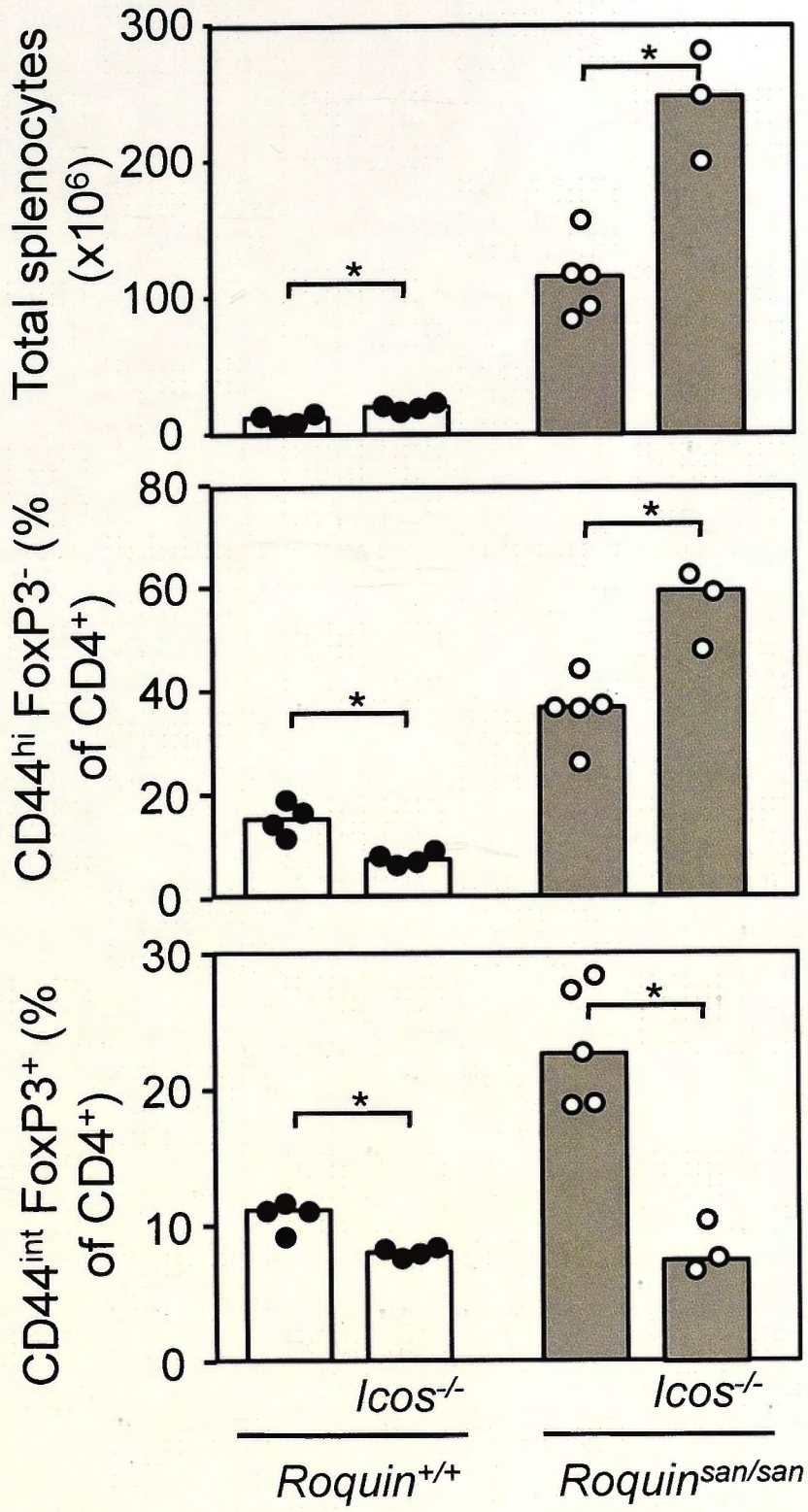


Figure 4.12. ICOS deficiency in *Roquin*$^{san/san}$ mice does not reduce hypercellularity.

Bar graphs enumerating total numbers of splenocytes (top), and frequencies of effector/memory (CD44^{hi} FoxP3^{+}; middle) and Treg (CD44^{int} FoxP3^{+}; bottom) cells from 8 to 10-wk-old *Roquin*$^{+/+}$, *Roquin*$^{+/+}$ *Icos*$^{-/-}$, *Roquin*$^{san/san}$ *Icos*$^{-/-}$ and *Roquin*$^{san/san}$ mice. These data are representative of three independent experiments with three to five mice per group.

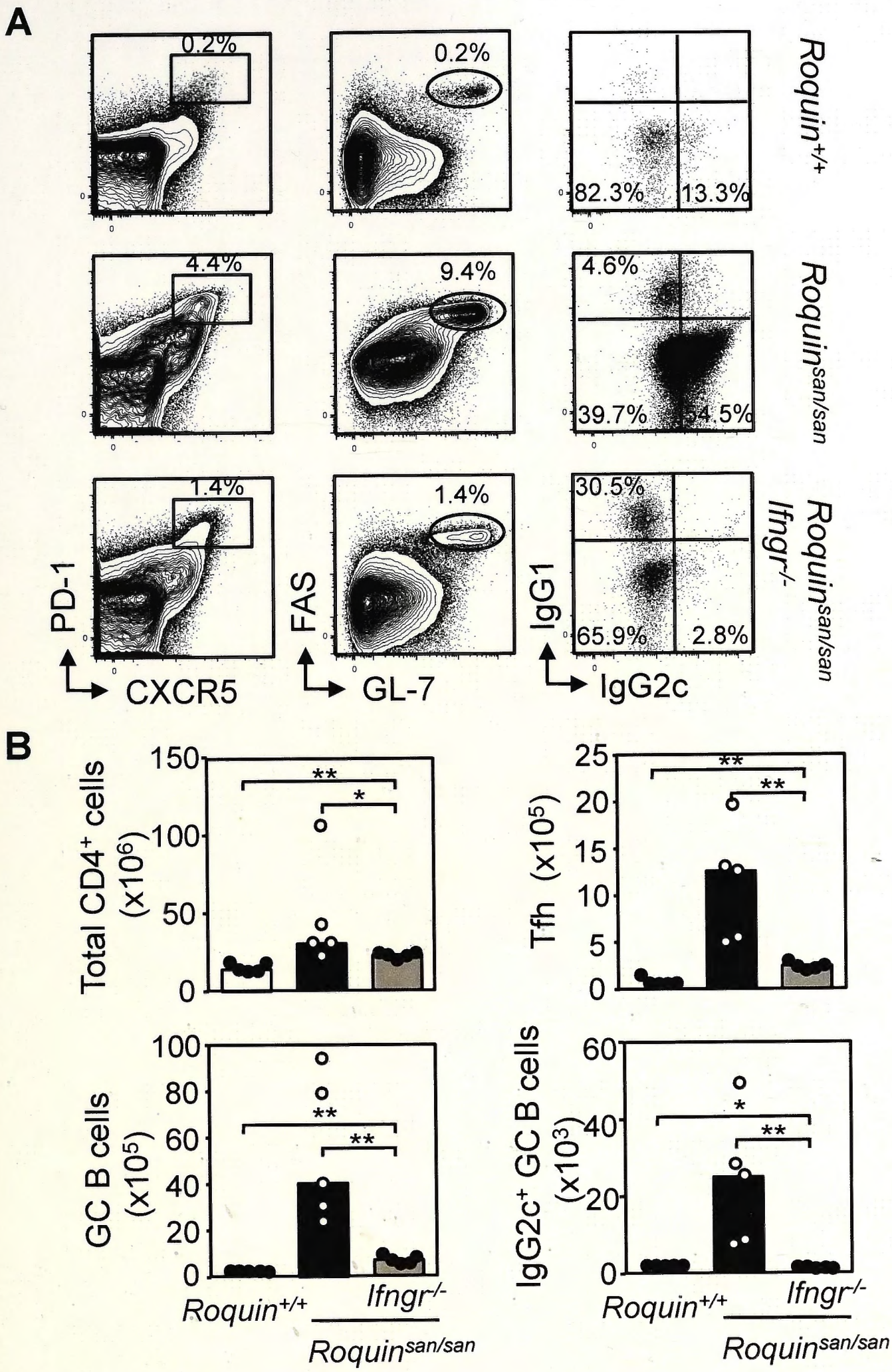


Figure 4.13. IFN-γR deficiency in *Roquin*$^{san/san}$ mice reduces Tfh and GC B cell formation.

Representative flow cytometric plots (**A**) and quantification of total numbers (**B**) of CD4$^+$ T cells, Tfh cells identified as CXCR5^{hi} PD-1^{hi} (gated on CD44^{hi} Foxp3$^-$ CD4$^+$ cells), GC B cells identified as GL-7$^+$ FAS$^+$ (gated on B220$^+$ cells) and IgG2c$^+$ cells (gated on GC B cells) from 8-wk-old *Roquin*$^{+/+}$, *Roquin*$^{san/san}$ and *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice. These data are representative of four independent experiments with five mice per group.

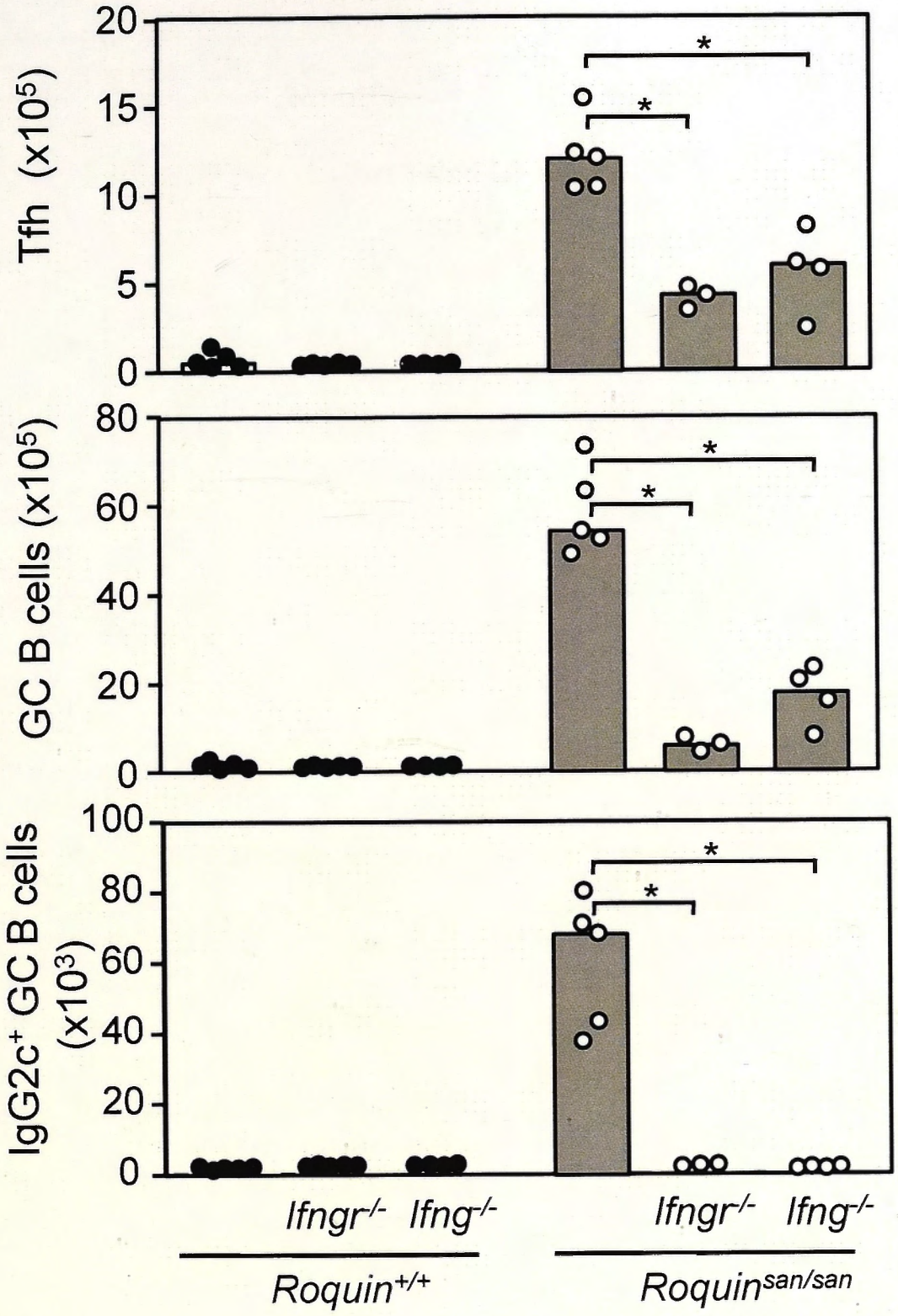


Figure 4.14. IFN-γ deficiency in *Roquin*$^{san/san}$ mice reduces Tfh and GC B cell formation.

Bar graphs showing total numbers of Tfh cells (top), GC B cells (middle) and IgG2c^{+} GC B cells (bottom) from 8 to 10-wk-old *Roquin*$^{+/+}$, *Roquin*$^{+/+}$ *Ifngr*$^{-/-}$, *Roquin*$^{+/+}$ *Ifng*$^{-/-}$, *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$, *Roquin*$^{san/san}$ *Ifng*$^{-/-}$ and *Roquin*$^{san/san}$ mice. These data are representative of three independent experiments with three to five mice per group.

C57BL/6 mice)[75, 366], IgG2c$^+$ GC B cells were also reduced or absent in *Roquin*$^{san/san}$ mice lacking IFN-γR or IFN-γ (**Figure 4.13A and 4.13B**).

Given our previous observation of a causal relationship between Tfh cell accumulation and lupus when *Roquin* is mutated[6], we investigated the consequences of IFN-γ signaling in *Roquin*$^{san/san}$ autoimmune disease. Assessment of renal histology revealed that all *Roquin*$^{san/san}$ mice had multiple mesangial electron-dense deposits, while only one out of six *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice had minor interstitial mesangial deposits compared to aged-matched 8-month-old *Roquin*$^{san/san}$ mice (**Figure 4.15A**). Lack of IFN-γR also reduced nephritis (**Figure 4.15A and 4.15B**) and prevented ANA formation (**Figures 4.16A and 4.16B**). These results contrast with the lack of contribution of IL-21 to the hypercellularity, Tfh and GC numbers, and autoantibodies in these mice[6].

We next investigated if disease can be ameliorated by blocking IFN-γ. For this, five week-old female mice, which had already developed ANAs, were treated with 500 μg of anti-IFN-γ mAb every 3 d for 3 weeks. At the end of this treatment, anti-IFN-γ treated mice had reduced numbers of effector/memory cells (**Figure 4.17A**), Tfh (**Figure 4.17B and 4.17C**) and GC B cells (**Figure 4.18A and 4.18B**), and significantly less ANAs than mice treated with isotype control (**Figure 4.18C**). Strikingly, autoantibody titers in anti-IFN-γ treated mice had reverted to the titers found in wild-type mice (**Figure 4.18C**). Together, these results demonstrate that IFN-γ overproduction leads to a pathogenic and lupus-inducing Tfh cell response and is required to sustain this aberrant response.

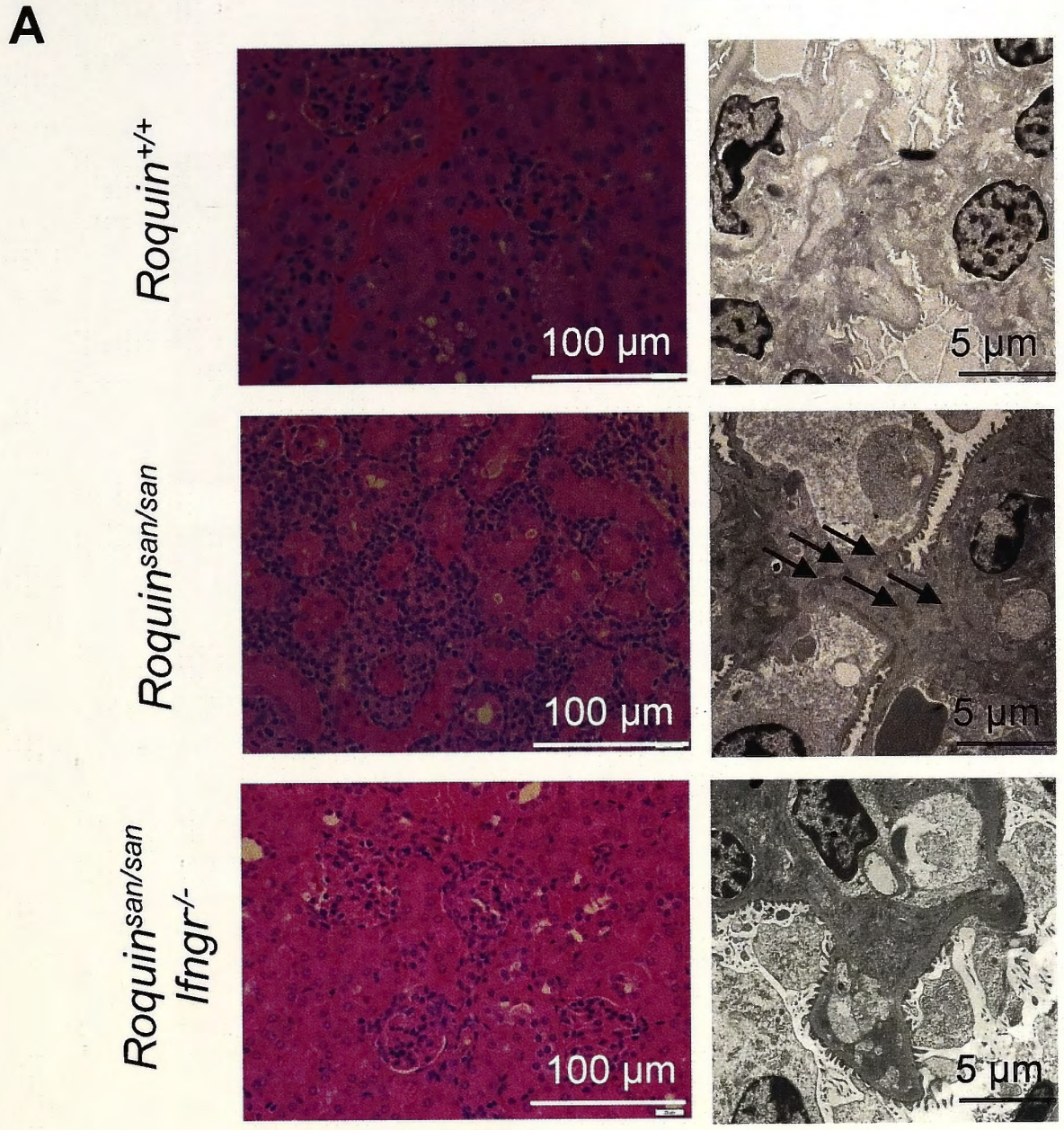


Figure 4.15. IFN-γR deficiency ameliorates the autoimmune phenotype of *Roquin*$^{san/san}$ mice.

Representative images of kidney sections (**A**) stained with H & E (left) or viewed under an electron microscope (right) and scores of nephritis severity (**B**) according to the criteria given in Table 4.1 from 6-mo-old *Roquin*$^{+/+}$, *Roquin*$^{san/san}$ and *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice. Electron-dense Ig deposits are indicated with arrows. These data are representative of two independent experiments with five to six mice per group. Severity of renal pathology was determined by Giles Walters.

