Human T cell cytokines in the regulation of B cell responses

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Statement of Originality

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

The results and analysis presented in this thesis are my own work, performed under the supervision of Professor Carola Garcia de Vinuesa, Dr Vicki Athanasopoulos and Professor David Fulcher unless otherwise stated in the text. This thesis is under 100, 000 words.

Katharine Bassett

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For Jordan, who put up with a lot.

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Abstract

Cytokines are essential communication molecules in the immune system, playing a role in the recruitment, function, survival, proliferation and suppression of leukocytes. Within the dynamic environment of the germinal centre, cytokines produced by T cells and other cells play essential roles in the growth, survival and selection of high-affinity B cells. Defects in cytokine signalling has been implicated in several autoimmune disorders and immune deficiencies. This study investigates the roles of T cell cytokines in follicular biology and autoimmunity through two distinct projects which were undertaken in parallel.

The first project is a high-risk project that concerns two families (A and B) affected by systemic lupus erythematosus (SLE), a complex and debilitating systemic autoimmune disease. Whole exome or genome sequencing of these two families identified different heterozygous single nucleotide variants (SNV) in the same domain of an RNA binding protein called zinc finger RNA binding protein 2 (ZFR2), whose role in the immune system has not been identified, although paralogs of this protein have been shown to regulate the gene expression of multiple cytokines, specifically interleukin-2 (IL-2). These patients have a unique cellular and molecular phenotype with an increase in plasma IL-2, an increase in regulatory T cells and an expanded $CD4^+$ $CD57^+$ cytotoxic T cell population. In this thesis we show that ZFR2 plays an important role in the immune system, specifically in promoting the activity of activator protein 1 (AP-1) and nuclear factor of activated T cells (NFAT). It also appears to play a role in mRNA splicing and IL-2 production. We also show that the SNV in Family A is a gain-of-function, leading to an increase in AP-1 and NFAT activity and IL-2 production. Furthermore,

we identified milder but similar cellular phenotypes in mice carrying the orthologous ZFR2 variant. From these results, we conclude that this variant is likely contributing to disease pathogenesis and systemic autoimmunity in these families.

The second project, which was initiated as a backup project, investigates the role of interleukin-32 (IL-32), a recently discovered cytokine that we found to be highly expressed in a subset of human germinal centre T cells that are similar to mouse T follicular regulatory (T_{FR}) cells. In the search for the function of IL-32 in the germinal centre reaction, we identified it induced rapid upregulation of PD-L1 on most B cell subsets. PD-L1 is one of the two ligands for PD-1, which is highly expressed on T follicular helper (T_{FH}) and very highly expressed on T_{FR} cells in the germinal centre. The interaction between PD-1 and PD-L1 has been shown to play several important roles in the germinal centre reaction; predominantly leading to the attenuation of T cell activation and the suppression of T_{FH} cell differentiation and help for immunoglobulin class switching. This study proposes a novel pathway by which IL-32, produced by human T_{FR}-like cells, increases B cell viability and induces B cells to become a previously described subset of regulatory B cells that acts independently of IL-10 and operates through PD-1/PD-L1 signalling and an elevated expression of BAFFR which acts as a BAFF 'sink' to sequester BAFF. Overall, this study provides a novel insight into the suppressive functions human T_{FR} cells and identifies a new regulatory pathway in human germinal centre reactions.

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Chapter One: Introduction

1.1 Preamble

All living organisms are continually exposed to harmful substances from the external environment. These harmful substances have acted as evolutionary pressures and have led to the emergence of various protective mechanisms. Most organisms possess physical barriers and chemical secretion that prevent pathogens from entering the body, however vertebrates also have a sophisticated mechanism of protection known as the immune system. If a pathogen breaches the physical barriers, it will face a diverse set of specialised immune cells and signalling molecules that have the ability to recognise and eliminate the pathogen. The immune system however is like a double-edged sword; it enables the body to recognise and destroy foreign pathogens, however the same systems and mechanisms can also cause destruction to the cells and tissues in our own body, leading to what are known as autoimmune disorders. Understanding the regulation of these immune cells, the pathogenic mechanisms that lead to the dysregulation of the immune response and the development of autoimmune disorders is crucial for the development of more effective vaccines and disease therapies.

1.2 The innate and adaptive immune system

1.2.1 The immune system

The mammalian immune system is a complex network of cells, receptors and molecular mediators that function together to protect the body from foreign pathogens such as bacteria, viruses, allergens or even abnormal cells that may result in cancer. Immune cells recognise foreign compounds called antigens which can induce antibody and cellular responses against such compounds or pathogens, and therefore they require the ability to distinguish self from non-self, a crucial process to vertebrate survival known as immunological tolerance. Development of tolerance towards self-derived antigens ensures that immune responses are directed only towards foreign antigens, preventing damage caused by the body mounting an immune response against self-antigen (Schwartz, 2012). The immune system in vertebrates can be considered as consisting of two branches; the innate and adaptive immune systems, a division that was classically based on whether the receptor recognising the foreign antigen is encoded in the germline deoxyribonucleic acid (DNA) (innate) or if gene rearrangement is required (adaptive) (Murphy and Weaver, 2016). Whilst the two systems differ in antigen specificity as well as activation kinetics, they possess many complementary mechanisms and cells that have functional characteristics of both systems. It should also be stated that the two systems actively interact and depend on each other, with activation of the adaptive immune system being controlled by components of the innate immune system (Janeway and Medzhitov, 2002, Iwasaki and Medzhitov, 2015, Jain and Pasare, 2017).

1.2.2 The innate immune system

The innate immune system is the body's first line of defence against pathogens, acting quickly to prevent the entry, establishment and spread of infection in a host. It depends upon germline-encoded receptors that recognise common features of many pathogens. These receptors can either recognise a foreign pathogen by identifying characteristics of the pathogen that are not in the host, or by the recognition of "missing-self", where the pathogen does not express markers expressed by the host. Whilst the innate immune response begins almost immediately after infection, it is unable to produce long-lasting immunity or memory responses for the host (Murphy and Weaver, 2016).