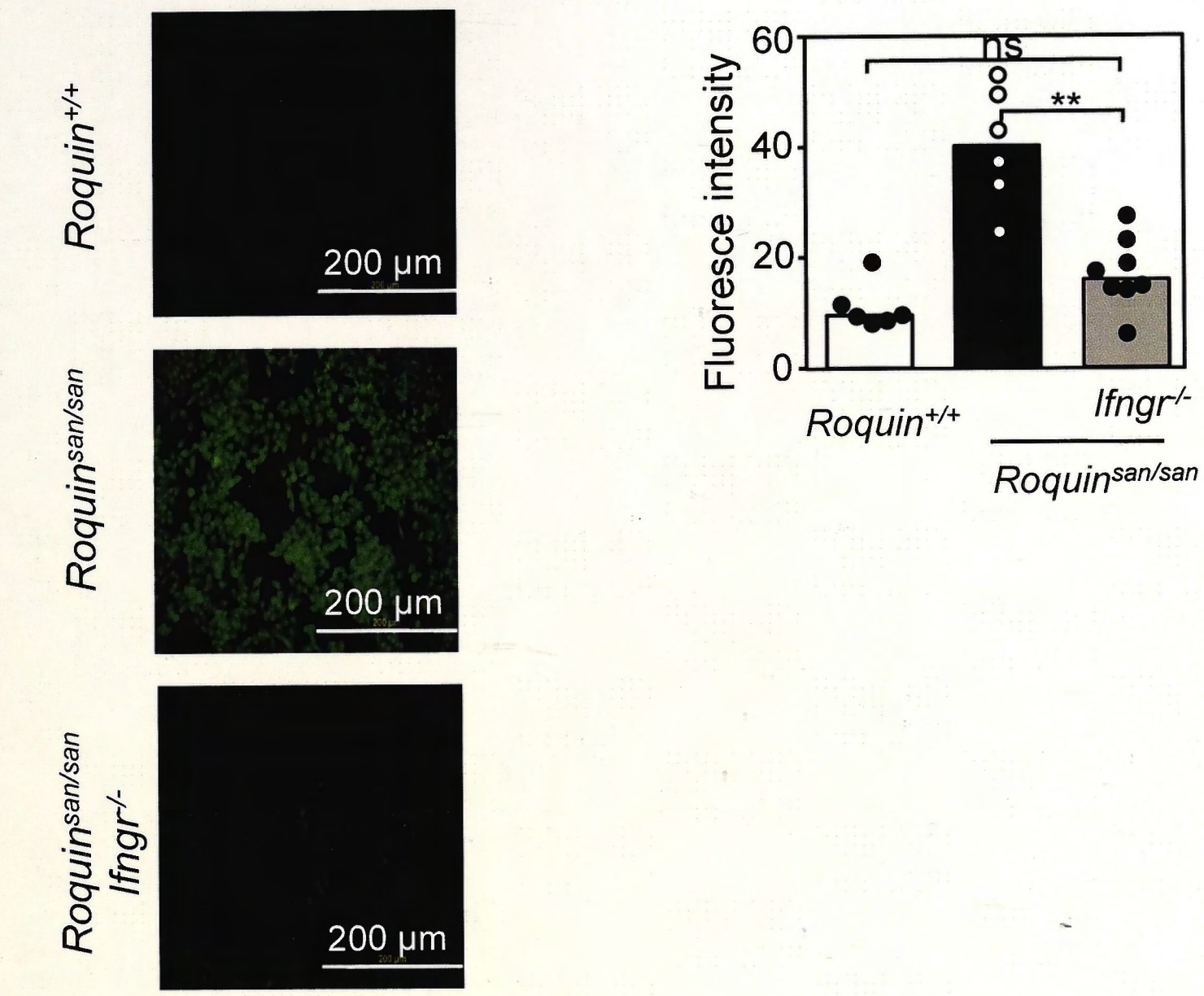


Figure 4.16. IFN-γR deficiency reduces ANAs in *Roquin*$^{san/san}$ mice.

Representative images (**A**) and quantification of fluorescence intensity (**B**) of ANA IgG autoantibodies detected using Hep-2 slides in serum from 8-wk-old *Roquin*$^{+/+}$, *Roquin*$^{san/san}$ and *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice. These data are representative of two independent experiments with six to eight mice per group.

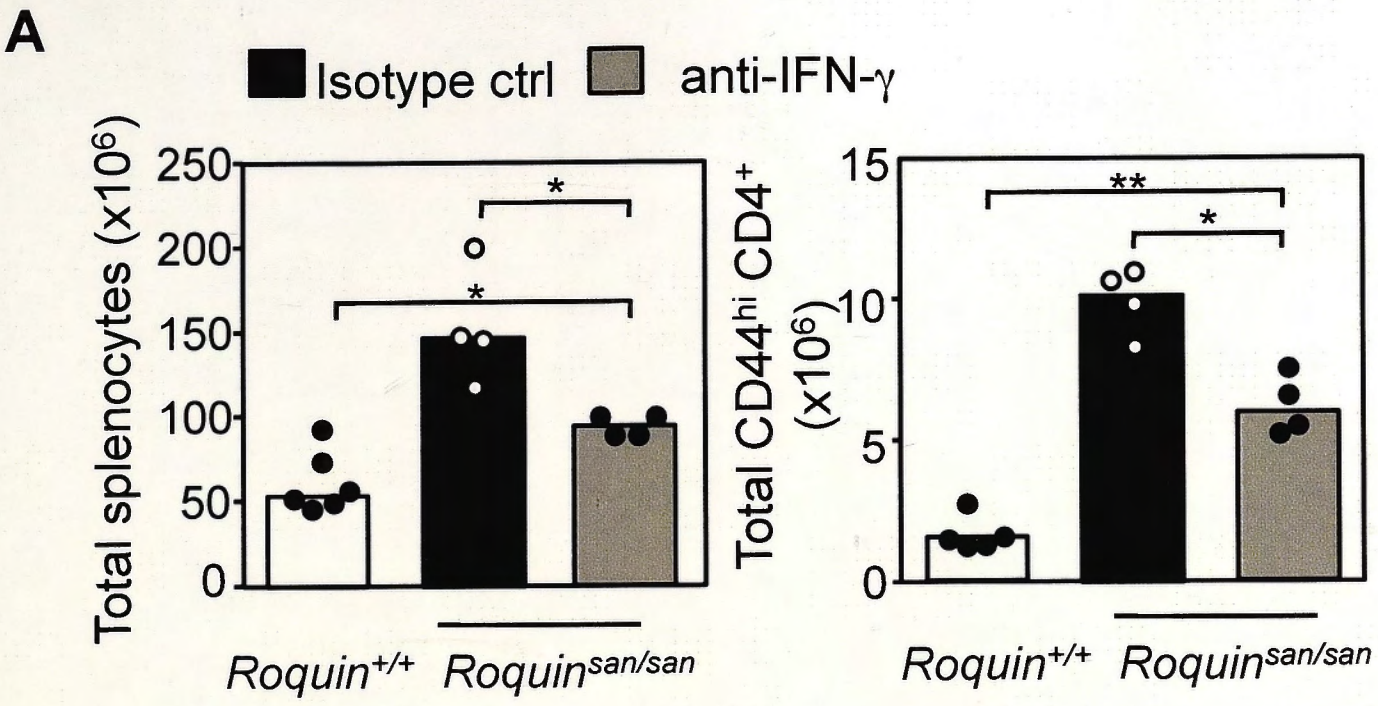


Figure 4.17. IFN-γ blockade reduces hypercellularity and Tfh cell accumulation in *Roquin*$^{san/san}$ mice.

A) Bar graphs showing total splenocytes (left) and effector/memory cells (right) of untreated *Roquin*$^{+/+}$ and treated female *Roquin*$^{san/san}$ mice with anti-IFN-γ or rat IgG1 isotype control every 3 d from week 5 to 8 of age.
B and C) Representative flow cytometric plots (B) and quantification (C) of percentages and total numbers of Tfh from untreated *Roquin*$^{+/+}$ and treated female *Roquin*$^{san/san}$ mice with anti-IFN-γ or rat IgG1 isotype control. These data are representative of two independent experiments with four to six mice per group.

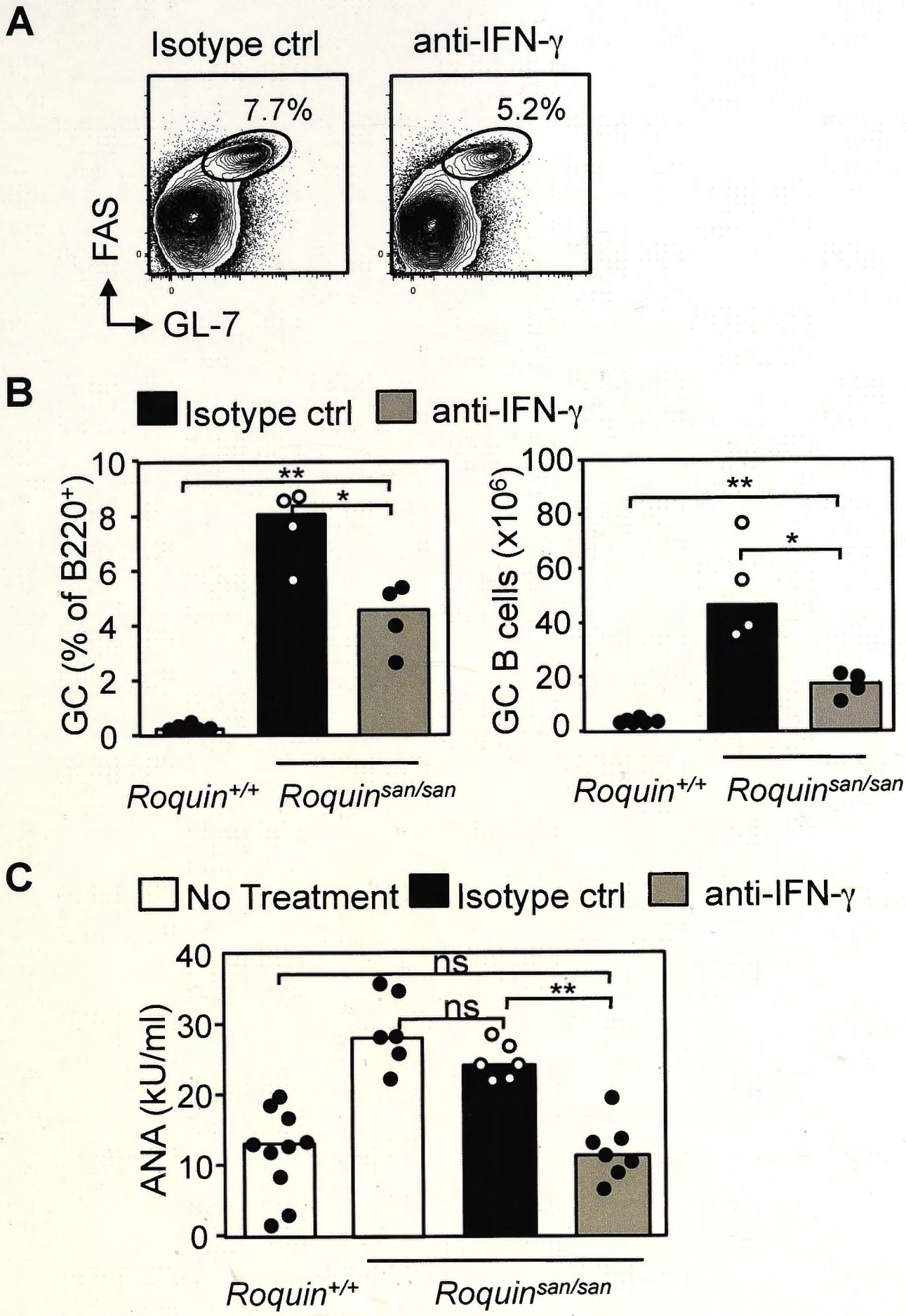


Figure 4.18. IFN-γ blockade reduces GC B cell accumulation and ANAs in *Roquin*$^{san/san}$ mice.

A and B) Representative flow cytometric plots (A) and quantification of percentages and total numbers (B) of GC B cells from untreated *Roquin*$^{+/+}$ and treated female *Roquin*$^{san/san}$ mice with anti-IFN-γ or rat IgG1 isotype control every 3 d from week 5 to 8 of age.
C) Concentration of ANA IgG autoantibodies in arbitrary activity units (U) according to the mouse ANA standards, in serum of *Roquin*$^{+/+}$ (untreated) and female *Roquin*$^{san/san}$ mice prior to treatment and after treatment with anti-IFN-γ mAb or rat IgG1 isotype control every 3 d from week 5 to 8 of age. These data are representative of two independent experiments with four to six mice per group.

Next we asked whether the aberrant Tfh response arising from an overactive IFN-γ response is unique to the *sanroque* mouse model or shared by other lupus-prone mice. *Fas*$^{-/-}$ mice develop early or late-onset lupus depending on whether they are bred on an MRL or B6 background, respectively[367, 368] and IFN-γ blockade experiment has demonstrated this treatment ameliorates lupus pathology[325]. MRL^{lpr} mice with defective FAS signaling also have expanded follicular helper T cells located at extrafollicular sites that are pathogenic[76]. Analysis of IFN-γ-producing cells in *Fas*$^{-/-}$ mice revealed a significant increase in IFN-γ-producing amongst total effector/memory (**Figure 4.19B, 4.19C**), non-Tfh and Tfh cells (**Figure 4.20A**). This was accompanied by a 3-fold increase in total effector/memory cells (CD4^{+} CD44^{hi}; **Figure 4.19A**), 9-fold increase in Tfh cells (PD-1^{hi} CXCR5^{hi}; **Figure 4.20B and Figure 4.20C**), 11-fold increase in GC B cells (**Figure 4.21A and 4.21B**) and increased ANAs compared with wild-type controls (**Figure 4.21C**). Our data together with evidence that SAP deficiency – known to selectively eliminate Tfh cells[6, 45, 172] – abrogates lupus in *Fas*$^{-/-}$ mice[369], further supports the notion that the pathogenic IFN-γ response is linked to the aberrant Tfh cell response.

Seeking further evidence that aberrant Tfh formation is not only associated with lupus-autoantibodies but is also a consequence rather than a cause of excessive IFN-γ signaling, we analyzed mice expressing a Bcl-2 transgene under the control of the Vav-promoter. These mice form a spontaneous and excessive GC response dependent on excessive T cell help[340]. Antinuclear antibodies were increased in VavP-*Bcl2* transgenic mice (**Figure 4.23C**), and this was associated with a 8-fold expansion in Tfh cells (**Figure 4.23A**) and a 5-fold increase in GC B cells (**Figure 4.23B**), but no difference in total effector/memory cells (**Figure 4.22A**). As expected

and in contrast to *sanroque* mice and *Fas*$^{-/-}$ mice, there was no significant increase in IFN-γ-producing cells (**Figure 4.22B and 4.22C**), consistent with aberrant Tfh expansion and survival being independent of cytokines and instead due to constitutive Bcl-2 expression. Together, these results support the evidence that Tfh cell expansion caused by IFN-γ overexpression - which can be recapitulated by a Bcl-2 transgene -, is pathogenic and causes autoantibody production.

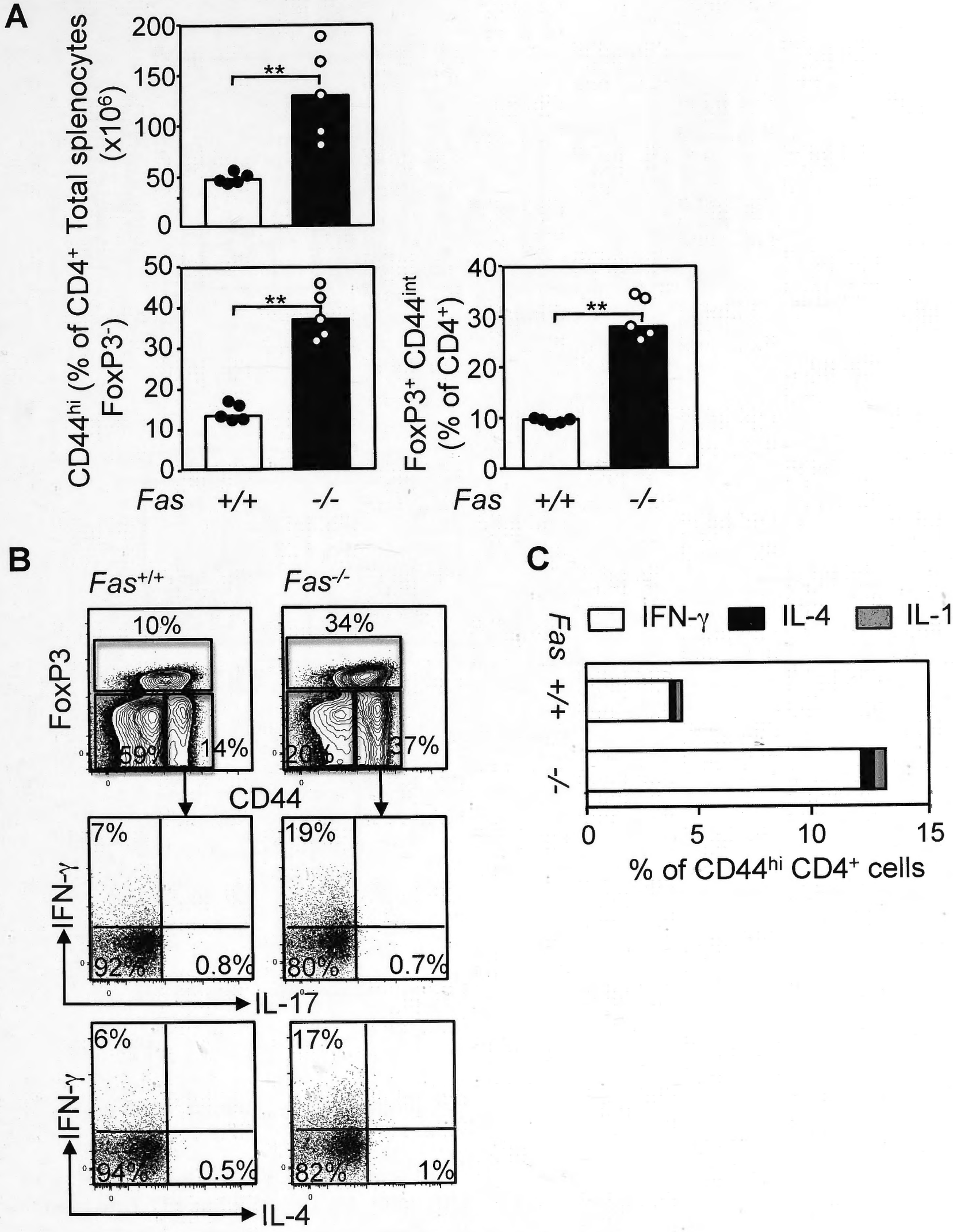
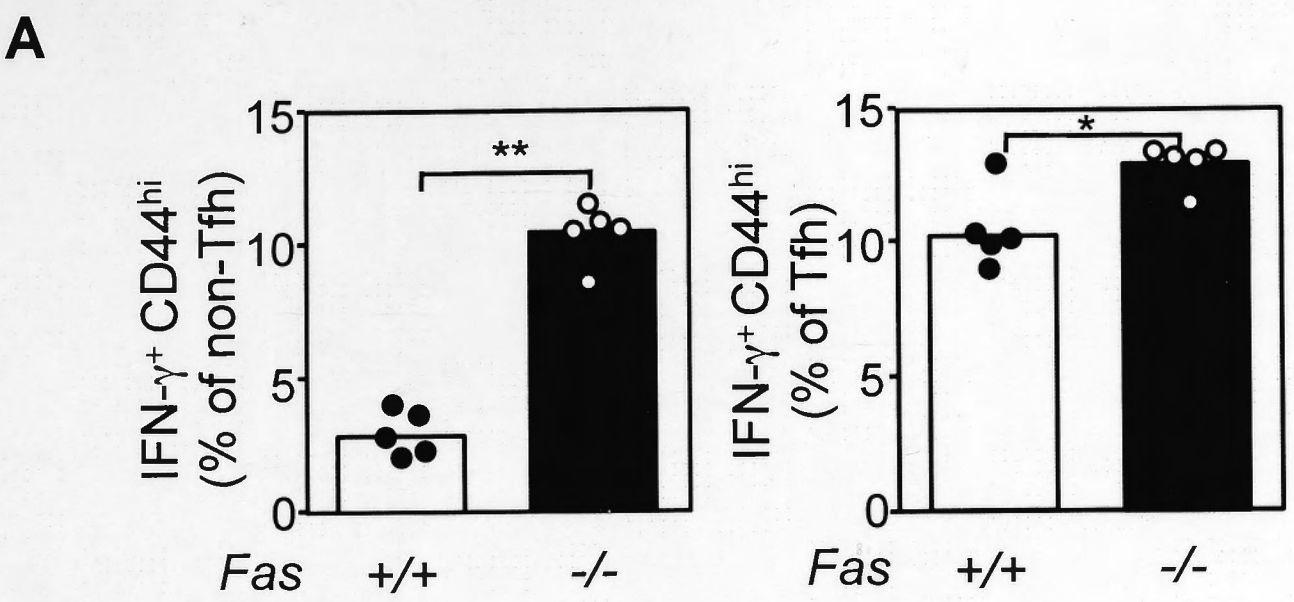
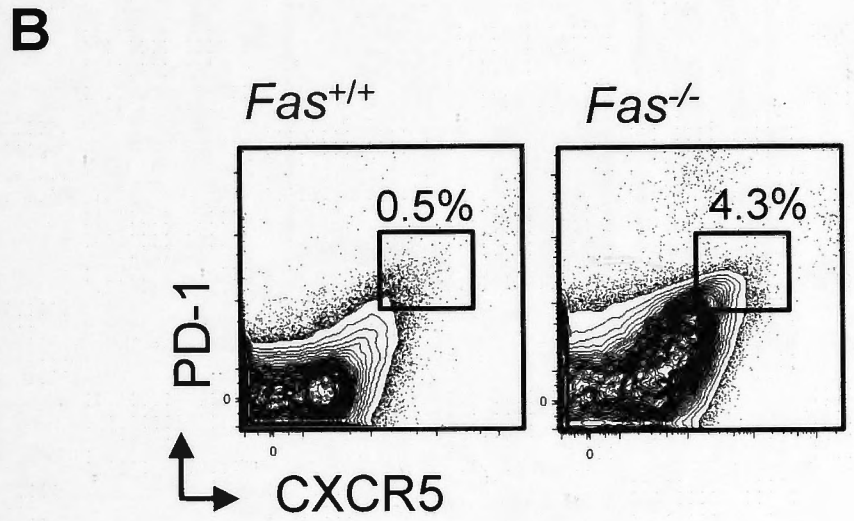


Figure 4.19. *Fas*$^{-/-}$ mice develop hypercellularity.

A) Quantification of total numbers of splenocytes (top), and frequencies of effector/memory (bottom left) and Treg (bottom right) cells from 10-wk-old *Fas*$^{-/-}$ and *Fas*$^{+/+}$ mice.
B and C) Representative flow cytometric plots (B) and quantification of percentages (C) showing IFN-γ-, IL-4- and IL-17-producing cells amongst CD4$^+$ CD44^{hi} cells from 10-wk-old *Fas*$^{-/-}$ and *Fas*$^{+/+}$ mice. These data are representative of two independent experiments with five mice per group.





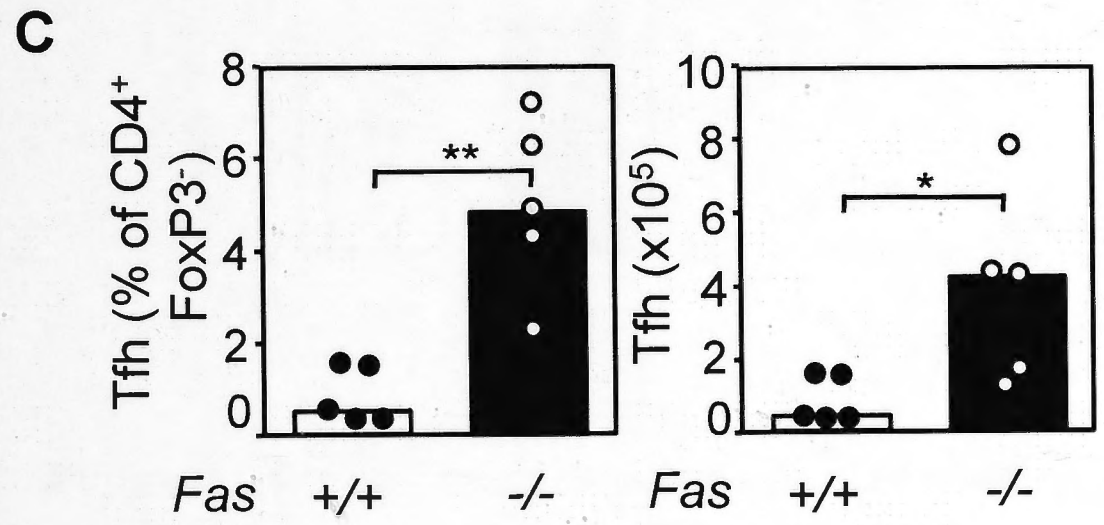


Figure 4.20. Increased numbers of IFN-γ-producing cells in *Fas*$^{-/-}$ mice.

A) Quantification of percentages of IFN-γ-producing cells amongst non-Tfh (left) and Tfh cells (right) from 10-wk-old *Fas*$^{-/-}$ and *Fas*$^{+/+}$ mice.
B and C) Representative flow cytometric plot (B) and quantification of percentages (C; left) and total numbers (C; right) of Tfh cells from 10-wk-old *Fas*$^{-/-}$ and *Fas*$^{+/+}$ mice. These data are representative of two independent experiments with five mice per group.

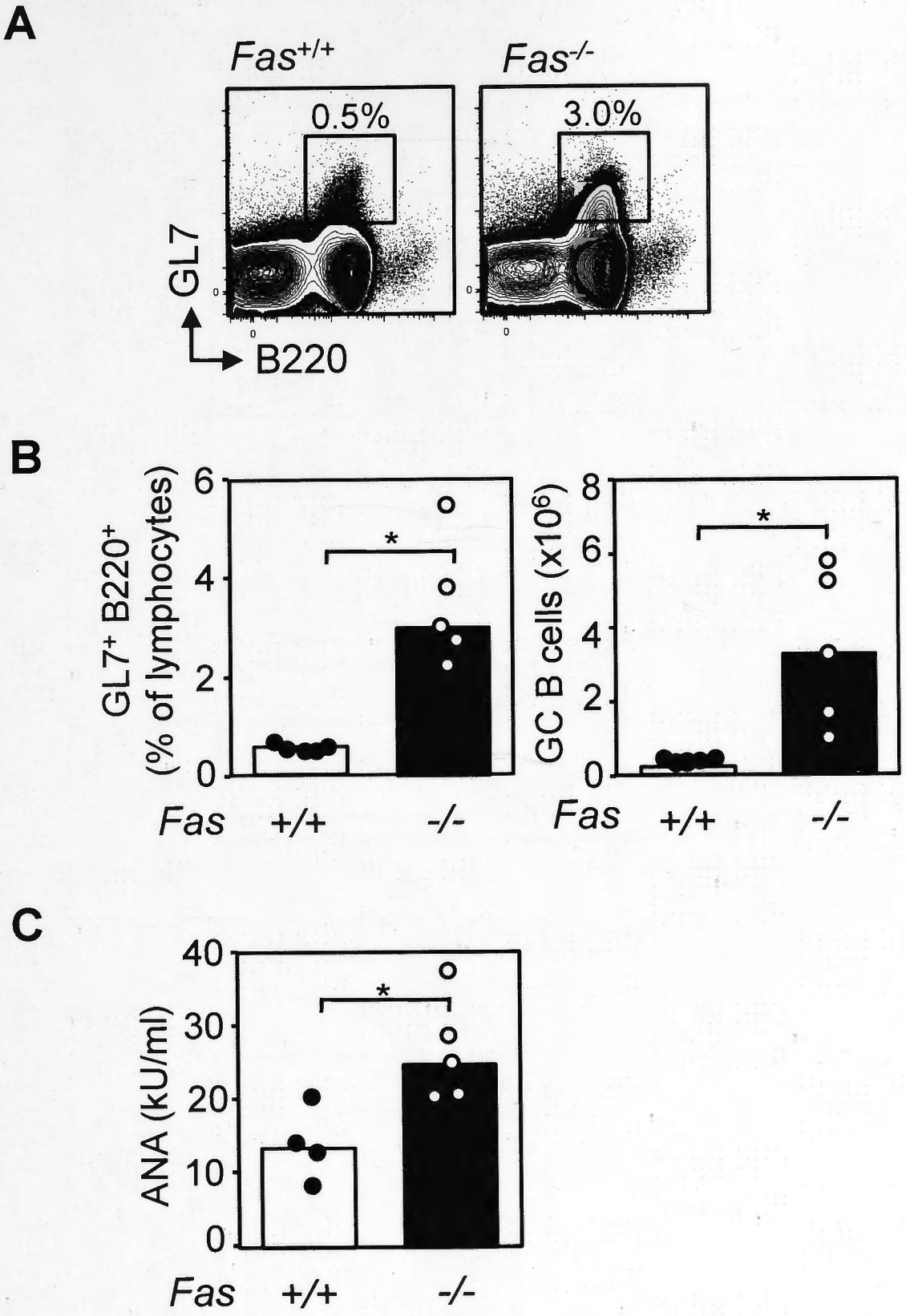


Figure 4.21. *Fas*$^{-/-}$ mice develop spontaneous GC and ANAs.

A and B) Representative flow cytometric plots (A) and quantification of percentages (B; left) and total numbers (B; right) of GC B cells from 10-wk-old *Fas*$^{-/-}$ and *Fas*$^{+/+}$ mice.
C) Concentration of ANA IgG autoantibodies in serum of 10-wk-old *Fas*$^{-/-}$ and *Fas*$^{+/+}$ mice. These data are representative of two independent experiments with five mice per group.

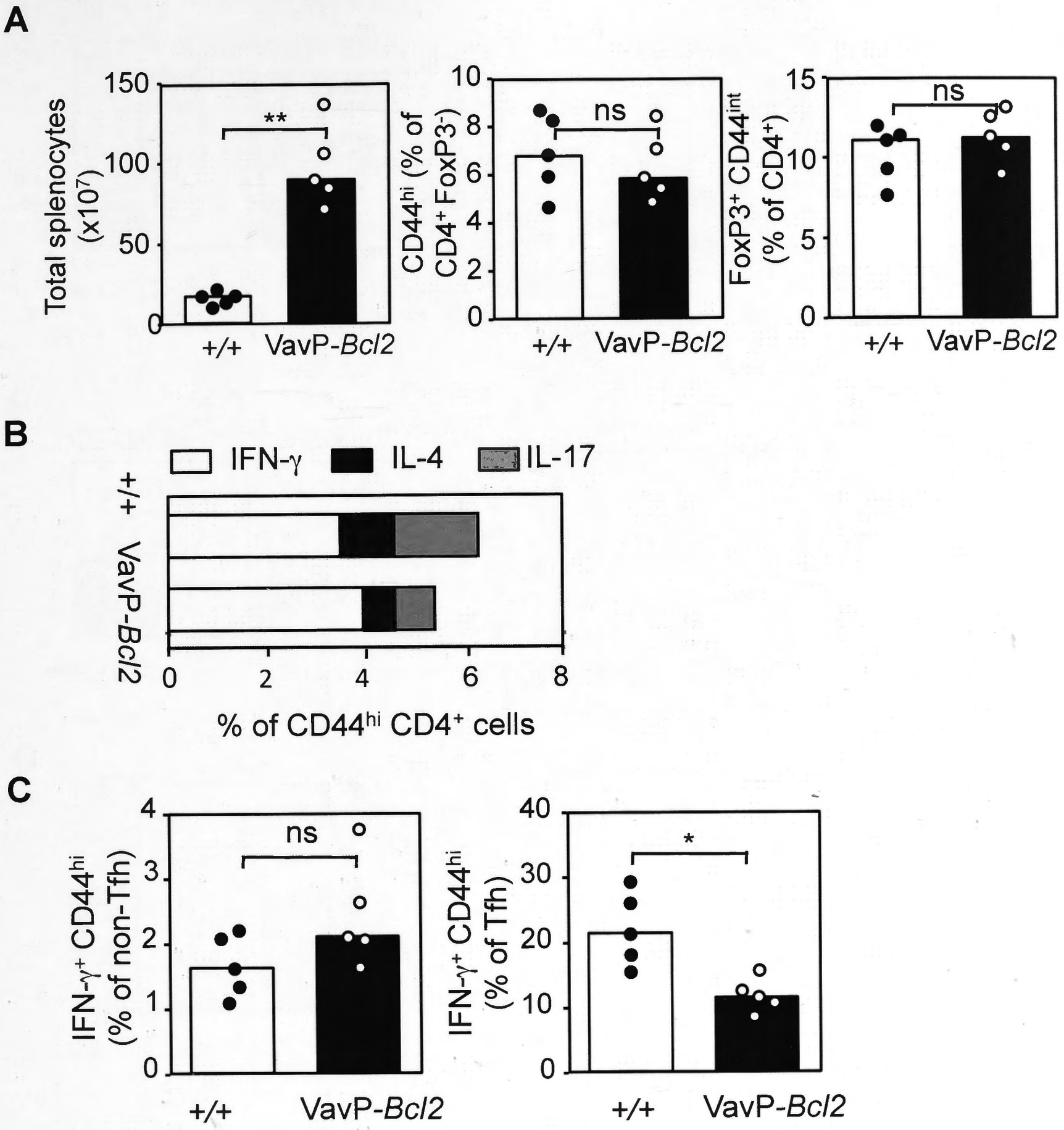


Figure 4.22. VavP-*Bcl2*$^{-/-}$ mice develop hypercellularity.

A) Quantification of total numbers of splenocytes (left), and frequencies of effector/memory (middle) and Treg (right) cells from 10-wk-old VavP-*Bcl2* and C57BL/6 mice.
B) Quantification of percentages showing IFN-γ-, IL-4- and IL-17-producing cells amongst CD4^{+} CD44^{hi} cells from 10-wk-old VavP-*Bcl2* and C57BL/6 mice.
C) Quantification of percentages of IFN-γ-producing cells amongst non-Tfh (left) and Tfh cells (right) from 10-wk-old VavP-*Bcl2* and C57BL/6 mice. These data are representative of two independent experiments with five mice per group.

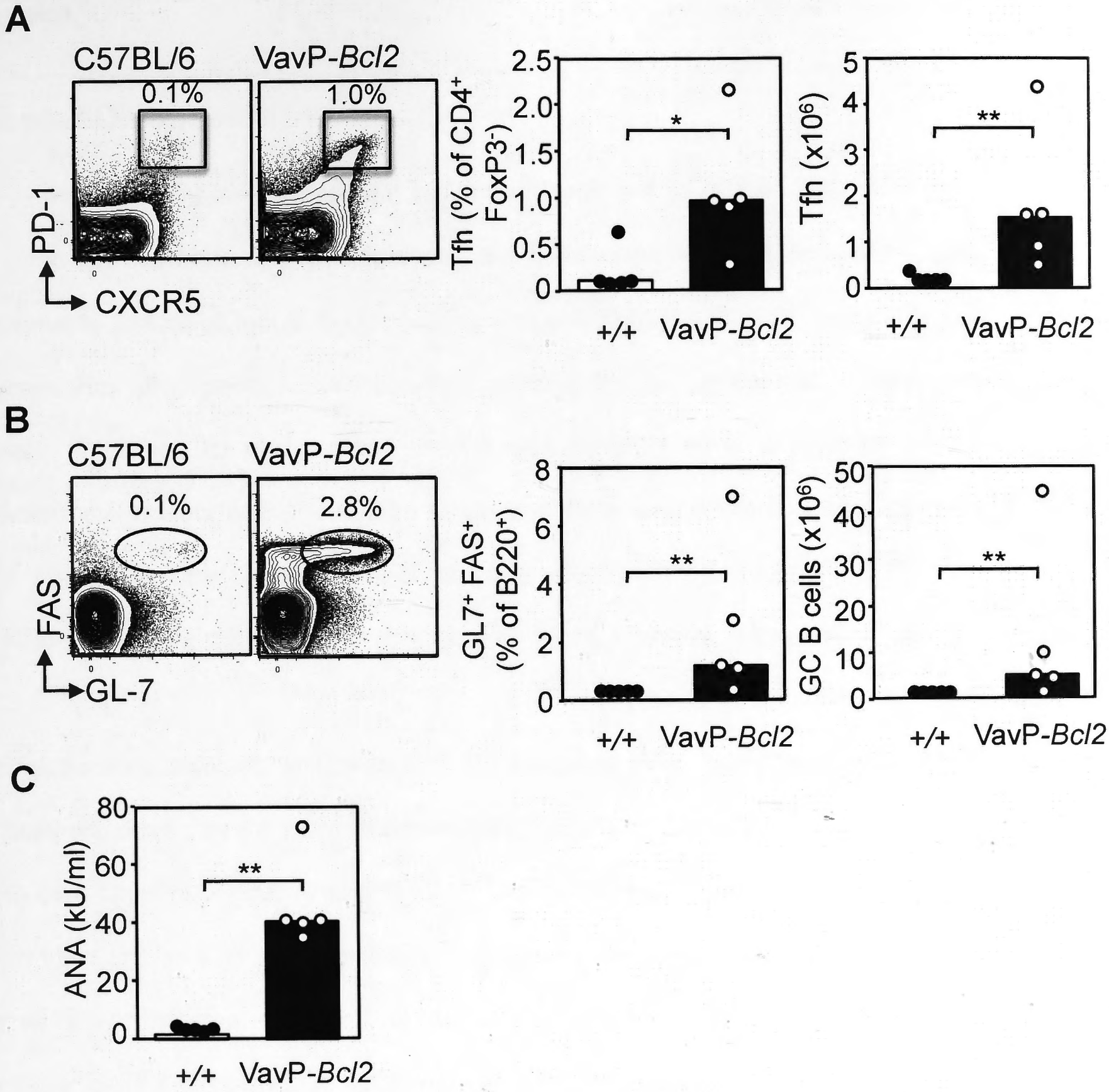


Figure 4.23. VavP-*Bcl2*$^{-/-}$ mice have increased Tfh, GC B cells and ANAs.