The cells of the innate immune system include natural killer (NK) cells, monocytes and macrophages, dendritic cells (DCs), and granulocytes (neutrophils, eosinophils, basophils and mast cells). These cells express pattern recognition receptors (PRRs) which identify pathogens by recognising pathogen-associated molecular patterns (PAMPs) (Takeuchi and Akira, 2010). Types of PRRs include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). PRRs can also recognise damage-associated molecular patterns (DAMPs) which are released from host cells following necrosis, damage or infection (Takeuchi and Akira, 2010). Activation of the PRRs leads to the release of chemical mediators such as chemokines and cytokines, which initiate inflammation and recruit more innate immune cells to the site of infection. The innate immune system is also responsible for activating the complement cascade, which is a system of plasma proteins that activate proteolytic reactions and coat microbial surfaces with fragments that are recognised by phagocytic receptors on macrophages (Noris and Remuzzi, 2013, Murphy and Weaver, 2016).

The complement system is an intricate network of serum proteins that plays a crucial role in innate immunity through the opsonisation/killing of pathogens, clearance of cellular debris and apoptotic cells, and recognition of foreign pathogens. Whilst the complement system typically acts as part of the innate immune system, it forms an important bridge between the innate and adaptive immune responses. The complement system removes immune complexes from circulation through binding to complement receptor 1 (CR1) expressed on erythrocytes. There are three main pathways to activate the complement system; the classical pathway which is activated through bound IgM and IgM molecules, the lectin pathway which is activated by binding of mannose through the mannose binding lectin (MBL) on the cell surface, and the alternative pathway which is activated by the constant hydrolysis of the complement component 3 (C3) (Ricklin et al., 2010).

When these innate immune defences are not sufficient to overcome an infection, the innate immune system will activate the adaptive immune system. This occurs when innate leukocytes such as macrophages or dendritic cells, also referred to as antigen presenting cells (APCs), recognise, ingest and process foreign particles within endosomes and lysosomes. These cells then present the digested material on major histocompatibility complex (MHC), a cell surface protein that is essential for regulating recognition of self and non-self -molecules. Material that has originated from outside the cell is presented on MHC-II, and material that originated from inside the cell is presented on MHC-I, however DCs have the ability to present extracellular material on MHC-I through cross-presentation (Heath and Carbone, 2001). The APCs then migrate to secondary lymphoid organs, such as tonsils, lymph nodes and spleen,

where they present the antigen to cells of the adaptive immune system, known as antigen presentation, thus setting the adaptive immune response into motion.

1.2.3 The adaptive immune system

The adaptive immune system, in contrast to the innate immune system, has a delayed response to pathogens, usually peaking 7-14 days after recognition of a foreign pathogen. The response is slow because the antigen must be taken up by the APCs and then introduced to the antigen-specific T (thymus-derived) and B (bursa- or bone-marrow-derived) lymphocytes; the major cellular components of the adaptive immune system. Unlike the innate immune system, the adaptive immune system is capable of recognising specific individual pathogens or antigens, and it generates increasingly potent and precise responses to that pathogen. The hallmark of the adaptive immune system is the preservation of the response through long-lived immunological memory, which allows for a rapid response upon re-exposure to the same pathogen - the fundamental principle of vaccination.

There are two broad classes of adaptive immune responses; antibody-mediated responses and cell-mediated immune responses, and the cells responsible for these types of responses are the B cells and T cells respectively. Antibody-mediated responses occur when B cells recognise antigens in the blood, lymph, or held within follicles of secondary tissues, leading to the production of antigen-specific antibodies which circulate throughout the body and specifically bind to foreign antigens to inactivate them or mark them for destruction (Murphy and Weaver, 2016). These antibodies are composed of different classes, and each class will mediate specific responses (further discussed in Section 1.3.5). The antibody-mediated

response also produces long-term immunological memory in the form of plasma cells and memory B cells (Murphy and Weaver, 2016, Effros, 2000).

Cell-mediated immune responses are independent of antibodies and are mediated by helper $CD4^+$ T cells reacting against foreign antigen that has been engulfed by APCs and presented on MHC-II, or $CD8^+$ T cells reacting against antigen presented on MHC-I (Murphy and Weaver, 2016). The binding of the T cell receptor (TCR) to the antigen-MHC complex is the first activating signal, and the second signal is provided by co-stimulatory molecules and cytokines, leading to the activation, proliferation and differentiation of the T cell. Helper T cells generally do not possess cytotoxic properties like the CD8 T cells (although some can as shown in this thesis), and upon activation secrete cytokines that activate cytotoxic $CD8^+$ T cells, macrophages, eosinophils or neutrophils to proliferate and eliminate the infected cells. (Murphy and Weaver, 2016, Kaiko et al., 2008). These elimination mechanisms can be destructive, so it is imperative that these adaptive immune cells are tolerised, and do not react to self-antigens.

1.3 The components of the immune system

1.3.1 Humoral Immunity

The humoral immune response is dependent on the production of antibodies. B cells and plasma cells produce antibodies that can neutralise pathogens, enable phagocytes to recognise a pathogen for engulfment and activate the complement system. As described above, the activation of these B cells and their differentiation into antibody-secreting cells is

trigged by antigens, and usually requires T cell help. The many factors that shape the outcome of an immune response will be discussed below.

1.3.2 Immunoglobulin

The production of immunoglobulin (Ig), also known as an antibody, distinguishes B cells from other cells of the immune system. Originally each B cell has the potential to generate millions of different Igs, but ultimately after activation, differentiation and maturation, each B cell will express Igs of a single specificity. These Igs are expressed in a membrane-bound form, associated with Igα and Igβ proteins, and this complex is known as a B-cell receptor (BCR). Igα and Igβ propagate cell signalling and activation using their immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails (Isakov, 1997). Once a B cell has terminally differentiated into antibody-secreting cells (ASC), also known as plasma cells, the Igs are secreted as soluble antibodies which can bind and neutralise antigens.