A) Representative flow cytometric plots (left) and quantification of percentages and total numbers (right) of Tfh cells from 10-wk-old VavP-*Bcl2* and C57BL/6 mice.
B) Representative flow cytometric plots (left) and quantification of percentages and total numbers (right) of GC B cells from 10-wk-old VavP-*Bcl2* and C57BL/6 mice.
C) Concentration of ANA IgG autoantibodies in serum of 10-wk-old VavP-*Bcl2* and C57BL/6 mice. These data are representative of two independent experiments with five mice per group.

Chapter Discussion

IFN-γ has been long associated with lupus pathology but its precise role in disease pathogenesis has been unclear. Here we have discovered how excessive IFN-γ acts in a reliable model of lupus: by promoting Tfh cell accumulation. Limiting Tfh cell numbers has emerged as an important mechanism to control GC output and maintain tolerance to nuclear antigens. These findings have significant clinical implications. Subsets of patients with lupus and other autoimmune disorders have been shown to bear biomarkers of an overactive Tfh/GC pathway[158, 232]. We speculate that IFN-γ neutralization may be particularly effective in this patient group.

The relationship between Th1 cells and Tfh cells has been controversial. Although Tfh cells are driven by their own transcriptional regulator, Bcl-6[136, 152, 153], description of Tfh cells capable of producing IFN-γ[291, 355] led to the hypothesis that Tfh cells could derive from Th1 as well as other T helper cell lineages[3]. More recently, the report of very early – at the time of the first or second division - and dichotomous polarization into either Bcl-6 expressing Tfh cells or Blimp-1-expressing non-Tfh cell effectors has suggested that Tfh cells originate independently from other effectors including Th1 cells[164, 165]. Conversely, A recent study by Liu et al. revealed substantial plasticity of all effector cell lineages in the early phase of Tfh cell differentiation *in vivo.* Both Th1 and Th2 cells, but only few Th17 cells were able to convert to Tfh cells[370].

A question that arises is whether expanded Th1 cells in *Roquin*$^{san/san}$ mice are also contributing to the lupus phenotype, independently of Tfh cells. Our previous experiments suggest this is unlikely: selective abrogation of Tfh formation via genetic deficiency in SAP – which did not reduce Th1 cells – in *sanroque* mice resulted in

complete abrogation of autoantibody formation and lupus disease, although it did not correct the splenomegaly or the hypergammaglobulinemia[6]. This together with the demonstration in the same study that passive transfer of *Roquin*$^{san/san}$ Tfh cells promotes spontaneous GC formation, suggests that Tfh cells but not Th1 cells are responsible for the aberrant B cell activity leading to autoantibody production and kidney disease in *Roquin*$^{san/san}$ mice. However, it is likely that Th1 cells, and not Tfh cells, are responsible for the hypercellularity of secondary lymphoid tissues, which is not an autoimmune manifestation *per se*. Importantly, the effect of IFN-γ described here acts independently of T-bet; in fact, T-bet deficiency in *Roquin*$^{san/san}$ mice did not ameliorate lupus. T-bet-independent IFN-γ production as observed in *Roquin*$^{san/san}$ has been shown to occur physiologically downstream of IL-12/STAT-4, BAFF, $CD8^+$ T cell-derived IFN-γ or γδ T cell-derived IFN-γ[264, 360, 371, 372]. We cannot totally exclude the possibility that IFN-γ may promote other cytokines and signalling pathways that contribute to the aberrant formation of GC B cell and Tfh cells in *Roquin*$^{san/san}$ mice. However, this is unlikely due to the fact that lack of IFN-γ signalling in *Roquin*$^{san/san}$ T cells virtually corrected the aberrant accumulation of Tfh and GC B cells in *Roquin*$^{san/san}$ mice.

As opposed to the amelioration of lymphoid organ cellularity observed by halving the gene dose of *Icos* in *Roquin*$^{san/san}$ CBAxB6 mice, complete ICOS deficiency failed to reduce splenomegaly. Interestingly, the pattern of the autoantibodies also changed, suggesting an additional mechanism of autoantibody formation is triggered by ICOS deficiency. The 4-fold reduction in the number of Treg cells in *Roquin*$^{san/san}$ *Icos*$^{-/-}$ mice together with impaired suppressive function of Treg cells lacking ICOS[363] are likely to account for the disease exacerbation. The corollary from this and our

previous study is that although excessive ICOS signaling contributes to Tfh cell expansion, it is not the primary cause of disease pathogenesis, which we show here stems from excessive *Ifng* mRNA production. This is consistent with the finding that IFN-γ deficiency alone could not completely correct Tfh cell numbers, suggesting other factors including ICOS overexpression lead to this phenotype. Our work reveals a second target of mRNA regulation by *Roquin*, only known to date to regulate *Icos* mRNA posttranscriptionally[248, 249, 253]. The increased *Ifng* mRNA half-life in *Roquin*$^{san/san}$ T cells compared to *Roquin*$^{+/+}$ controls, together with the demonstrated T-cell autonomous nature of *Ifng* mRNA accumulation from the naïve T cell stage, suggest *Ifng* and *Icos* mRNA may be deregulated by *Roquin*^{san} by a similar mechanism.

In this chapter, we have identified a GC tolerance checkpoint: posttranscriptional control of *Ifng* mRNA that prevents excessive Tfh formation. In the *Roquin*$^{san/san}$ model of lupus, impaired *Ifng* mRNA decay leads to aberrant Tfh cell formation and spontaneous GCs, and lupus pathology. In addition, IFN-γ blockade or deficiency of *Roquin*$^{san/san}$ mice prevent Tfh accumulation and all autoimmune manifestations, highlighting a direct causal role for this cytokine in lupus disease due to overactive Tfh cell activity. It is important to note that it is only in the presence of excessive IFN-γ that Tfh cells are dysregulated; IFN-γ deficiency did not impair a Tfh response against immunization with foreign antigen (data not shown). This emphasizes the importance of posttranscriptional control to specifically prevent accumulation of *Ifng* mRNA after its induction, in the prevention of autoimmunity.

Chapter 5 – Excessive Interferon-gamma Promotes Bcl-6 Overexpression in Tfh cells and Proliferation of Effector T cells

Chapter Introduction

Tfh cell formation is driven by the transcription factor Bcl-6, which controls expression of key Tfh molecules including PD-1 and CXCR5[136, 152, 153]. Recently, extrinsic mechanisms that control Tfh homeostasis and in turn GC output have been described; these include the inhibitory effects of plasma cells[36] and specialized CD4 and CD8 follicular regulatory T cells[138, 139, 373]. Little is known about cell-intrinsic mechanisms that repress Tfh formation to prevent autoimmunity. Thus, understanding the mechanisms that control Tfh cell homeostasis or lead to their dysregulation will be important for deciphering the pathogenesis of the growing list of autoantibody-mediated diseases[5].

In the previous chapter, we have shown that excessive IFN-γ caused by impaired posttranscriptional regulation of ROQUIN^{M199R} changes the quality of the T cell response by inducing the accumulation of Tfh cells; this in turn leads to aberrant GC formation, autoantibody production and lupus pathology. The next important question arising is: How does IFN-γ influence Tfh cell accumulation? There are several possibilities by which IFN-γ can enhance Tfh formation and/or accumulation: 1) IFN-γ can act directly on T cells and induce Tfh cell formation via for example modulation of Bcl-6 and/or increased proliferation or survival of Tfh cells or their precursors. 2)

IFN-γ-produced by other cells such as myeloid cells or NK cells can have an indirect impact on Tfh cells by providing an inflammatory environment that fosters B and T cell activation leading to differentiation of the latter cells into IFN-γ-producing T cells. In turn, these cells may contribute to the Tfh cell precursor pool. In addition, IFN-γ production by T cells may also provide a positive feedback loop to sustain more myeloid cell activation/proliferation. 3) IFN-γ may act on B cells to induce B cell proliferation, survival, and enhanced T cell priming. All of this together would promote Tfh formation and provide a niche that favors Tfh cell and GC B cell survival. Indirect effects of IFN-γ on B cells could also be mediated via stimulation of myeloid cells to release BAFF[334, 360]. To adequately address these questions, we have taken advantage of mixed bone chimeras that specifically lack T or B cells, and passive transfer experiments into *Rag1*$^{-/-}$ mice. Understanding the molecular and cellular basis of Tfh-driven lupus is critical for the development of specific therapies in affected individuals.

To our knowledge, direct effects of IFN-γ on Bcl-6 expression by T cells have not been reported, with the exception of an *in vitro* experiment showing that rIFNg was able to induce transient upregulation of Bcl-6 in Jurkat cells and murine T cells[374]. Nevertheless, whether IFN-γ has an effect on Tfh cells and Bcl-6 expression *in vivo* has not yet been documented. Dichotomous functions of IFN-γ in promoting proliferation or inducing apoptosis of differentiated CD4+ T cells have been reported. In an autoimmune/inflammatory setting, the ability of IFN-γ to induce CD4^{+} T cell proliferation has been reported in patients with multiple sclerosis, acute myeloid leukemia (AML) and B cell-type chronic lymphocytic leukemia (B-CLL); it has also been reported in human myeloid leukemia cell lines[375-377]. Interestingly, IFN-γ is also

required for apoptosis of expanded $CD4^+$ T cell clones during infection and mice lacking IFN-γ or IFN-γR exhibit impaired apoptosis and delayed contraction of activated antigen-specific T cells[378-381].

Despite numerous studies strongly implicating IFN-γ in SLE pathogenesis and IFN-γ mAb (AMG811) treatment recently entering phase I clinical trials[382], the precise pathogenic mechanism by which this cytokine contributes to disease is poorly understood. Here we have demonstrated a fundamentally different mechanism of action of this important cytokine: failure to repress *Ifng* posttranscriptionally by ROQUIN acts in early effector $CD4^+$ T cells in a cell-intrinsic manner to enhance Bcl-6 expression and increase their proliferation, leading to aberrant Tfh cell accumulation, pathogenic GCs, autoantibodies and end-organ damage.

Results

T cell-specific IFN-γR deficiency is sufficient to reduce Tfh accumulation

To investigate whether IFN-γ was acting predominantly on $CD4^+$ cells to induce the aberrant Tfh cell and GC phenotype, we generated chimeric mice in which only T cells would lack IFN-γR signaling. For this, sublethally irradiated *Rag1*$^{-/-}$ recipient mice were reconstituted with a 70%:30% mix of *Roquin*$^{san/san}$ *Tcrα*$^{-/-}$ and *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ bone marrow cells. A control set of chimeras with intact IFN-γR signaling was constructed using a 70%:30% mix of *Roquin*$^{san/san}$ *Tcrα*$^{-/-}$ and Roquin$^{san/san}$ bone marrow cells. Sets of 100% chimeras reconstituted with either *Roquin*$^{+/+}$, *Roquin*$^{san/san}$ or *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ bone marrow were also used as controls.

Selective deficiency in IFN-γR in T cells reduced Tfh accumulation in *Roquin*$^{san/san}$ mice to the levels found in *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice and *Roquin*$^{+/+}$ mice indicating that increased IFN-γR signaling in T cells leads to Tfh cell accumulation in *Roquin*$^{san/san}$ mice (**Figure 5.1**). IFN-γR signaling in T cells also contributed to the increase in GC B cells observed in *Roquin*$^{san/san}$ mice: fewer GL-7^+ FAS^+ B cells were observed in *Roquin*$^{san/san}$ chimeras lacking IFN-γR only in T cells although GC B cells were higher than in mice lacking IFN-γR signaling in all cells (**Figure 5.1**).

To formally test whether excessive IFN-γ signaling also acts directly on GC B cells, we set up mixed chimeras in which only B cells would lack IFN-γR signaling by reconstituting sublethally-irradiated *Rag1*$^{-/-}$ recipient mice with a 70%:30% mix of *Roquin*$^{san/san}$ *Igα*$^{ken/ken}$ and *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ bone marrow cells. Although both Tfh

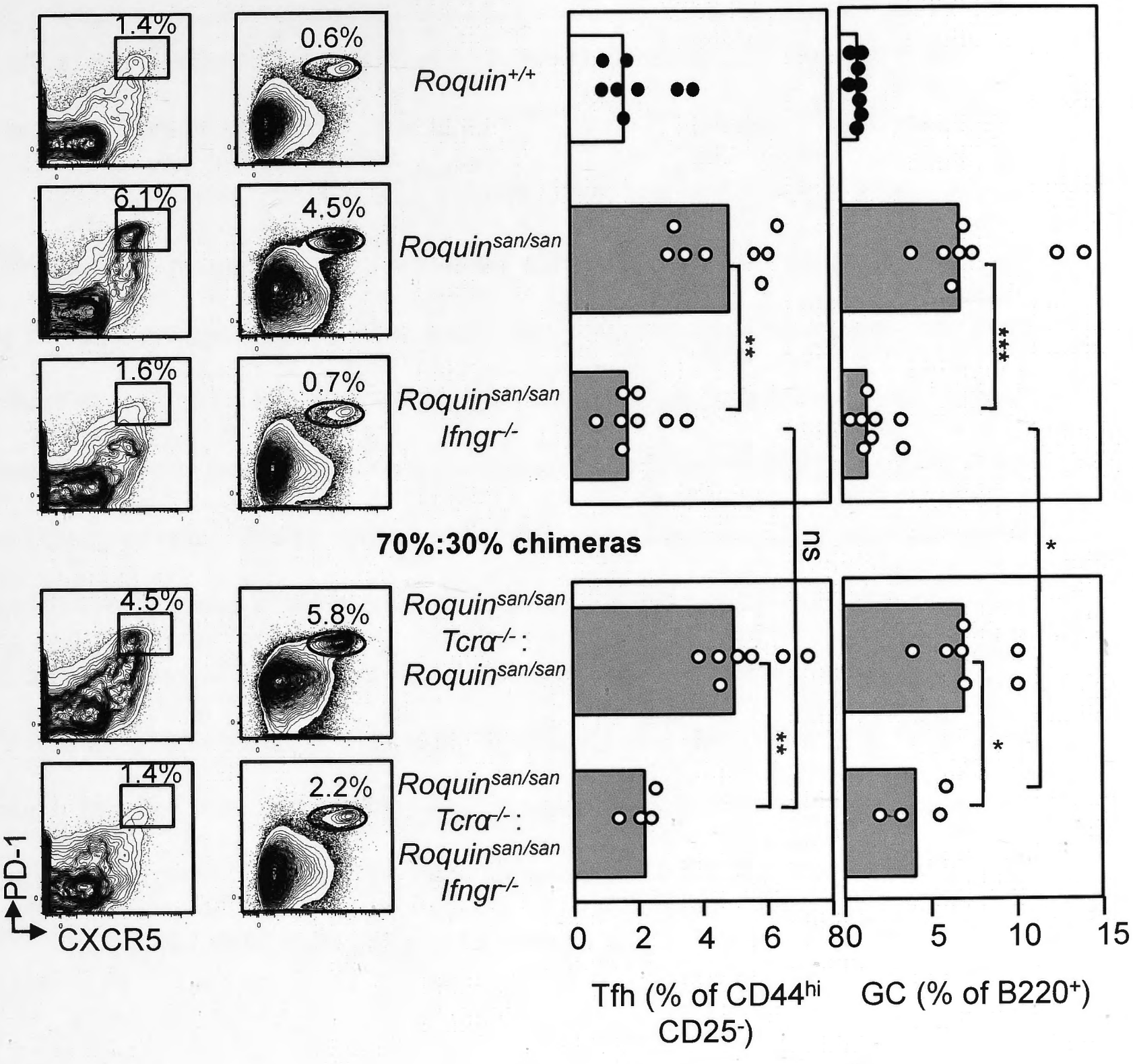


Figure 5.1. IFN-γR signaling in T cells is required for Tfh and GC B cell accumulation in *Roquin*$^{san/san}$ mice.

Representative flow cytometric plots (left) and quantification of percentages (right) of Tfh cells identified as CXCR5^{hi} PD-1^{hi} (gated on CD44^{hi} CD25^{-} CD4^{+} cells) and GC B cells cells identified as GL-7^{+} FAS^{+} (gated on B220^{+} cells) from mixed bone chimeras of indicated genotypes 10 weeks after reconstitution. These data are representative of two independent experiments with four to eight mice per group in each experiment.

and GC B cells were reduced in these chimeras (**Figure 5.2**), the same reduction was seen in control recipients of *Roquin*$^{san/san}$ *Igα*$^{ken/ken}$: *Roquin*$^{san/san}$ bone marrow cells. The number of B220^{+} cells was ~ 5-fold lower, but no difference in total number of CD4^{+} cells, in recipients of bone marrow containing *Igα*$^{ken/ken}$ cells compared with other chimeric groups (**Figure 5.3A and 5.3B**), suggesting that the correction of the Tfh and GC phenotype is a consequence of the significantly reduced B cell numbers in these chimeric mice and these chimeras cannot be used to draw conclusions on B cell-intrinsic effects. ANAs were not measured because the irradiation and reconstitution process induces autoantibodies. IFN-γR signaling is known to exert important effects on myeloid cells. All myeloid cell subsets were found to be expanded in total numbers in *Roquin*$^{san/san}$ spleens, but this effect was T cell-driven because myeloid cell expansion and splenic hypercellularity were completely corrected in *Roquin*$^{san/san}$ *Rag1*$^{-/-}$ mice (**Figure 5.4A-C**) but not in *Roquin*$^{san/san}$ *Igα*$^{ken/ken}$ (B cell-deficient) mice (data not shown).