1.3.3 Structure of immunoglobulin

Igs consist of two identical polypeptide heavy chains (approximately 55-57 kDa) and two identical light chains (25 kDa), which are linked by disulphide bonds (Figure 1.1). Each heavy and light chain possesses a variable domain at the N-terminus which is important for the recognition of antigens. Each chain also possesses three short sequences of amino acids which mediate antigen recognition, known as the complementarity-determining regions (CDRs), which are highly variable between different Igs allowing them to bind an infinite diversity of antigenic structures (Murphy and Weaver, 2016). Both the heavy and light chains possess constant (C) domains, and the amino acid sequence of these domains defines the Ig isotype

(Murphy and Weaver, 2016). The light chain C domains are either kappa (***κ***) or lambda (***λ***), and the heavy chain possesses multiple C domains depending on the antibody isotype.

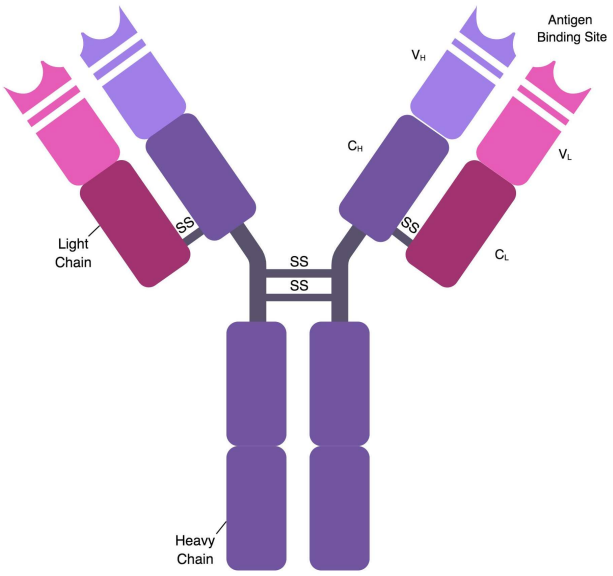


Figure 1.1. Structure of an immunoglobulin (adapted from Janeway's immunobiology, (Murphy and Weaver, 2016)).

1.3.4 Diversity of immunoglobulin

The antigen binding site of an antibody is highly diverse, brought about by the rearrangement of gene segments coding for the variable region of both the heavy and light chains, and the imprecise assembly of gene segments which leads to insertions, deletions and changes in amino acids. Igs can then undergo further class switching and somatic hypermutation after seeing antigen, leading to high affinity antibody production.

The heavy chain and light chain that form the Ig are encoded at loci located on different chromosomes. The variable region of the heavy chain is coded by a variable (V), joining (J)

and diversity (D) segment of DNA, and the light chain has a V and J segment (Murphy and Weaver, 2016). The V, D and J segments are separated by introns, and therefore the DNA sequences must be assembled together to produce each segment by a process known as V(D)J recombination. This recombination is initiated by recombination-activating gene (RAG) 1 and RAG2, which together as a complex recognise highly conserved recombination signal sequences (RSSs). These RSSs are positioned 3' of each V gene segment, 5' of each J segment and on both sides of each D segment, and the RAG proteins nick the DNA at these sequences, bind them and bring the targeted segments close together. Terminal deoxynucleotidyl transferase (TdT) will then add nucleotides at the V(D)J joints, further increasing the diversity of the antibody repertoire (Murphy and Weaver, 2016, Gauss and Lieber, 1996).

1.3.5 Class switch recombination and immunoglobulin classes

The V(D)J rearrangements of the heavy and light chain gene segments generate a clonally-unique immature B cell repertoire that can now bind antigen. Initially alternative splicing of the mRNA leads to the production of IgM and IgD isotypes, however when an immune response is triggered, the B cells will start to undergo class switch recombination (CSR). The class of Ig produced depends on the cytokines and other factors that the B cell is exposed to. Immunoglobulins are divided into five classes: IgM, IgG, IgA, IgD and IgE (Murphy and Weaver, 2016). Each of these different classes of antibody mediate specific responses, and will localise to a different place in the body to serve particular role. Pentameric IgM is found in the blood and is a component of the first line of defence in the immune system, activating the complement system (Feinstein et al., 1986). IgG is the most abundant antibody in the circulation, found in the blood and extracellular fluid, and has the ability to neutralise

pathogens, activate complement and mediate opsonisation for phagocytosis. In humans, IgG can be further divided into four subclasses named in order of decreasing abundance IgG1, IgG2, IgG3 and IgG4 (Schur, 1988), with each subset possessing unique antigen binding, placental transfer, immune complex formation, complement activation and binding, and half-life. IgA is the major class of antibody present in mucosal secretions and exists in two subclasses, IgA1 and IgA2 (Kawamura et al., 1992), with IgA1 present predominantly in serum and IgA2 predominantly in secretory lymphoid tissues. IgA monomers are mostly found in the blood and extracellular fluid (Mestecky et al., 1986), and dimers are produced locally at mucosal epithelial surfaces (Novak et al., 2005). Finally, IgE is a monomeric antibody that plays important roles in defence against helminths, and when produced in excess can lead to allergies. It is produced primarily in mucosal lymphoid tissues such as the tonsils (Eckl-Dorna et al., 2012).

1.3.6 Somatic hypermutation

After antigen encounter, the diversity of the Ig proteins can be increased by the V gene segments undergoing somatic hypermutation (SHM). This process occurs after recently primed T cells activate cognate B cells via interaction of cluster of differentiation 40 (CD40) on T cells with CD40-ligand (CD40L) on B cells. Primed B cells upregulate the expression of activation-induced cytidine deaminase (AID), which deaminates cytosine to uracil at hot spot motifs (Bachl et al., 1997). Point mutations are then introduced by an error-prone DNA polymerase during the replication and repair of the uracil residues (Murphy and Weaver, 2016), with the hope of producing B cells with antibodies of higher specificity for an antigen. Nevertheless, B cells can also lose specificity for the immunising antigen or become self-

reactive through the process of SHM. Thus, stringent selection after SHM is required, in the competitive microenvironment of the germinal centre (further discussed in Section 1.4).