Excessive IFN-γ signaling enhances Bcl-6 expression and increases proliferation of early CD4^{+} T cell effectors

IFN-γ has been described to have paradoxical pro-proliferative and pro-apoptotic effects in CD4^{+} and CD8^{+} T cells[383, 384]. Intracellular staining with Ki-67 and Annexin V/7AAD was used to investigate proliferation and survival, respectively. Lack of IFN-γR signaling did not have any effects on Tfh cell proliferation (**Figure 5.5B**), but significantly decreased the proportion of cycling naïve (CD44^{lo} FoxP3^{-}) and non-Tfh

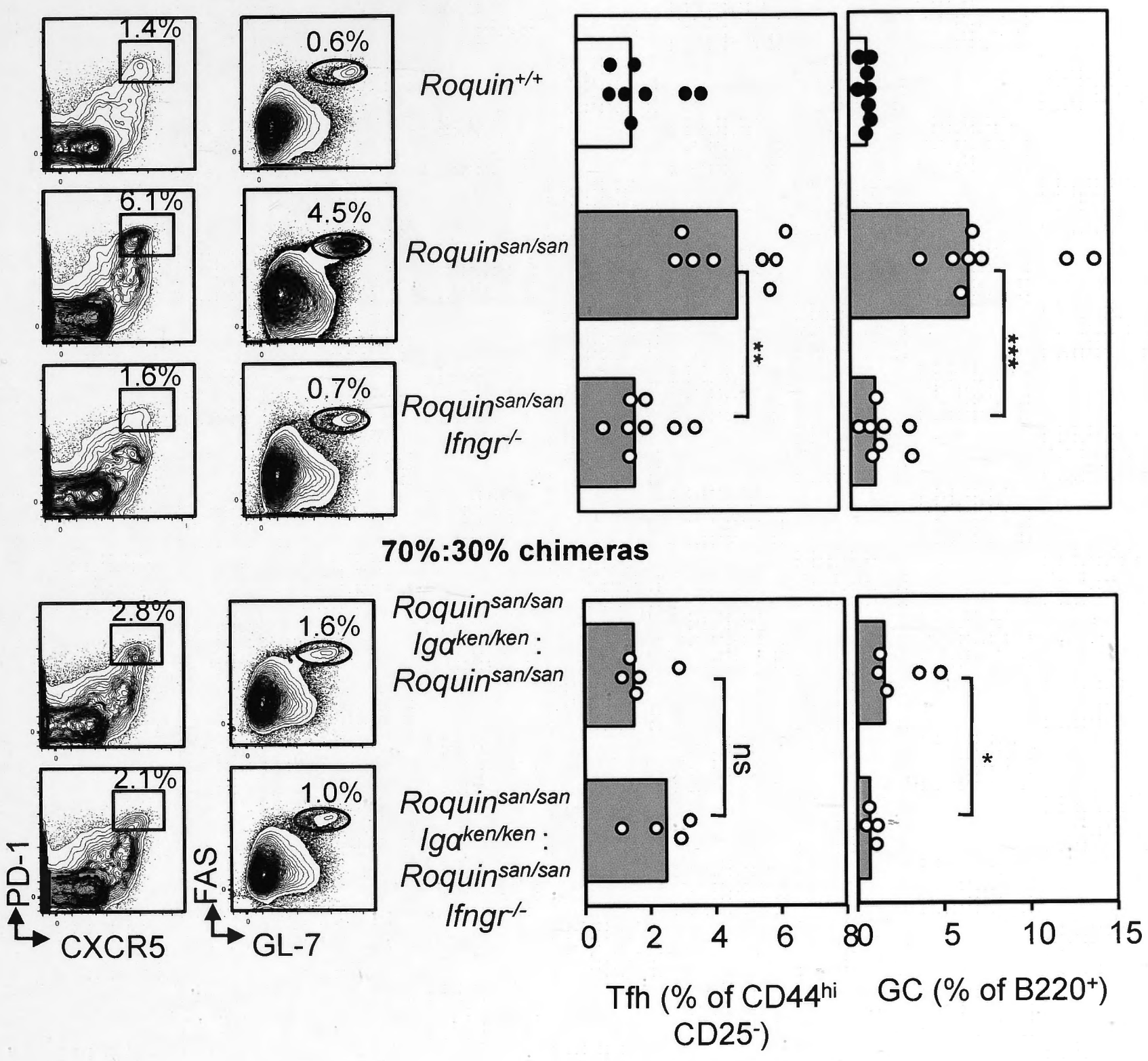


Figure 5.2. Reduced Tfh and GC B cell numbers in chimeras constructed with B cell-deficient bone marrow.

Representative flow cytometric plots (left) and quantification of the percentages (right) of Tfh and GC B cells from mixed bone chimeras of indicated genotypes 10 weeks after reconstitution (top panel is reproduced from Figure 5.1). These data are representative of two independent experiments with four to eight mice per group in each experiment.

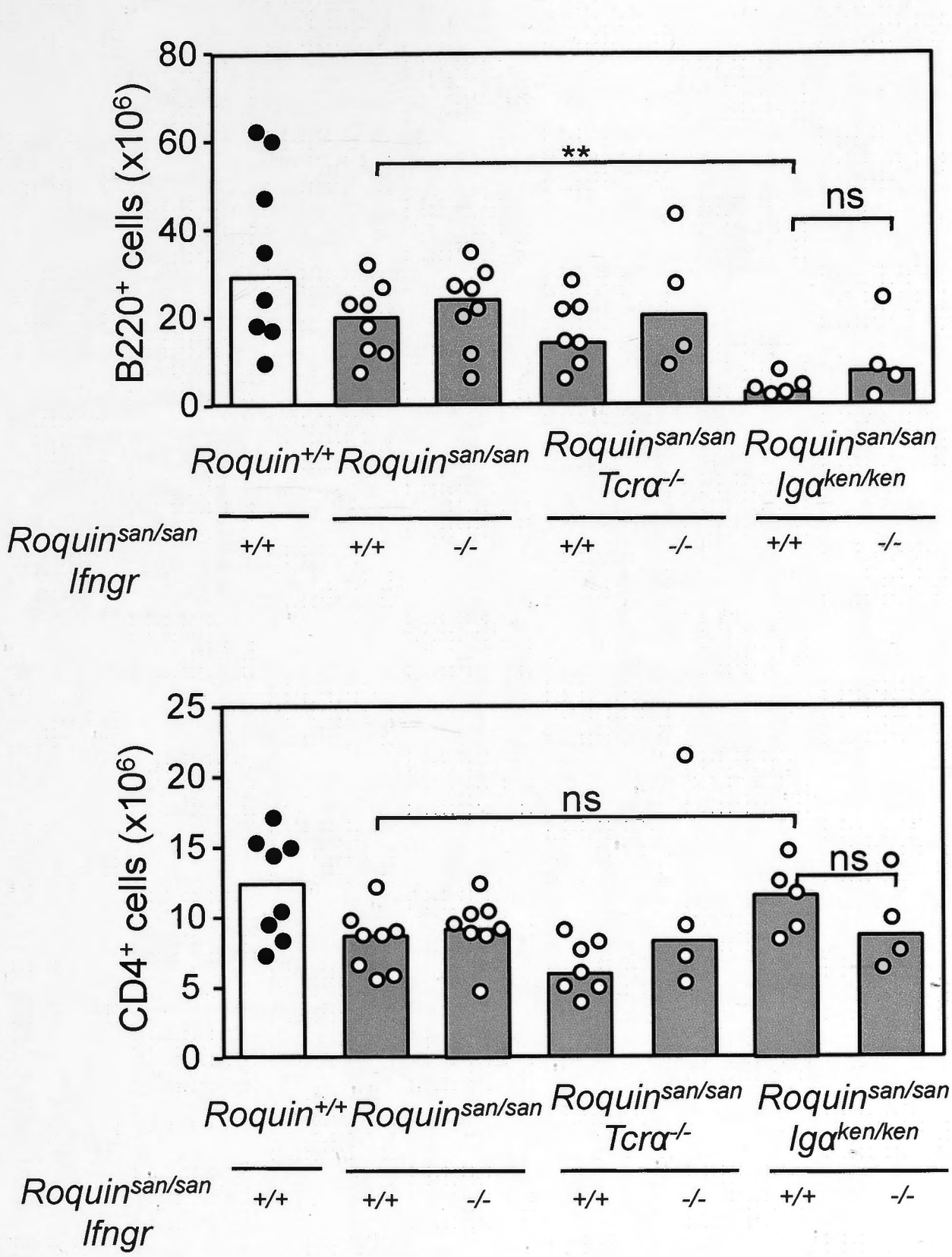


Figure 5.3. Reduced Tfh and GC B cell numbers in chimeras constructed with B cell-deficient bone marrow is associated with decreased total B cell numbers.

Bar graphs showing total numbers of $B220^+$ (**A**) and $CD4^+$ (**B**) cells in the chimeric mice shown in Figures 5.1 and 5.2. These data are representative of two independent experiments with four to eight mice per group in each experiment.

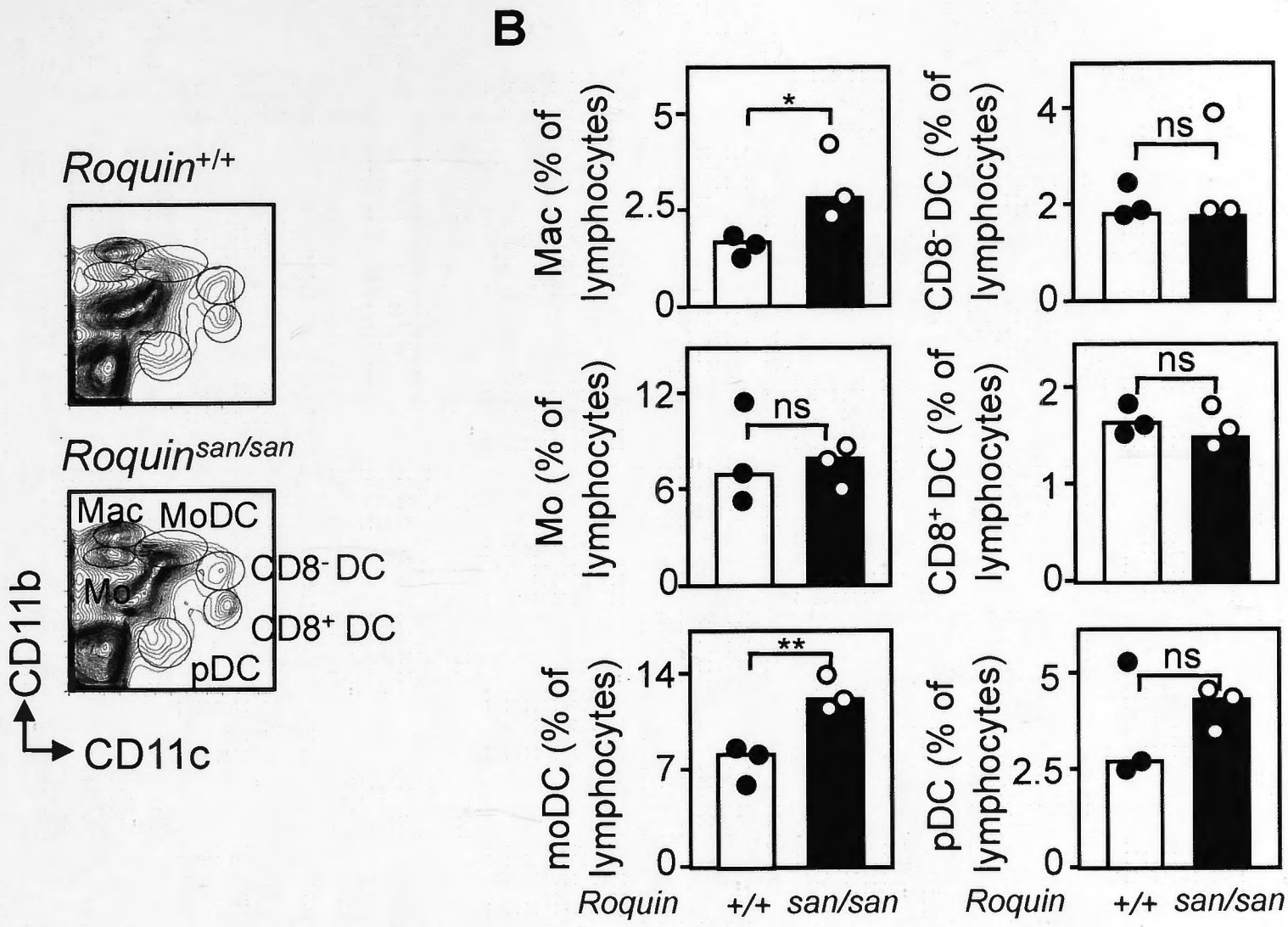


Figure 5.4. Myeloid cell expansion and splenic hypercellularity in *Roquin*$^{san/san}$ mice are completely corrected in *Roquin*$^{san/san}$ *Rag1*$^{-/-}$ mice.

A and B) Representative flow cytometric plots of CD11b versus CD11c (A) and quantification of all myeloid subsets (B) from 8 to 10-wk-old *Roquin*$^{+/+}$ and *Roquin*$^{san/san}$ mice.
C) Bar graphs showing total numbers of splenocytes (left) and CD11b^{+} cells (right) from 8 to 10-wk-old *Roquin*$^{+/+}$, *Roquin*$^{+/+}$ *Rag1*$^{-/-}$, *Roquin*$^{san/san}$ *Rag1*$^{-/-}$ and *Roquin*$^{san/san}$ mice. These data are representative of two independent experiments with three to five mice per group. This experiment was performed by Pheh-Ping Chang.

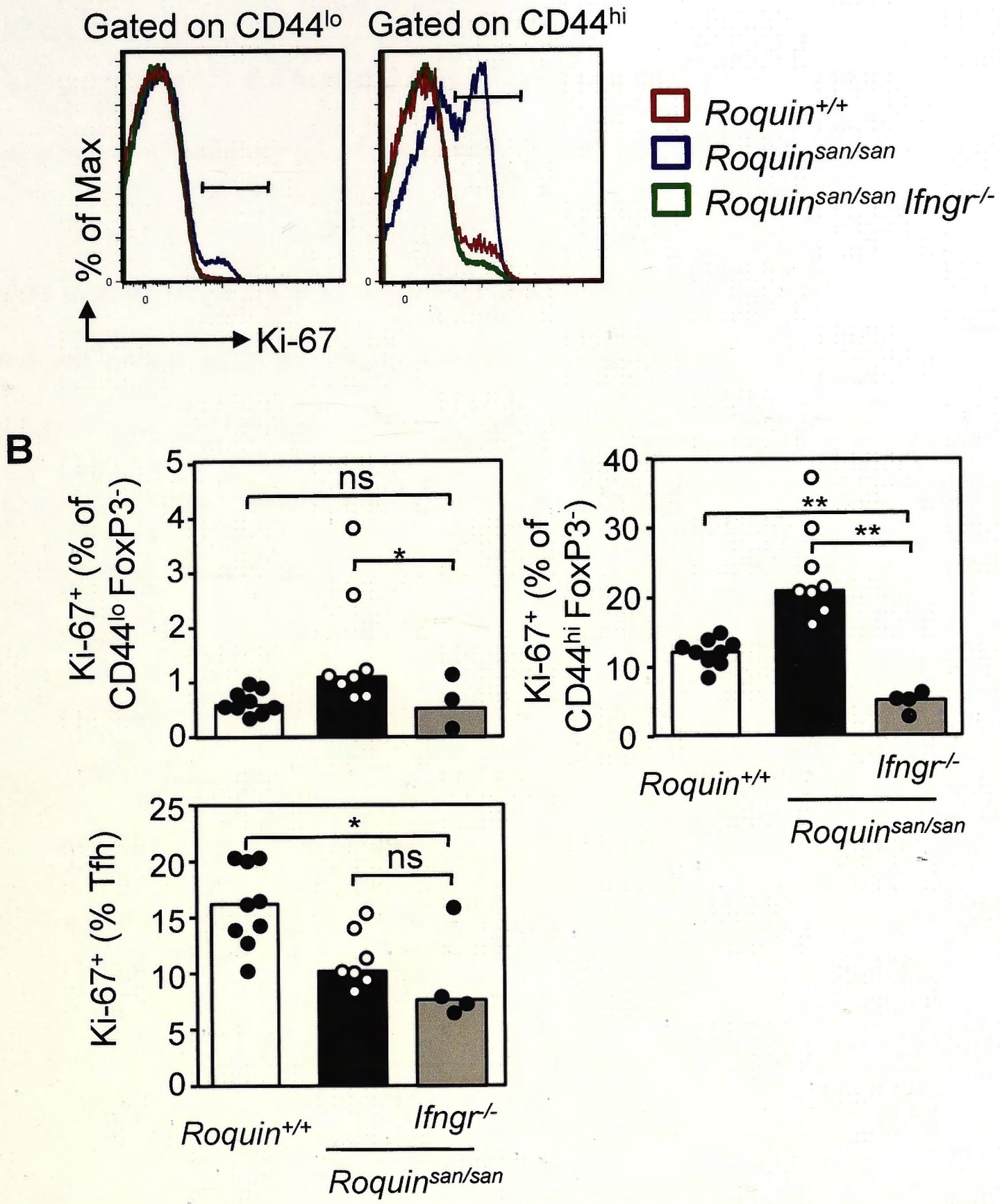


Figure 5.5. IFN-γR signaling enhances proliferation of effector/memory T cells.

A) Histograms showing Ki-67 expression (gated on $CD4^+$ cells) from 8-wk-old *Roquin*$^{+/+}$, *Roquin*$^{san/san}$, *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice.
B) Quantification of percentages of Ki-67$^+$ cells amongst naïve ($CD44^{lo}$ $FoxP3^-$; top left), effector/memory ($CD44^{hi}$ $FoxP3^-$; top right) $CD4^+$ cells, and Tfh cells (bottom) from 8-wk-old *Roquin*$^{+/+}$, *Roquin*$^{san/san}$, *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice. These data are representative of three independent experiments with four to nice mice per group.

cell effectors (**Figure 5.5A and 5.5B**). IFN-γR deficiency did not have any influence in the survival of naïve or effector/memory T cells (**Figure 5.6**).

Several studies have shown that Bcl-6 is required for the formation of Tfh cells[136, 152, 153], so we asked whether excessive IFN-γR signaling altered Bcl-6 expression in *Roquin*$^{san/san}$ mice. *Roquin*$^{san/san}$ mice were found to express significantly higher amounts of Bcl-6 (an isotype control for the Bcl-6 stain is shown in **Figure 5.7B**). Bcl-6 overexpression was observed in both PD-1^{hi} CXCR5^{hi} GC Tfh cells and PD-1^{int} CXCR5^{+} cells that include Tfh cell precursors[178]; **Figure 5.7A**). Strikingly, lack of IFN-γR signaling completely normalized Bcl-6 expression in both Tfh-related subsets in *Roquin*$^{san/san}$ mice (**Figure 5.7A and 5.7C**). Bcl-6 expression was not altered in *Roquin*$^{san/san}$ naïve (CD44^{lo}) or non-Tfh (CXCR5^{lo} PD-1^{lo}) cells (**Figure 5.8A**). Also, IFN-γR signaling had no effect on Bcl-6 expression in GC B cells (**Figure 5.8B**).