1.3.7 B cell development

The generation of B cells occurs primarily in the fetal liver prior to birth, and during the postnatal period it continues in the bone marrow (Murphy and Weaver, 2016, Melchers, 1995). During this development, hematopoietic stem cells (HSCs), which are characterised by an absence of lineage markers and the ability to self-renew, gradually acquire specialised functions associated with B cells. The initial event that drives the HSC down the B cell lineage pathway is when it loses its ability to self-renew and differentiates into a multipotent progenitor (MPP) cell (Figure 1.2). From here the cells will start to express higher levels of cluster of differentiation antigen 135 (CD135) and become lymphoid-primed multipotent progenitors (LMPPs). These LMPPs can then start to express genes that are exclusively associated with lymphoid cells, such as the RAG proteins, and are termed common lymphoid progenitors (CLPs). CLPs will mainly differentiate into B, T or NK cells depending on the signals from bone marrow stromal cells, and from here the decision to progress towards one lineage over another will depend on the expression and silencing of regulatory genes and transcription factors (Nowick and Stubbs, 2010).

The earliest identifiable stage of B cell development is the pre-pro-B cell stage, where they express many transcription factors involved in B cell development as well as surface markers such as interleukin-7 receptor alpha (IL-7Rα). Whilst these pre-pro-B cells will predominantly differentiate into B cells, they are not yet committed to the B cell lineage and can still

differentiate into other cell types. As the pro-B cells develop, they start to express CD19, CD79a (Igα) and CD79b (Igβ), as well as RAG1, RAG2 and TdT. B-Cell Lineage Specific Activator (BSAP) is induced at the pro-B cell stage and plays an important role in the commitment of cells to the B cell lineage, activating the transcription of B cell lineage-associated genes such as CD19, and is maintained throughout B cell development and maturation. At the end of pro-B cell development V(D)J rearrangement of the heavy chain is complete. These cells are termed pre-B cells, and they possess a pre-B cell receptor consisting of a rearranged heavy chain and surrogate light chains (SLCs) Igα and Igβ which stabilise the receptor (Murphy and Weaver, 2016, Radaev et al., 2010). Heavy chains that are unable to associate with the SLCs will not form a pre-BCR and will undergo apoptosis. The complex of the pre-B cell receptor with Igα, Igβ and CD19 make a functional signalling complex allowing the B cell to proliferate and cease further heavy chain rearrangements (Murphy and Weaver, 2016).

The pre-B cells then begin rearrangement of the light chain through RAG-mediated V-J rearrangement of the kappa or lambda locus, and when the light chain is successfully rearranged and paired with the heavy chain it is termed a B cell receptor (BCR). These cells are termed immature B cells and express IgM, the product of a successful heavy and light chain product. This BCR can now associate with Igα and Igβ receptors and CD19, and cells that are unable to make this association and create a signal transducing BCR will undergo apoptosis. These immature B cells will then test their reactivity on the surrounding tissue in the bone marrow, a process known as negative selection, to prevent self-reactive B cells from exiting the bone marrow. If a B cell reacts to a self-antigen it will be signalled to undergo receptor editing and rearrange its light chain (Casellas et al., 2001, Gay et al., 1993, Tiegs et al., 1993, Melamed et al., 1998, Nemazee and Burki, 1989). A B cell can undergo several

rounds of receptor editing, however if it uses up all the V-J segments and is unable to become self-tolerant it will undergo apoptosis (known as clonal deletion). If the BCR is weakly cross-linked with self-antigen, a state of unresponsiveness or anergy can occur, meaning the cell is still viable and can exit the bone marrow but cannot be activated upon antigen encounter (Goodnow et al., 1989, Goodnow and Basten, 1989).

Rearrangement of the heavy and light chain gene segments generates a clonally-unique immature B cell repertoire that can bind antigen. These B cells express an increased density of IgM on their surface (the BCR), and after exiting the bone marrow they also acquire IgD (Vitetta et al., 1975, Kearney et al., 1977, Fu et al., 1974). To complete their development, these immature B cells migrate from the bone marrow into the spleen as transitional B cells (transitioning from immature to mature B cells) and can be subdivided into transitional 1 (T1), transitional 2 (T2) and transitional 3 (T3) B cells. These transitional B cells can complete their development by differentiating into mature naïve B cells which can become activated and divide rapidly in response to antigen (Su and Rawlings, 2002, Martin et al., 2016, Chung et al., 2003).

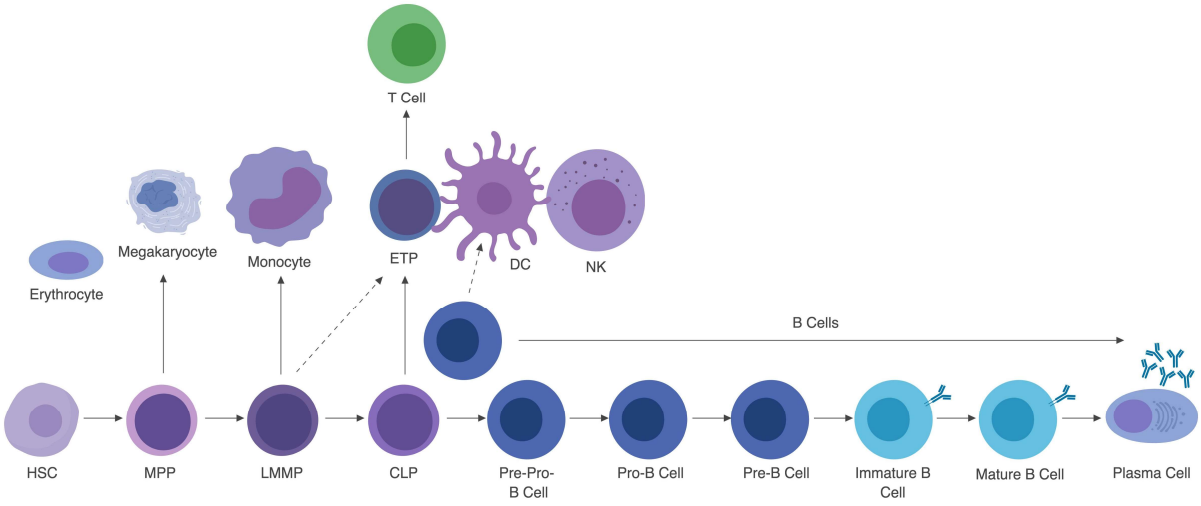


Figure 1.2. B cell differentiation pathway (adapted from (Nagasawa, 2006)).

1.3.8 B cell subtypes, activation and immunological memory

In humans there are many different types of B cells, and these subsets differ slightly to those in mice. As mentioned above, when the B cells leave the bone marrow they leave as transitional B cells, and then develop into mature naïve B cells which recirculate between the blood and the secondary lymphoid organs. Depending on the type of B cell and the type of antigen, there are several activation pathways that the B cell can take.

B cell activation requires the reception of two distinct signals through particular cell surface receptors. The first signal (signal 1) is delivered when the BCR engages with its cognate antigen, with costimulation through cell surface CD19 and CD21. The second signal (signal 2) can be delivered by two methods; with assistance from T cells (thymus-dependent activation) or from the B cell responding directly to the antigen independently of T cell help (thymus-independent activation) (Murphy and Weaver, 2016, Parker, 1993). The type of response

depends on the antigen, and therefore antigens can also be classified as T-dependent or T-independent. T-independent responses can be further divided into type 1 and type 2 responses, where type 1 B cell activation is independent of BCR engagement and can be accomplished through TLR activation (for example LPS binding to TLR4), and type 2 responses which are dependent on BCR engagement and cross-linking of BCRs (Richards and Clark, 2009, Murphy and Weaver, 2016). In T-independent responses a second signal for activation can come from another cell, not just the antigen, such as a dendritic cell, eosinophil or neutrophil (Chang et al., 2011), however in T-dependent responses this second signal is typically given by a T helper cell or NK T cell (Chang et al., 2011).

Following activation, the B cells proliferate and class-switch the BCR constant region from IgM/IgD into another isotype (IgG, IgA or IgE). They can differentiate outside follicles as extrafollicular plasma cells, or they can enter follicles and become germinal centre B (GCB) cells. These GCB cells, after undergoing rounds of proliferation, SHM and selection, can further differentiate into long-lived antibody secreting plasma cells, or into memory B cells (Victora and Nussenzweig, 2012, Allen et al., 2007, Tarlinton and Smith, 2000). The generation of long-lived plasma cells and memory B cells that produce high-affinity isotype-switched antibodies to foreign antigens is fundamental to producing long-lasting immunological memory. This allows the immune system to mount a faster and more efficient immune response upon re-encounter of the pathogen; the biological foundation of vaccinations. Germinal centres will be further discussed in Section 1.4.

Following an inflammatory response, anti-inflammatory mediators and cytokines, such as IL-10, are produced by suppressive/regulatory cells (Medzhitov, 2008). One of the B cell

populations that has been associated with inhibition of excess inflammation are the regulatory B cells (B_{regs}), a population of cells with immunosuppressive capabilities that supress inflammation and T cell differentiation mainly through the production of IL-10, TGF$\boldsymbol{\beta}$ and IL-35 (Rosser and Mauri, 2015). Whilst our understanding of these cells has expanded over the last decade, we are still trying to understand the biology and phenotypes of these cells, including how these cells acquire regulatory function. Furthermore, no specific marker for regulatory B cells has been identified, and whilst IL-10 has commonly been used as a marker for these suppressive cells, regulatory B cell subsets that act independently of IL-10 have also been identified (Ray et al., 2015, Ray et al., 2012, Khan et al., 2015), making it challenging to study these cells.

1.3.9 T cell development

The thymus is a primary lymphoid organ that supports the establishment, development and maintenance of T cells, and is pivotal during early development as it establishes the T cell repertoire that will protect the body for its lifetime. The thymus is divided into two main regions; the outer cortical region and the inner medullary region. Each region possesses unique stromal cells which are essential for T cell development, comprising of DCs, macrophages, endothelium cells, fibroblasts and thymic epithelial cells (TEC) (Anderson et al., 1996), which can be further subdivided into cortical TECs (cTEC) and medullary TECs (mTEC) (Suster and Rosai, 1992), named based on their anatomical position in the cortical and inner medullary regions of the thymus.

Like B cells, thymocytes develop from self-renewing HSC originally derived from the fetal liver during embryogenesis and then postnatally from the bone marrow (van Ewijk et al., 1988), which then differentiate into short-lived MPPs and then CLPs (which have the ability to generate both T and B cell lineages). The CLPs then differentiate into thymus seeding progenitors (TSPs), which still have the ability to differentiate into T, B and NK cells, and are not committed to the T cell lineage until the cell expresses a variety of markers including CD7 (Hao et al., 2008), however this is still not well understood.

Once committed to the T cell lineage, immature thymocytes are double negative (DN) for CD4 and CD8 expression, and they begin the process of TCR rearrangement by breaking and joining gene segments at the TCR$\boldsymbol{\beta}$ locus (Danska et al., 1990). Upon successful expression of a TCR$\boldsymbol{\beta}$ chain, it forms a complex with the germline-encoded pre-TCR$\boldsymbol{\alpha}$ protein and CD3 molecules (γ, δ, ε, ζ), termed the pre-TCR. This complex transduces signals that trigger the DN thymocyte to undergo proliferation, with unsuccessful pairing leading to apoptosis (von Boehmer and Fehling, 1997). Double negative thymocytes will then upregulate both CD4 and CD8, and become double positive (DP) thymocytes that initiate gene rearrangement in the TCR$\boldsymbol{\alpha}$ locus to generate aa TCR$\boldsymbol{\alpha}$ chain (Goldrath and Bevan, 1999). Once rearranged the $\boldsymbol{\alpha}$ and $\boldsymbol{\beta}$ chains can bind to form a mature TCR$\boldsymbol{\alpha\beta}$.