To investigate whether excessive IFN-γR signaling in T cells is sufficient to drive autoantibody formation and enhance Bcl-6 expression, we transferred 5 x 10^6 *Roquin*$^{san/san}$ T cells sufficient or deficient in IFN-γR signaling together with 7 x 10^6 wild-type B cells into *Rag1*$^{-/-}$ mice. Eight weeks after transfer, ANAs were higher in recipients of *Roquin*$^{san/san}$ T cells than in recipients of *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ T cells; the latter were comparable to recipients of wild-type T cells (**Figure 5.9C**). The increase in autoantibodies in recipients of *Roquin*$^{san/san}$ T cells was paralleled by a substantial increase in Tfh cells (**Figure 5.9A and 5.9B**), which expressed higher amounts of Bcl-6 (**Figure 5.9B**). Together these results suggest that overproduction of IFN-γ and IFN-γR signaling in T cells are the main drivers of the Tfh cell accumulation and

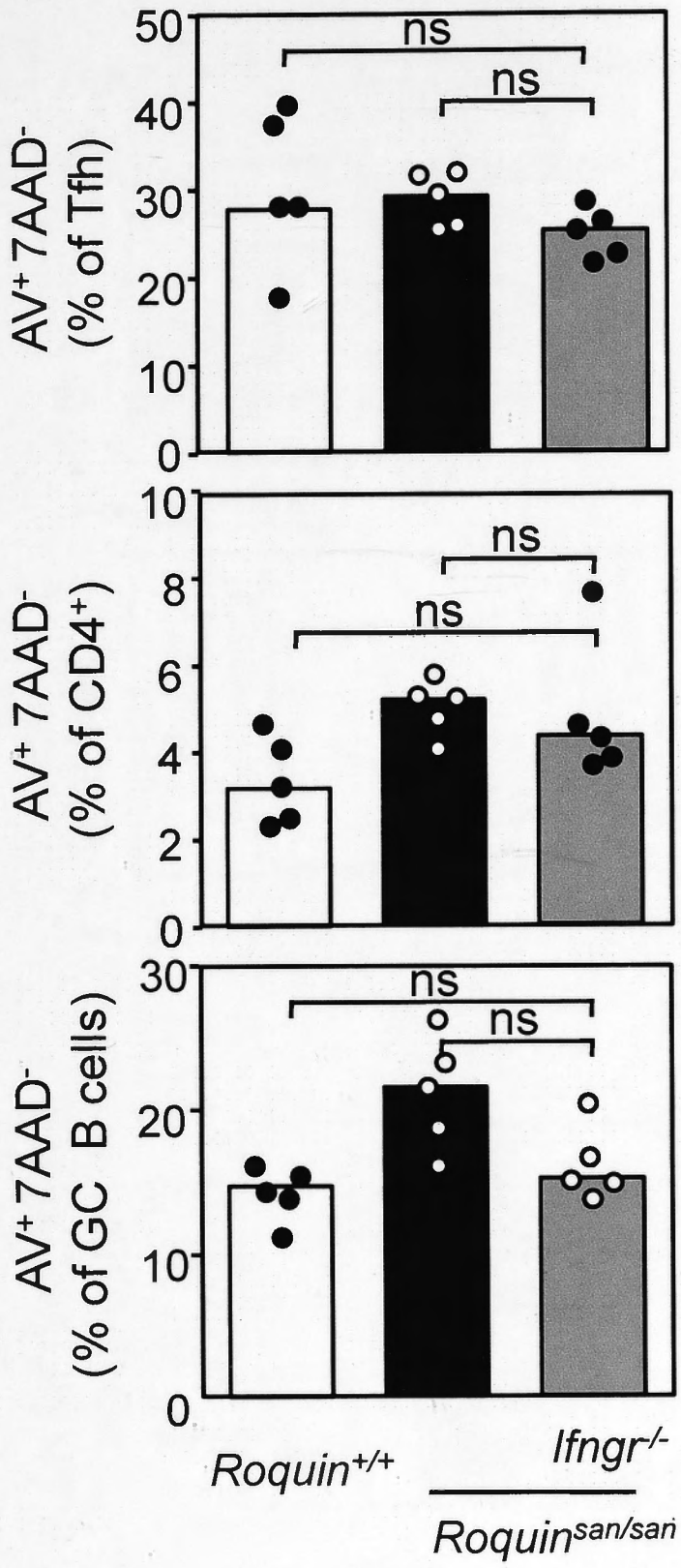


Figure 5.6. IFN-γR deficiency in *Roquin*$^{san/san}$ mice does not affect survival.

Quantification of frequencies of apoptotic cells identified as Annexin-V (AV)$^+$ 7AAD$^-$ cells amongst Tfh (top), total CD4$^+$ (middle) and GC B cells (bottom) from 8-wk-old *Roquin*$^{+/+}$, *Roquin*$^{san/san}$ and *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice. These data are representative of two independent experiments with five mice per group.

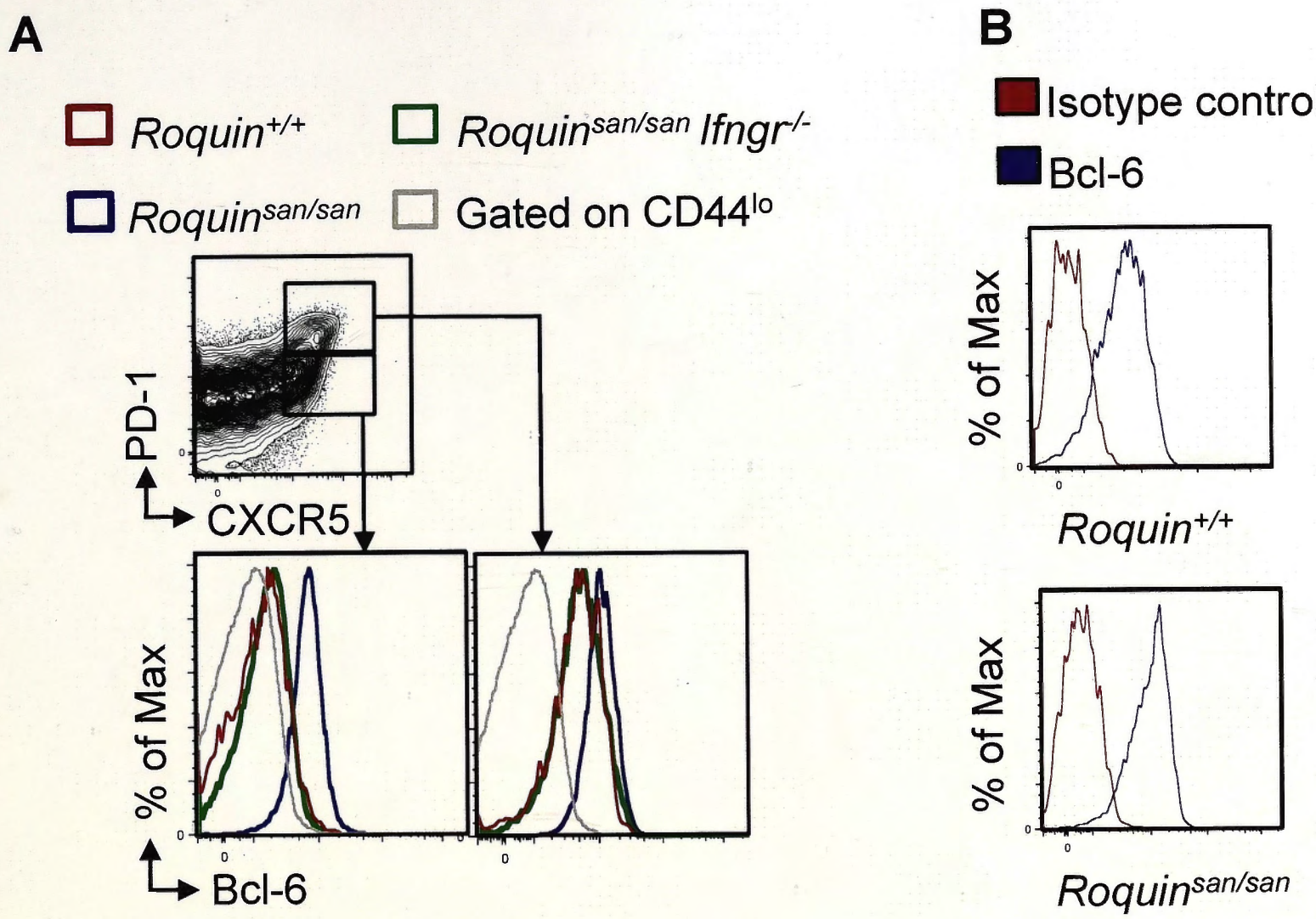


Figure 5.7. IFN-γR signaling enhances Bcl-6 expression of Tfh cells.

A) Histograms of Bcl-6 expression in naïve ($CD44^{lo}$ $Foxp3^{-}$), pre-Tfh identified as $CXCR5^{hi}$ $PD\text{-}1^{int}$ and Tfh cells (gated on CD4+ T cells) from 8-wk-old *Roquin*$^{+/+}$, *Roquin*$^{san/san}$, *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice.
B) Isotype control of Bcl-6 staining in *Roquin*$^{+/+}$ and *Roquin*$^{san/san}$ mice.
C) Bar graphs of Bcl-6 expression in pre-Tfh and Tfh cells from 8-wk-old *Roquin*$^{+/+}$, *Roquin*$^{san/san}$, *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ mice. These data are representative of two independent experiments with four to eight mice per group.

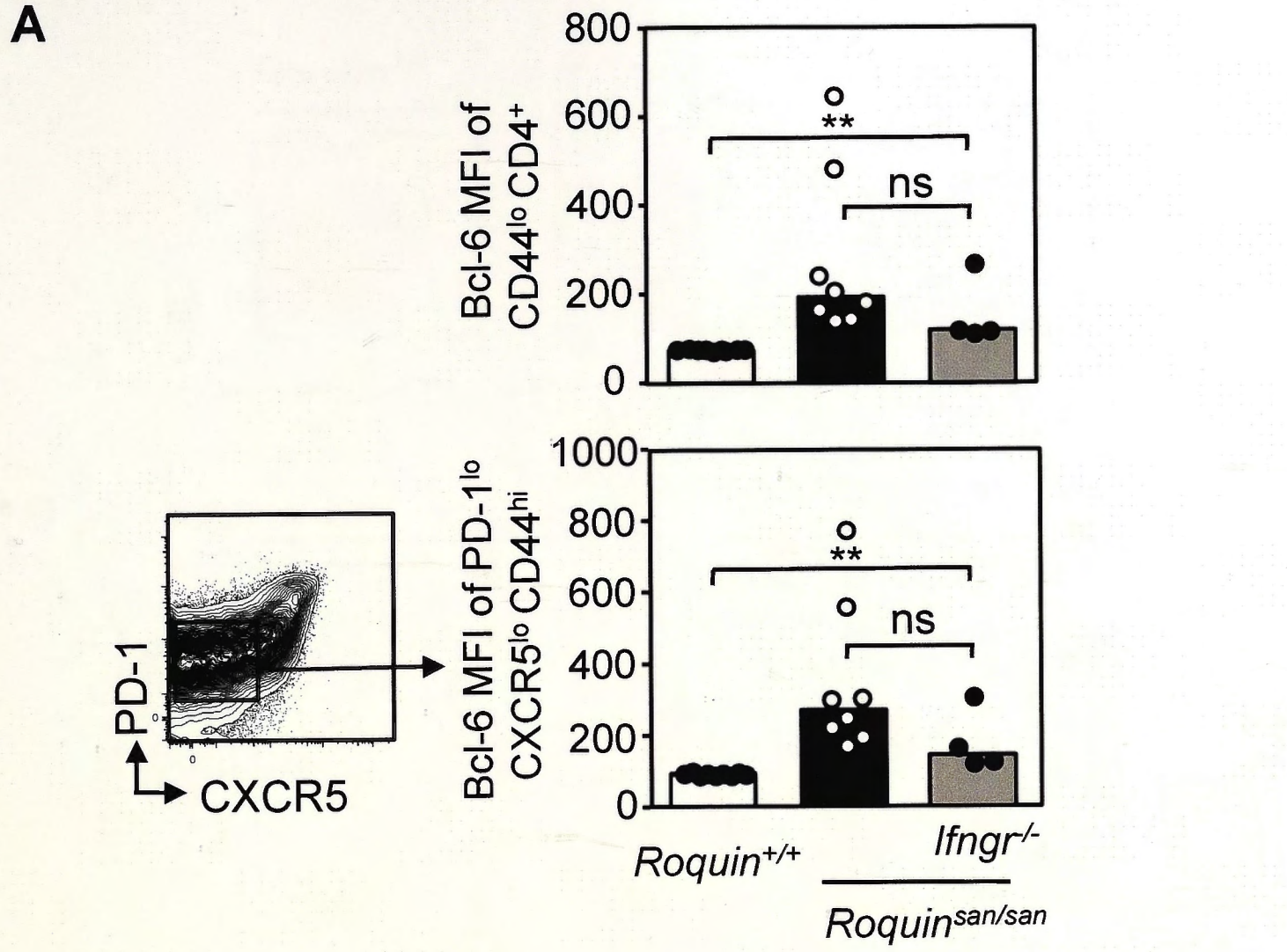


Figure 5.8. IFN-γR deficiency in *Roquin*$^{san/san}$ mice does not affect Bcl-6 expression of non-Tfh effector/memory cells and GC B cells.

A) Gating strategy and bar graphs showing Bcl-6 expression in naïve ($CD44^{lo}$ $CD4^{+}$; top), non-Tfh effector/memory cells ($CXCR5^{lo}$ $PD\text{-}1^{lo}$ $CD44^{hi}$ $CD4^{+}$; bottom)
B) bar graphs showing Bcl-6 expression in GC (top) and non-GC B cells (bottom). These data are representative of two independent experiments with four to eight mice per group.

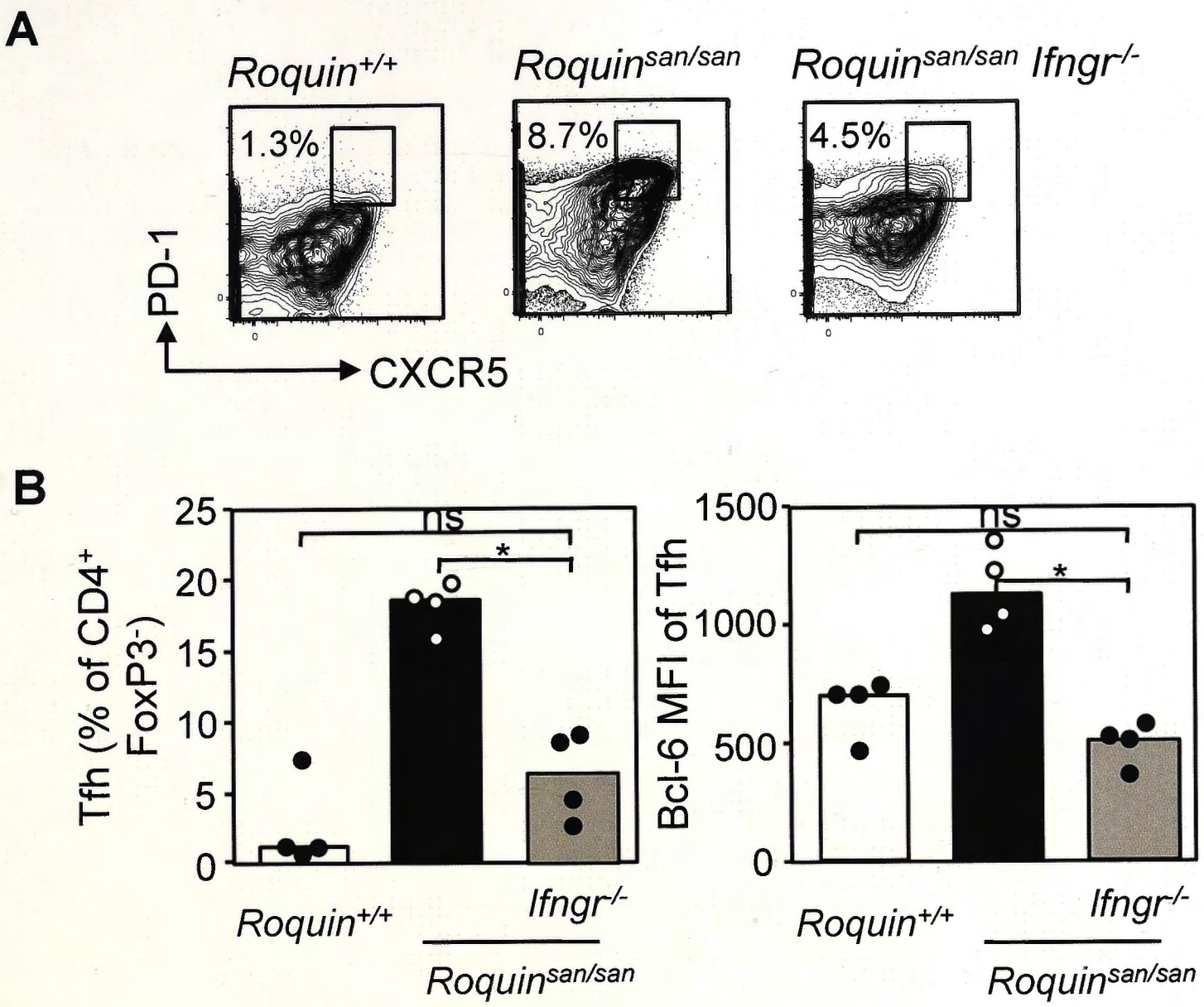


Figure 5.9. IFN-γR signaling in T cells is sufficient to drive autoantibody formation.

A and B) Representative flow cytometric plots (A) and quantification (B) of percentages of Tfh cells (left), Bcl-6 expression (right) in *Rag1*$^{-/-}$ mice transferred with 5 x 10^6 sorted *Roquin*$^{+/+}$, *Roquin*$^{san/san}$ or *Roquin*$^{san/san}$ *Ifngr*$^{-/-}$ CD4$^+$ T cells and 7 x 10^6 sorted *Roquin*$^{+/+}$ B220$^+$ cells 8 weeks after adoptive cell transfer.

C) Concentration of ANA IgG autoantibodies in serum of *Rag1*$^{-/-}$ mice as described in panel A and B. These data are representative of two independent experiments with four mice per group.

autoimmune phenotype, but a contribution of IFN-γR signaling in myeloid cells and B cells cannot be excluded and is likely to accelerate or exacerbate the disease.

We confirmed previous findings demonstrating that 5 ng/ml rIFN-γ can induce *bcl6* mRNA in anti-CD3 activated T cells within 24 h, regardless of CD28 costimulation (**Figure 5.10A**). Bcl-6 upregulation was maintained for 72 h in the presence of CD28 costimulation to levels comparable those induced by rIL-6, but not in the absence of CD28 (**Figure 5.10B**). Collectively, these data suggest that IFN-γ promotes proliferation of activated naïve T cells and enhances Bcl-6 expression in Tfh cells and their precursors.

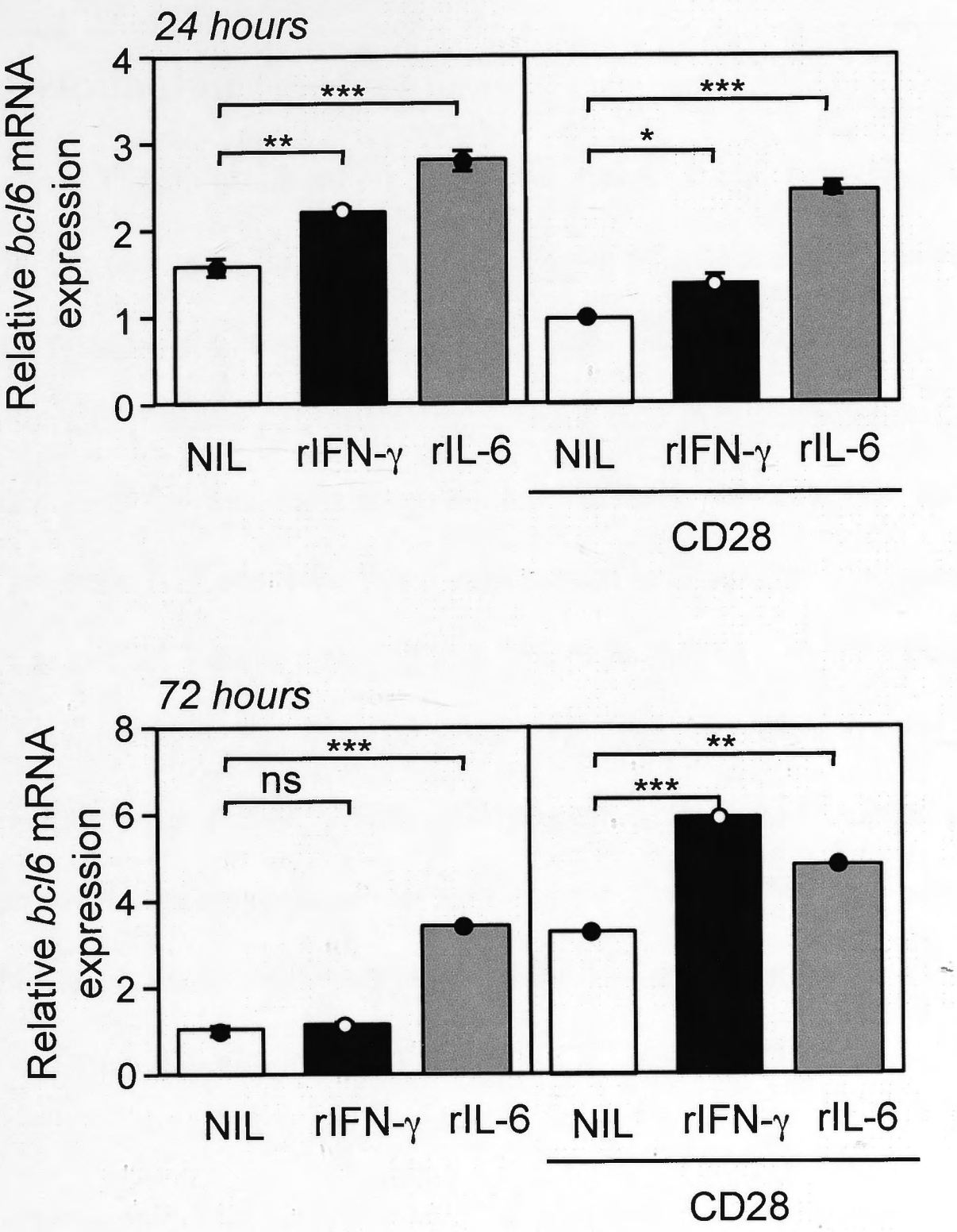


Figure 5.10. Recombinant IFN-γ can induce *bcl6* mRNA in *ex vivo* T cells.