It is at this point that the DP cells are subjected to central tolerance to eliminate thymocytes with TCRs that recognise self-antigen. Central tolerance is achieved by the processes of non-selection (neglect), positive selection and negative selection. T cells must be able to recognise MHC and peptide in order to mount an immune response, meaning that they must be able to bind to self-MHC with a moderate affinity. Double positive T cells test their affinity with self-

MHC expressing cortical epithelial cells, resulting in low affinity cells not receiving sufficient TCR mediated signalling and undergoing apoptosis due to neglect. Negative selection removes those thymocytes with TCRs that bind too strongly to peptides bound to self-MHC, as activation of these cells in the periphery could lead to autoimmunity (Sprent and Webb, 1987, Benoist and Mathis, 1989, Benoist and Mathis, 1999, Sprent, 1993, Sebzda et al., 1999). These cells are signalled to either undergo apoptosis, continue rearrangement of the TCR, or a small proportion will become regulatory T cells (Sakaguchi et al., 2008, Sakaguchi, 2008, Sakaguchi, 2006, Sakaguchi, 2011, Benoist and Mathis, 2012). Positive selection preserves those thymocytes whose TCRs recognise peptides bound to self-MHC appropriately, and survival signals are delivered to positively select these thymocytes to proliferate and develop.

1.3.10 T cell subsets

T cells can be identified by surface CD3 expression and are comprised of two major groups; helper $CD4^+$ T cells and cytotoxic $CD8^+$ T cells, named based on their predominant helper and cytotoxic functionality respectively. Helper $CD4^+$ T cells support the immune response by generating cytokines that activate neighbouring cells, and chemokines to recruit other immune cells to the targeted site. $CD8^+$ T cells also produce cytokines, however their function is largely focused on eliminating pathogen-infected cells by delivering cytotoxic granules into the cytosol. The type of T cell response produced is largely dependent on the type of invading pathogen, as the interaction between the PRRs on innate immune cells and the pathogen results in a specific production of cytokines.

Naïve $CD4^+$ T cells expressing a unique TCR only commit to a specific T cell subset following TCR engagement and the integration of molecular signals downstream of cytokine receptors. Depending on the cytokine milieu, these T cells can be programmed to become a variety of effector cells which each have different mechanisms for eliminating pathogens. The major subsets of T helper (Th) cells include T_H1, T_H2, T_H17, T_H9 and T_{FH}, each of which target particular infections and display a unique cytokine profile (see Figure 1.3).

When a naïve $CD8^+$ T cell recognises antigen on MHC-I in the presence of IFN$\boldsymbol{\alpha/\beta}$ and IL-2, they will differentiate into cytotoxic T cells(Pipkin et al., 2010). These cells will then deliver large amounts of proinflammatory cytokines and secretory vesicles containing perforin and granzyme across the synapse to lyse the infected cell (Johnson et al., 2003). It is however important to note that there are many overlapping characteristics between CD4 and CD8 T cells, for example CD8 T cells can also influence cytokine production in a similar way to CD4 T cells, producing documented subsets such as Tc2s and Tc17s (Mittrucker et al., 2014), and CD4 T cells can also have cytotoxic properties (Marshall and Swain, 2011).

During an infection the T cells will proliferate and differentiate into the T cell subsets described above to clear an infection, and when the infection has been cleared the majority (around 90-95%) of these T cells will die. A subset of T cells called memory T cells remains after the infection, and just like with memory B cells, these memory T cells have memory for the pathogen they just encountered and can provide protection for decades. This enables an increased proliferative potential and more rapid effector response upon reinfection by the same pathogen. For both CD4 and CD8 T cells there are central memory and effector memory T cells, and the more recently described resident memory T cells which are non-recirculating

memory T cells that persist in the absence of antigens in epithelial barrier tissues such as the gastrointestinal tract (Clark, 2015). The central memory cells are characterised by their increased potential for proliferation on reencounter of pathogen, and the effector memory cells display a rapid effector function (granzyme and interferon production) but have a limited proliferative potential. The central memory cells also home to secondary lymphoid organs as they constitutively express the CCR7 ligands CCL19 and CCL21, allowing for them to be well situated if reinfection occurs. In contrast, the effector memory cells do not express CCR7, and therefore move throughout the non-lymphoid tissues, enabling them to be the first responders at peripheral sites where reinfection could occur. This means that the effector memory cells can control initial exposure at the site of infection, allowing time for the central memory cells to proliferate and create more effectors. These central and effector memory cells are at opposite ends of a spectrum of memory T cell subsets and thus there are many more subsets than this simplified dichotomy.

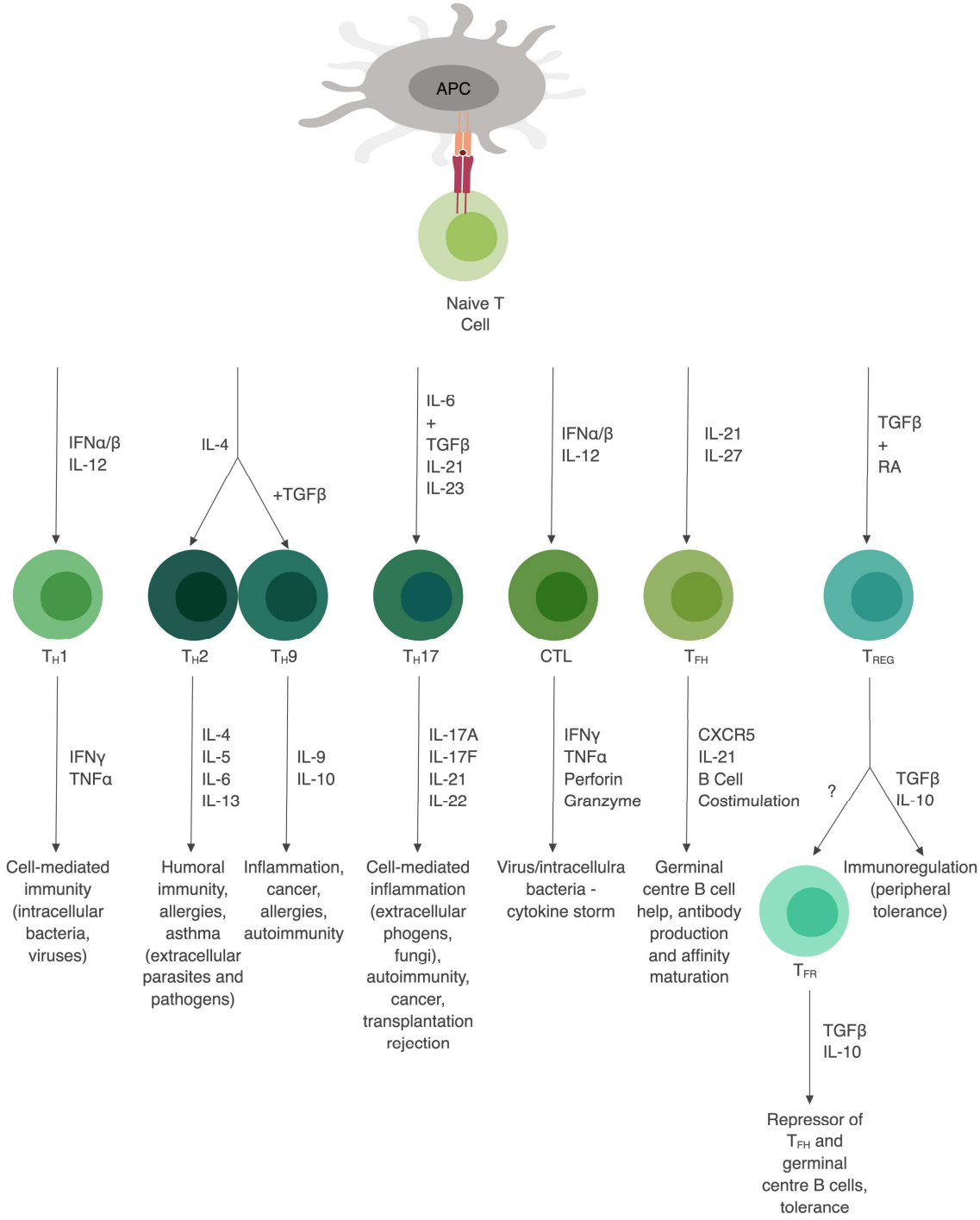


Figure 1.3. T cell differentiation pathway (adapted from (Nurieva and Chung, 2010)).

1.4 Long-lived antibody production and the germinal centre reaction

1.4.1 The germinal centre reaction

The germinal centre is a complex and highly dynamic microanatomical niche that was first described in 1884 by Walther Flemming as an area with high mitotic activity in secondary lymphoid organs (Nieuwenhuis and Opstelten, 1984). Despite the fact his original proposed function of the germinal centre was eventually disproven, his work prompted study that is continuing today. Since then, our knowledge of germinal centres has increased substantially, and it is widely accepted that germinal centres are crucial for humoral immunity, as they generate high-affinity antibody-producing plasma cells and memory cells which provide long-lived highly protective antibody responses. Germinal centres form in secondary lymphoid organs within B cell follicles. These B cell follicles are populated by naïve B cells and are in direct contact with the T cell-rich areas (the T cell zone). The border between the B cell follicle and the T cell zone known as the T-B border.

Mature naïve B cells continually recirculate through the body, searching for signs of infection. Upon antigen recognition via the BCR, the naive B cell will become activated and rapidly upregulate chemokine receptor 7 (CCR7) and EBI2. This enables the B cell to migrate and congregate at the T-B border where its ligand chemokine ligand 21 (CCL21) is expressed, in search for T cell help (Veerman et al., 2007, Forster et al., 1999, Haynes et al., 2007). Here at the T-B border, the B cell will encounter and form long-lived interactions with antigen-stimulated cognate T_{FH} cells, a specialised subset of $CD4^+$ T cell that expresses a high level of CXCR5, a chemokine receptor that attracts T cells to the B cell follicles in response to CXCL13 ligand produced by Follicular Dendritic Cells (FDCs). The interaction between CD40 on the B

cell and CD40L on the T_{FH}, the secretion of cytokines by T cell, and the subsequent downregulation of EBI2 and CCR7 is crucial for B cell proliferation, enabling the B and T_{FH} cells to move from the periphery into the centre of the follicle, establishing the germinal centre. Some of these activated B cells however will not enter the germinal centre reaction, taking the extrafollicular route of differentiation and form short-lived plasma cells and memory B cells which produce low-affinity antibody in the extra-follicular foci (Garside et al., 1998, Okada et al., 2005, Qi et al., 2014, Kerfoot et al., 2011) (Figure 1.4).

In 1930 it was shown that germinal centres are divided into two regions; the dark zone and the light zone (Röhlich, 1930). B cells which take the germinal centre route will rapidly upregulate BCL6, move towards the centre of the follicle, and begin to rapidly divide and fill the follicle as B blasts. They then move towards the area closer to the T cell zone known as the dark zone, where they become centroblasts (also referred to as dark zone cells). In the dark zone, the centroblasts proliferate and undergo SHM of the immunoglobulin variable region genes (Victora and Nussenzweig, 2012, Allen et al., 2007). These centroblasts will cease proliferation and give rise to centrocytes which populate the light zone (thus centrocytes are also referred to as light zone cells) and interact with antigen trapped on FDCs. Centrocytes take up antigen, process it and present it on MHC class II to T_{FH} cells and probably to T follicular regulatory (T_{FR}) cells (MacLennan, 1994, Chung et al., 2011a, Linterman et al., 2011b, Wollenberg et al., 2011b, Sage and Sharpe, 2015a), a recently defined specialised subset of regulatory T cells (T_{regs}) that will be further discussed below. B cells that have been provided with survival signals are positively selected and can re-cycle to the dark zone to divide and undergo further rounds of somatic hypermutation to produce antibodies with higher affinity, or exit the germinal centre reaction as long-lived memory cells or plasma cells. These high-

affinity memory B cells will then circulate the body or home to secondary lymphoid organs, and the long-lived plasma cells will home to the bone marrow and secrete antibody for months to years (Tarlinton et al., 2008, Wrammert and Ahmed, 2008). Once the process of affinity maturation is over, the germinal centre collapses through a poorly-understood process (Allen et al., 2007).