Quantitative RT-PCR analysis of *bcl6* mRNA expression in sorted $CD44^{lo}$ $CD4^{+}$ T cells from 8-wk-old $Roquin^{+/+}$ mice activated for 24 (**A**) and 72 hours (**B**) with anti-CD3, anti-IL-4, anti-TGFβ in the presence or absence of CD28 and rIFN-γ or rIL-6. These data represent mean values ± standard deviation with three biological replicates in two independent experiments.

Chapter Discussion

IFN-γ promotes T cell proliferation from the naive stage and augments Bcl-6 expression in Tfh cells and their precursors. These effects are likely to enhance the formation and possibly the maintenance of Tfh cells: Bcl-6 transgenic mice develop T cell-derived tumours bearing Tfh markers[385]. Bcl-6 acts in a dose-dependent manner in the regulation of downstream targets and Tfh cells hemizygous in Bcl-6 form decreased Tfh cells[136]. Excessive Bcl-6 expression is predicted to maintain Blimp-1 levels low in activated T cells, favoring this fate over that of non-Tfh effectors[164]. We and others have shown that incubation of anti-CD3-activated T cells with IFN-γ induces upregulation of Bcl-6[374]. This upregulation is thought to occur via pSTAT1 binding to an interferon-responsive element in exon 1 of *Bcl6*[374]. The pro-proliferative effect of IFN-γ is probably a consequence of this cytokine's ability to enhance T cell costimulation and/or antigen presentation by pDC[386] and mDCs[387].

Our work also illuminates fundamental Tfh cell biology: IFN-γ deficiency did not impair a Tfh response against immunization with foreign antigen (data not shown); nevertheless, excessive IFN-γ led to Tfh accumulation. Our findings underscore the potential danger of excessive IFN-γ production during viral infection, and therefore, the importance of IFN-γ regulation to offset this risk of developing GC-driven autoimmunity. Our observation that costimulation is required to maintain IFN-γ-driven Bcl-6 expression is consistent with the notion that uncontrolled or chronic stimulation with foreign pathogens may trigger a pathogenic autoimmune response. This observation is also consistent with our previous findings showing CD28-deficiency prevented spontaneous Tfh cells and GC B cell formation, and lupus formation in

sanroque mice. Furthermore, our results provide an explanation for the intriguing observation made by us and others that, unlike sustained high production of IL-4 and IL-21 as Tfh cells terminally differentiate, IFN-γ production is lowered on a per cell basis in GC Tfh cells compared to their precursors (reviewed in[154]). The added layer of IFN-γ repression revealed by our study - control of *Ifng* mRNA stability in T cells - emerges as a potent mechanism to prevent accumulation of Tfh cells in the context of infection, and as key checkpoint to prevent GC-derived autoimmunity.

There is renewed interest in cytokine therapy in human SLE. Ongoing clinical trials with therapies directed at blocking TNF, IFN-α and IFN-γ (reviewed in[359]) hold the promise to further improve disease course and prognosis. One of the main stumbling blocks for successful therapy is the marked clinical heterogeneity of SLE disease, likely to be underpinned by more than one pathway to disease. Cytokine profiles themselves vary significantly from individual to individual and at different disease stages[359]. Recently, subsets of patients with lupus and other autoimmune disorders have been shown to bear biomarkers of an overactive Tfh/GC pathway[158, 232]. Our study highlights the potential of IFN-γ blockade in this subset of SLE patients.

In summary, our studies indicate that the control of IFN-γR signaling in T cells is important at two levels in the generation of Tfh cells: excessive IFN-γ promotes T cell proliferation from the earliest stages – increased Ki-67 staining was already evident in cells with an otherwise naive phenotype-, and augments Bcl-6 expression in Tfh cells and their precursors. Both of these are likely to promote spontaneous Tfh formation in *Roquin*$^{san/san}$ mice. This in turn leads to aberrant GC formation and autoantibody production and subsequently to the development of SLE.

Chapter 6

General Discussion

Chapter 6 –General Discussion

Preamble

In recent years, the GC response has received significant attention and has been extensively studied, but the extrafollicular response has been relatively neglected, at least in part due to the lack of accurate methods to distinguish plasma cells arising from follicular versus extrafollicular pathways. Chapter 3 in this thesis provides some new insights into the nature of T cell help required for extrafollicular B cell differentiation. Our results reveal a requirement of Bcl-6 expression by T cells for extrafollicular antibody responses. By using three different mouse models in which extrafollicular plasma cells can be unequivocally distinguished from those of GC origin. We demonstrated that pre-GC Tfh cells, identified by their expression of Bcl-6 and intermediate levels of PD-1 expression, are necessary for B cell priming to induce extrafollicular antibody responses. Bcl-6-expressing T cells were seen at the T-B border soon after T cell priming, prior to GC formation (**Figure 6.1**). IL-21 was also required for the early extrafollicular response. These pre-Tfh cells precede GC Tfh cells, which are found within the GCs. Pre-Tfh cells were able to induce B cell Ig switching to IgG2a (IgG2c in C57BL/6) in purely extrafollicular responses to *Salmonella*.

In the second part of the thesis, our work evaluated the effect of excessive IFN-γ production in driving SLE pathology. Previous work from our laboratory showed that in *sanroque* mice, the dysregulation and resulting overexpression of some costimulatory molecules such as ICOS contributed to aberrant accumulation of Tfh

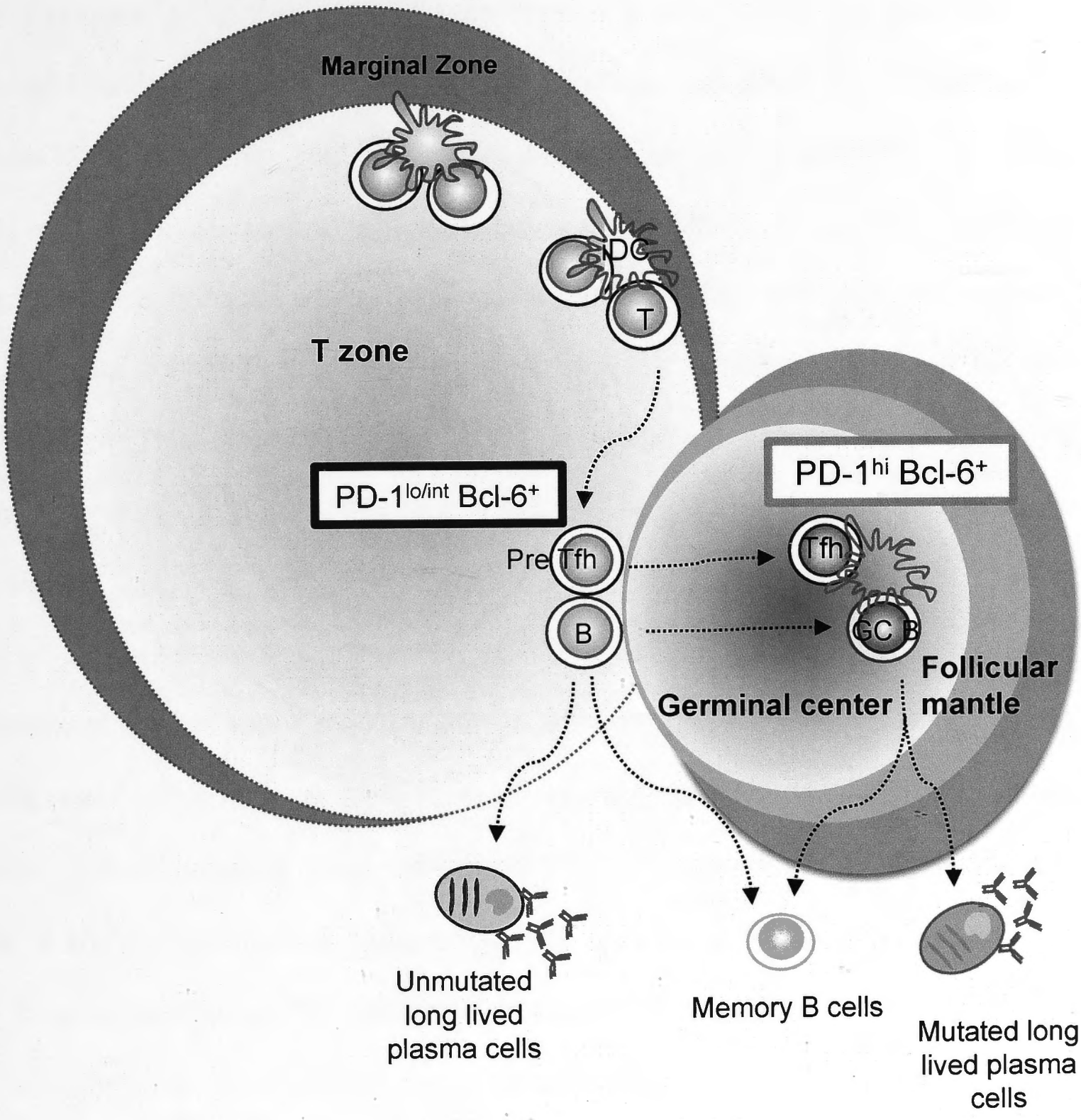


Figure 6.1. Bcl-6 expression by T cells is required for the extrafollicular pathway.

Antigen-specific T cells become activated after priming by the interdigitating DCs (iDCs) in the T-cell area. These cells then migrate to the T-B border to interact with antigen-specific B cells. At the extrafollicular sites, these cells acquire the phenotypic markers Bcl-6^{+} PD-1$^{int/lo}$ and become Pre-Tfh cells. Pre-Tfh cells precede Bcl-6^{+} PD-1^{hi} T (Tfh) cells within GC and are able to prime B cells and drive their differentiation into extrafollicular plasmablasts or memory B cells.

cells. Halving the gene dose of ICOS corrected, to a large extent, the splenomegaly and lymphadenopathy of *sanroque* mice[249]. However, we show in this thesis that complete ICOS deficiency had a different effect, aggravating autoimmunity. It not only failed to rescue several autoimmune manifestations of *sanroque* such as splenomegaly, lymphodenopathy and ANAs, but it also changed the disease phenotype with a shift from production of cytoplasmic antibodies to purely nuclear autoantibodies. This was possibly due to the observed reduction in Treg cells with a reciprocal increase in T cell effectors – ICOS has been previously shown to be important for Treg cell formation and function. We thus hypothesized that other signaling pathways were likely to be involved in the *sanroque* Tfh cell-driven autoimmunity. Given the excessive IFN-γ production by *sanroque* T cells, this pathway was probed as a likely disease-causing candidate. To our surprise, *sanroque* IFN-γR deficient mice developed minimal pathology. Data presented in chapter 4 and 5 of this thesis demonstrate that excessive IFN-γ signaling in T cells leads to accumulation of Tfh cells and maintains spontaneous GC, autoantibody formation and lupus development. We also showed that increased IFN-γR signaling causes Bcl-6 overexpression in Tfh cells, and their precursors, and promoted the proliferation of early effector cells, resulting in an increased precursor pool for Tfh cell differentiation (**Figure 6.2**).

Our work thus reveals yet another mechanism for IFN-γ pathogenicity in lupus. This adds to the described ability of IFN-γ to induce switching to IgG2a (IgG2c in C57BL/6) in mice, known to promote myeloid cell activation and end-organ damage in lupus. Although IFN-γ plays a profound role in disease development, as shown by antibody blockade and genetic manipulation studies, we cannot exclude the

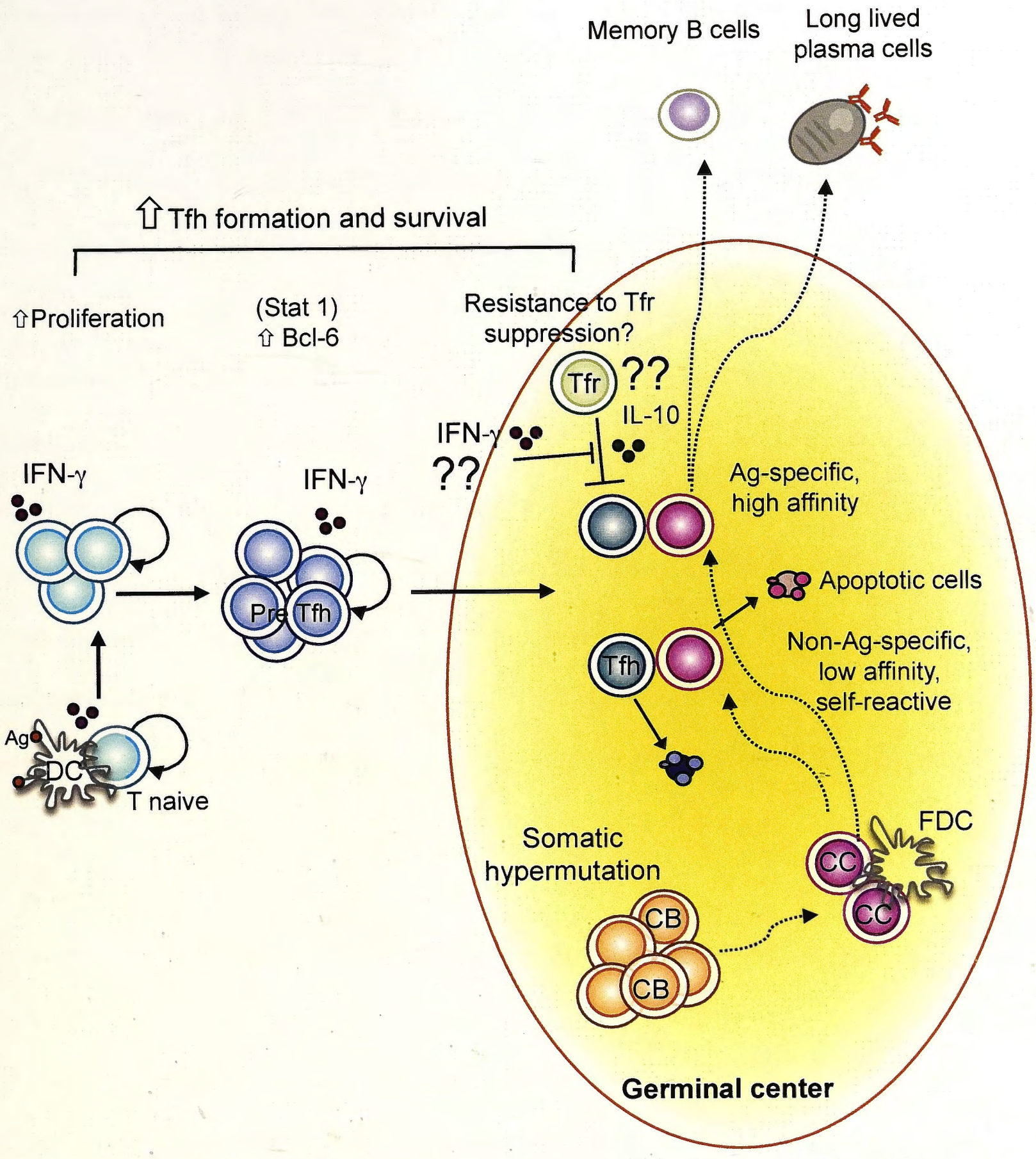


Figure 6.2. Excessive IFN-γR signaling leads to dysregulated Tfh cell formation.

Increased IFN-γR signaling due to impaired IFN-γ mRNA repression by ROQUIN leads to IFN-γ overproduction in *sanroque* T cells. Excessive IFN-γR signaling promotes the proliferation of early effector cells and causes Bcl-6 overexpression in Tfh cells and their precursors, probably via STAT1 signaling and may also cause resistance to Tfr suppression (remains to be tested). All together, these lead to Tfh cell accumulation, spontaneous GC, autoantibody formation and lupus development in *sanroque* mice.

possibility of other genes overexpressed by *sanroque* T cells such as Tnfsf11/RANKL, Maf, Nfatc1, Fyn, Ox40, and others contributing to *sanroque* pathology[227]. This is suggested by our observation that disease is substantially reduced in *sanroque* IFN-γR-deficient mice but not totally eliminated. Intriguingly, protection from disease as seen in *sanroque Ifngr*$^{-/-}$ mice was not observed in *sanroque Tbx21*$^{Du/Du}$ (lacking functional T-bet). However, in these mice, T cells still overexpressed IFN-γ, presumably independent of T-bet transcription, as a consequence of failed posttranscriptional repression conferred by ROQUIN^{san}. It is also possible that overproduction of other cytokines, for example, type I IFN may also lead to similar findings since both IFN-γ and type I IFN share the same signalling pathways via STAT1 and STAT3. Indeed, type I IFN has been shown to induce IL-6 secretion by DCs and thus supported the generation of lymph node-resident Tfh cells[191].

In this thesis, I have proposed a novel tolerance checkpoint to prevent autoimmunity: ROQUIN-mediated repression of *Ifng* mRNA to limit IFN-γ production. Limiting IFN-γ emerges as an important brake in the control of Tfh cell numbers, and ultimately in preventing the development of SLE. Therapies that block IFN-γ action or production may thus be useful for SLE patients with an overactive Tfh pathway.

These observations also raise some interesting questions. First, how important is pre-Tfh-derived IFN-γ in extrafollicular antibody responses? Does IFN-γ secreted by other cell types contribute in *sanroque* autoimmunity? Does IFN-γ/IFN-γR signaling lower the threshold for T cell differentiation into Tfh cells? Why do *sanroque* mice show a more exacerbated phenotype than *Roquin* knockout mice?

Does IFN-γ promote extrafollicular antibody responses in lupus?

An obvious role for IFN-γ in pathogenic extrafollicular antibody responses is the induction of switching to IgG2a (IgG2c in C57BL/6), shown to be more pathogenic than other Ig isotypes[335]. Signals to switch for either follicular or extrafollicualr antibody responses are likely to be delivered by pre-Tfh cells, judging by the early detection of switch transcripts in activated B cells and the comparable rates of switching in both pathways of B cell differentiation[30, 31]. Although IFN-γ-induced switching occurs in mice, there is some evidence that in humans this might not be the case. Human circulating IFN-γ-producing $CD4^+$ T cells that are either $CXCR5^+$ or $CXCR5^-$ are unable to prime B cells to produce Ig, unlike $CXCR5^+$ T cells skewed to Th2 or Th17 cells[158]. Circulating Th17-like and Th2-like $CXCR5^+$ cells were equally capable to induce naïve B cells to produce Ig via IL-21.

Despite the evidence that GC are unique microenvironments where B cells undergo somatic mutation to produce high-affinity autoantibodies, there is evidence that in MRL^{lpr} lupus-prone mice, SHM and autoantibody formation occurs at extrafollicular sites[50, 388, 389]. Within GCs, Tfh cells contribute to the survival and selection of mutated B cells. Less is known about the T cell help requirements for autoreactive B cells generated outside GC and there have been conflicting reports on this issue. T cell help was reported to be dispensable for *in vivo* activation of expansion and differentiation of autoreactive B cells in MRL^{lpr} mice; activation being dependent on endogenous B cell-intrinsic MyD88/TLR7 or 9 signals[114]. However, other evidence pointed to a role for Tfh-like cells located within extrafollicular plasma cell foci for

plasma cell survival and autoantibody production through the provision of IL-21 and CD40L signals in MRL^{lpr} mice[76, 261]. There is also some recent evidence that T cell help can also boost TLR-mediated B cell activation *in vivo*[390]. Unlike the pre-Tfh cells described here, the extrafollicular Tfh-like cells in MRL^{lpr} mice lack CXCR5 expression, which is probably related to their positioning requirements[76].

IFN-γ has been shown in chapter 5 of this thesis to promote Bcl-6 expression and increase Tfh differentiation and accumulation in *sanroque* mice. Tfh-like PSGL^{lo} T cells from MRL^{lpr} mice also secrete IFN-γ[76]; it is possible that IFN-γ promotes formation and/or accumulation of these cells at extrafollicular sites, although this remains to be tested. High levels of circulating IFN-γ, increased expression of IFN-γ and IFN-γR signaling pathway related genes such as IL-12 and STAT1, and increased IFN-γ-producing CD4$^+$ T cells have been documented in blood and/or kidney tissue of MRL^{lpr} mice[309-312, 314-317, 391, 392]. Also, deficiency of IFN-γ or IFN-γR signaling in MRL^{lpr} mice has been shown to reduce autoantibodies and glomerulonephritis[318-320, 338]. Furthermore, depletion of IFN-γ using cDNA encoding IFN-γ/Fc receptor is sufficient to control the progression of SLE disease[325]. Since excessive IFN-γ enhances Bcl-6 expression, this may assist stabilising the Tfh cell fate and promoting persistence of Tfh cells in GC particularly in the context of autoimmunity. It will be important to determine IFN-γ-production by single pre-Tfh cells in autoimmune-prone mice and examine the location of IFN-γ-producing cells in secondary lymphoid tissues. IFN-γ and Bcl-6 dual reporter mice would be required for precise analysis. Also, improved phenotypic markers to identify pre-Tfh cells would be needed.

Does IFN-γ secreted by other cell types contribute to *sanroque* pathology?

Besides T cells, it is possible that B cells and NK cells are other important sources of IFN-γ in *sanroque* mice. Although *sanroque* $CD4^+$ T cell transfer alone into RAG mice lead to increased Tfh cells in recipient mice compared with transfers of wild-type $CD4^+$ T cells, formal investigation of potentially additive effects of B or NK cell-derived IFN-γ was not undertaken. The role of B cells in modulating immunity to pathogens by means other than via antibody production has been controversial. Some studies have shown that B cells are dispensable for the generation and maintenance of antigen-specific T cells responses[393, 394]. Nevertheless, there is accumulating evidence that B cells play important roles in T cell maturation. In this thesis, I have summarised the evidence that B cells promote differentiation of Tfh cells via provision of SLAM-family members[6, 45, 172] and ICOS ligands[185]. Also, IFN-γ produced by B cells has been shown to promote T cell priming[395]: follicular B cells produce IFN-γ when stimulated with TLR ligands or during infection with *Salmonella*[396]. In mixed bone marrow chimeras in which only B cells lack MyD88 signaling, secretion of IFN-γ by Th1 cells was reduced and GC formation was impaired, suggesting that production of IFN-γ by B cells triggered by MyD88 signals is required for generating an optimal IFN-γ-secreting effector T cell and GC responses[395]. To date, it is unknown to what extent IFN-γ produced by B cells is required for Tfh cell formation including maximal expression and/or stabilization of Bcl-6.

In the context of autoimmunity, important roles for B cells in autoantigen presentation to T cells have been demonstrated in NOD mice[397, 398]. Recent clinical data has shown that B cell depletion with rituximab (anti-CD20 mAB) is an effective therapy for several T cell-mediated autoimmune diseases including multiple sclerosis, type 1 diabetes, rheumatoid arthritis and others[399]. The efficacy of B cells depletion therapy does not always correlate with changes in the levels of autoantibodies; this suggests suggesting additional roles for B cells in the regulation of T cell-mediated immune responses independently of autoantibody production. Thus, it is plausible that IFN-γ produced by B cells may also contribute to the *sanroque* phenotype.

The role of DCs in SLE remains uncertain. Some studies have shown a critical role of myeloid cells for development of autoimmunity in lupus mice models including *Lyn*$^{-/-}$, *Fas*$^{-/-}$ and c-mer$^{-/-}$ (mice lacking the memberane tyrosine kinase c-mer) and B6.Sle3 mice, mainly attributed to their ability to present antigen to autoreactive T cells, secrete pro-inflammatory cytokines, promote of B cell autoantibody formation and phagocytose apoptotic cells[360, 400-402]. A recent study by Teichmann et al. showed that in MRL^{lpr} mice, DCs are dispensable for the initial activation of B and T cells, but are required to promote expansion and differentiation of T cells as the disease progresses; the net effect of DC depletion was amelioration of disease[403]. Myeloid cell dysregulation is likely to play a role in *sanroque* pathology, if only because of the extensive expansion of DCs, monocytes and macrophages in *sanroque*. There is also an intriguing link described between IFN-γ, myeloid cell activation and lupus. IFN-γ can stimulate myeloid cells to release BAFF[334], a well-known trigger of B cell activation and lupus disease[120]. In turn, BAFF can induce the differentiation of T helper cells to release more IFN-γ. IFN-γ, in a positive feedback

loop, is likely to sustain myeloid cells proliferation/activation and BAFF production[334, 360]. In lupus-like *Lyn*$^{-/-}$ mice, IFN-γ and BAFF further amplify activation and survival of self-reactive B cells clones leading to autoimmunity and organ damage[360, 404, 405]. However, *Lyn*$^{-/-}$ mice do not show increased Tfh cells (Tarlinton, unpublished data), suggesting that IFN-γ signaling in myeloid cells may exacerbate but cannot initiate an overactive Tfh pathway.

IFN-γ/IFN-γR signaling lower the threshold for T cell differentiation to Tfh cells

Sanroque pre-Tfh and Tfh cells cells express Bcl-6 above the amounts seen in wild-type T cells, and this Bcl-6 overexpression is dependent on IFN-γ signaling. Thus we concluded that excessive IFN-γ signaling could enhance Tfh differentiation. It is unclear if physiological levels of IFN-γ seen in the context of immunization or infection play a role in promoting Tfh responses. Our *in vitro* cultures with IFN-γ mAb confirms and extends previous work showing rIFN-γ could induce Bcl-6 expression *in vitro* in Jurkat cells and in human *ex vivo* T cell cultures[374], but whether this is of relevance *in vivo* remains unknown. Adoptive transfer of *sanroque* T cells sufficient or deficient in IFN-γR into *Rag1*$^{-/-}$ mice demonstrated a requirement for IFN-γ to sustain Bcl-6 expression *in vivo*. Nevertheless, we only observed minimal reduction in Tfh formation when we immunized IFN-γ$^{-/-}$ mice with a foreign antigen - SRBC. During chronic LCMV infection, it has been shown that Th1 cells could convert into Tfh cells suggesting that chronic antigen exposure led to prolonged TCR stimulation and sustained Bcl-6 expression[406]. In an analogous manner we could speculate that

in the presence of persistent self-antigens (i.e. continued antigenic stimulation), excess IFN-γ produced by *sanroque* T cells enhances Bcl-6 expression, leading to more naïve T cells being primed to differentiate into Tfh cells. This lowered threshold for Tfh cell differentiation would in turn promote the anti-self B cell response. It is possible that similar effects might be observed in response to viruses that elicit potent IFN-γ responses, but not in response to antigens that may not reach the threshold levels of IFN-γ production to increase Bcl-6 levels. It would be interesting to compare the total amount of IFN-γ produced by recently activated T cells in response to a range of immunogens and adjuvants.

We also observed that IFN-γ promotes proliferation of all effector cells. *sanroque Ifngr*$^{-/-}$ *mice* showed decreased proliferation of naïve and effector cells including Tfh precursors. Nevertheless, and for reasons we do not fully understand, deficiency in IFN-γ did not reduce T cell survival as shown by comparable percentages of pro-apoptotic cells amongst the different $CD4^+$ T cell subsets.

Why do *sanroque* mice but not *Roquin*$^{-/-}$ mice develop autoimmunity?

Unlike homozygosity for *Roquin*^{san}, genetic ablation of the entire ROQUIN protein (comprised of RING and ROQ domains; a CCCH-type zinc finger and a proline-rich domain) does not cause an autoimmune phenotype[254]. Why does a single substitution within ROQUIN's RNA-binding ROQ domain – M199R - cause such severe dysregulation of Tfh cells and autoimmunity that cannot be recapitulated by

the loss of ROQUIN protein? Previous work including that from our laboratory showed that ROQUIN localized to cytoplasmic stress granules and P-bodies to bind to target mRNAs such as to promote mRNA degradation[248, 249, 253]. ROQUIN^{M199R} can still localize to stress granules, but it fails to repress its target mRNA leading to overexpression of some mRNAs including ICOS and IFN-γ, which contribute to the lupus-causing accumulation of Tfh cells. We hypothesize that "san" allele may represent a "niche-filling" loss- of-function allele that selectively inactivates the normal mRNA-regulating function of ROQUIN but preserves its scaffold function, thus preventing MNAB (encoded by *Rc3h2*) protein, the only ROQUIN paralogue in mammals, from compensating and repressing shared target mRNAs[407]. We have recently generated mice lacking the RING domain of ROQUIN. These *Roquin*^{RINGLESS} mice express a truncated ROQUIN protein that fails to localize to stress granules and are phenotypically similar to *Roquin*$^{-/-}$ mice. Unlike the M199R ROQ mutation, *Roquin*^{RINGLESS} exerts a minimal effect on *Icos* mRNA stability and does not increase Tfh cell numbers[407]. We have also generated *Mnab*^{RINGLESS} mice; MNAB RING deficiency alone does not cause an observable T cell phenotype[407]. By contrast, mice doubly defective in ROQUIN and MNAB in T cells (MNAB and ROQUIN RINGLESS) failed to repress ICOS mRNA and had elevated numbers of Tfh cells and GCs[407]. ROQUIN and MNAB appear to be overlapping functions and can compensate for each other. The RING –deleted form of ROQUIN in *Roquin*^{RINGLESS} mice fails to localize to stress granules and allows compensation by MNAB. By contrast, *Roquin*^{san} still localizes to stress granules, and despite being unable to degrade mRNA, we speculate that it retains important protein scaffold functions that prevent MNAB compensation.

Another piece in the puzzle is our recent observation of ROQUIN's ability to interact with and inhibit AMP-activated protein kinase (AMPK), resulting in mTOR activation[408]. Unrestrained AMPK activation in *Roquin*^{RINGLESS} mice significantly dampens Tfh formation. Presumably, given that *sanroque* mice express an intact RING domain, it is possible that ROQUIN M199R preserves the AMPK inhibitory function, further amplifying the aberrant Tfh formation.

Roquin^{san} failed repression of *Ifng* mRNA leads to excessive IFN-γ production. Although I have not tested the direct interaction of *Ifng* mRNA with ROQUIN due to time constraints, I would expect that *Ifng* mRNA is repressed by ROQUIN via a mechanisms analogous to that described for *Icos* – through direct binding and induction of mRNA decay. Increases in both *Ifng* and *Icos* mRNA transcripts are likely to contribute to the excessive Tfh phenotype. It will be important nevertheless to test experimentally that ROQUIN binds to *Ifng* mRNA and represses it directly.

Plasticity of Tfh and Th1 cells

Amongst the important questions in this field is whether Tfh cells represent a T cell subset derived directly from a naïve T cell, or whether they develop from other effector subsets such as Th1, Th2 and Th17 that have received appropriate additional signals from B cells. Two recent studies have shown that Tfh differentiation starts very early – at the first or second cell division of $CD4^+$ T cells (2 days after LCMV infection), immediately after DC priming and facilitated by DC-derived ICOS signals[164, 165]. These results suggest that Tfh cells are derived directly

from naïve T cells and that Tfh differentiation starts concurrently with other cell lineages[164, 165]. Nevertheless, it is still possible that some Tfh cells emerge from other T cell effectors; this would be consistent with the growing appreciation that Tfh cells are heterogeous and can secrete large amounts of the signature cytokines of Th1 and Th2 effectors, such as IFN-γ and IL-4, respectively, in the context of infections[291, 409, 410]. Some support for the existence of plasticity between Tfh and other T cell subsets has also been provided by recent ChIP-Seq analysis, showing positive epigenetic markings on *Tbx21*, *Gata3* and *Rorc* in *ex vivo* Tfh cells and *vice versa* (on *bcl6* in non-Tfh cells)[411]. This suggests that Bcl-6 was induced in Th1, Th2 and Th17 cells and that these cells could be reprogrammed or converted into Tfh cells. In human peripheral blood, $CXCR5^+$ Tfh cells also express chemokine receptors that are characteristic of different T cell subsets: CXCR3, CCR4, and CCR6, characteristic of Th1, Th2 and Th17 cells, respectively[158]. An example of T cell conversion has also been shown in the gut: Treg cells can convert to $Bcl6^+$ Tfh cells under inflammatory conditions in Peyer's patch[412], downregulating FoxP3. Th1 cells have also been shown to convert into Tfh cells in the context of chronic LCMV infection[406].

Further examples of coexpression of different effector cytokines and conversion of one T cell subset to another have also been demonstrated in autoimmune models. For example, purified Th17 cells could switch to Th1 cells (expressing T-bet and secreting IFN-γ) when transferred into NOD/SCID mice upon induction of autoimmune diabetes[413]. IL-17 and IL-4-producing Th2 cells that coexpress GATA3 and RORγt have been identified in the influx of inflammatory leukocytes and implicated in the exacerbation of chronic allergic asthma[414]. Therefore, the

relationship between Tfh cells and other T cell subsets appears complex and modulated by multiple factors including tissues analyzed, tolerance status and microenvironment in general.

Clinical use of monoclonal antibodies against IFN-γ

Systemic autoimmune disease such as lupus and rheumatoid arthritis are progressive, leading to severe organ dysfunctions, and commonly associated with increased mortality. Effective therapies for these diseases are limited, one of the major obstacles being disease heterogeneity. There are different mechanisms for SLE disease initiation with various effector T cell subsets being implicated in different pathogenic routes. Thus, it is likely that different subsets of patients may be responsive to different therapeutic strategies. For example, IL-6, IL-17, IL-23 and IL-21 derived from Th17-associated cells have been implicated in SLE pathogenesis[258, 415]. Here, we propose a mechanism by which excessive IFN-γ triggers SLE by promoting pathogenic accumulation of Tfh cells and driving spontaneous GC formation that leads to autoantibody formation. It is noteworthy that our blockade experiments using anti-IFN-γ (Chapter 4) had therapeutic benefit in *sanroque* mice. Anti-IFN-γ mAb are currently in Phase I clinical trials for SLE. Likewise, ICOSL, CD40-CD40L, and IL21R-Fc blocking treatments in mice and human patients showed a certain therapeutic beneficial with some improvement in disease activity; the main effects were a decreased in pathogenic autoantibody formation and immune-complex mediated glomerulonephritis. All these treatments have direct or indirect blocking effects on Tfh cell numbers or function (i.e. the delivery of Tfh-derived signals to B cells), strongly suggesting a causative role of Tfh in SLE

development[259, 416-419]. Given the prominent role for IFN-γ in fighting infection, great caution is also needed because complete IFN-γ blockade may cause immunodeficiency and inability to clear viruses and intracellular bacteria. For example, patients with genetic deficiencies in IFN-γR develop disseminated tuberculosis or atypical mycobacterial infections. Deficiencies in STAT1 also lead to disseminated mycobacteriosis early in life along with several mycobacterial and viral infections such as Bacillus Calmette-Guerin (BCG) infection, herpetic skin infection and interstitial pneumonia by cytomegalovirus[420-422].

This study provides new insights on how blocking IFN-γ may act to delay progression in lupus patients. An understanding of the underlying molecular mechanisms of dysregulation of cytokine signaling in lupus disease will allow us to identify novel biomarkers to stratify SLE patients according to the disease-causing signaling pathways and predict responses to different treatments. Circulating Tfh-like cells and serum IFN-γ may turn out to be useful biomarkers for identification of SLE patients with an overactive Tfh/GC pathway, which appears to correlate with disease severity[232]. The insights gained in this thesis into novel mechanisms by which IFN-γ contributes to SLE development may help to refine the readouts and patient selection in SLE clinical trials using IFN-γ blockade.

Chapter 7

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Appendix

Supplementary table for Chapter 1

Appendix 1.1. Criteria for the classification of SLE.

The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have SLE if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

	Criteria	Definition
1	Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds.
2	Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions.
3	Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation.
4	Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician.
5	Arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion.
6	Serositis	a) Pleuritis--convincing history of pleuritic pain or rubbing heard

		by a physician or evidence of pleural effusion *OR* b) Pericarditis--documented by ECG or rub or evidence of pericardial effusion.
7	Renal disorder	a) Persistent proteinuria greater than 0.5 grams per day or grater than 3+ if quantitation not performed *OR* b) Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed.
8	Neurologic disorder	a) Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance *OR* b) Psychosis--in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance.
9	Hematologic disorder	a) Hemolytic anemia--with reticulocytosis *OR* b) Leukopenia--less than 4,000/mm<>3<> total on 2 or more occasions *OR*

		c) Lyphopenia--less than 1,500/mm<>3<> on 2 or more occasions *OR* d) Thrombocytopenia--less than 100,000/mm<>3<> in the absence of offending drugs.
10	Immunologic disorder	a) Positive LE cell preparation *OR* b) Anti-DNA: antibody to native DNA in abnormal titer *OR* c) Anti-Sm: presence of antibody to Sm nuclear antigen *OR* d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test.
11	Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome.

Supplementary table for Chapter 2

Appendix 2.1. Scoring of nephritis severity.

Kidney nephritis was scored according to the indicated scale of disease severity.

	Glomeruli		Tubulointerstitium	
Score	**Cells**	**Matrix**	**Cells**	**Matrix**
0	NAD	NAD	NAD	NAD
1	Hypercellularity only	Mesangial matrix increase	Very mild	Very mild
2	Proliferative or fibrinoid GN without crescents	Mild scarring	Mild	Mild
3	GN with fibrinoid or crescents in < 50% of glomeruli	Moderate scarring	Moderate	Moderate
4	GN with fibrinoid or crescents in > 50% of glomeruli	Fibrous obliteration	Severe	Severe

NAD, no abnormality detected