Recent studies have demonstrated that centrocytes and centroblasts can be distinguished based on the expression of CD83 or CD86 with CXCR4, with those B cells expressing CXCR4 localising in the dark zone (centroblasts) and those not expressing CD83 or CD86 localising in the light zone (centrocytes) (Victora and Nussenzweig, 2012). The movement of these cells between the dark zone and the light zone of the germinal centre is orchestrated by the expression of various chemokines. CXCL12 is expressed highly in the dark zone compared to the light zone, and thus the expression of the receptor for CXCL12, CXCR4, is higher on centroblasts than centrocytes and follicular B cells (Allen et al., 2004). Furthermore, CXCL13 is more abundant in the light zone compared to the dark zone, and its receptor CXCR5 is highly expressed on centrocytes, T_{FH} and T_{FR} cells (Allen et al., 2004, Stone et al., 2015).

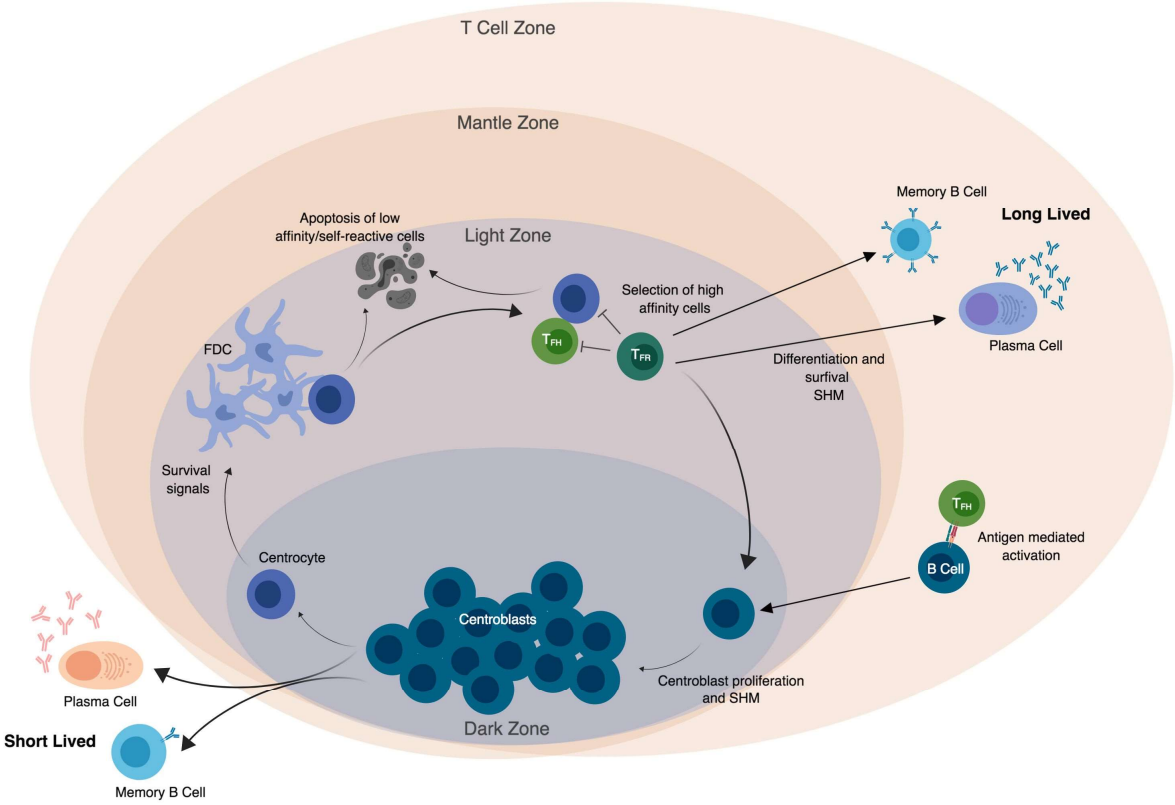


Figure 1.4. The germinal centre reaction (adapted from (Klein and Dalla-Favera, 2008)).

1.4.2 T_{FH} and T_{FR} cells in the germinal centre reaction

Regulation within the germinal centre reaction is crucial, as the antibodies that are produced will persist for years. T_{FH} cells are a population of BCL6^{+} PD-1^{hi} CXCR5^{hi} T cells that help B cells and promote affinity maturation by providing proliferation, survival and differentiation signals, first to B cells at the T:B border that have recently bound antigen, then to centrocytes in the germinal centre. Help is delivered via CD40-CD40L interactions and cytokines such as IL-21 and IL-4 (Vinuesa et al., 2016, Vinuesa et al., 2005b, King and Mohrs, 2009, Crotty, 2011). Deficiency in T_{FH} cells leads to absent germinal centres and compromised antibody responses (Vinuesa et al., 2016, Crotty, 2014). Limiting T_{FH} cell help appears to be crucial for long-lived high-affinity antibody responses (Victora et al., 2010) and abnormal accumulation of T_{FH} cells has been shown to lead to autoantibodies (Linterman et al., 2009, Vinuesa et al., 2005b,

Simpson et al., 2010). More recently, a population of PD1^{hi} CXCR5^{hi} follicular T cells that are also express forkhead box P3 (FOXP3) were identified, and named T_{FR} cells (Linterman et al., 2011c, Wollenberg et al., 2011a, Chung et al., 2011b).

T_{FR} cells share many phenotypic characteristics regulatory T cells (T_{regs}), and are thought to be derived from $T_{regs,}$ however in addition to FOXP3 and beta-interferon gene positive-regulatory domain 1 (Blimp1), mouse T_{FR} cells also express Bcl6 and co-opt the follicular differentiation pathway, allowing them to localise in germinal centres. T_{FR} cells have been shown to limit the magnitude of germinal centre reactions and antigen-specific antibody production, although the mechanism for this remains unknown (Linterman et al., 2011a, Wollenberg et al., 2011b, Chung et al., 2011a, Aloulou et al., 2016, Sage et al., 2014, Wing et al., 2014). They can also limit the emergence of non-antigen specific and self-reactive antibodies (Linterman et al., 2009, Fu et al., 2018). It is possible that T_{FR} cells inhibit the initial T-B cell interactions at the T-B border, which would potentially inhibit initial germinal centre formation, but this has not been proved experimentally. Another theory is that T_{FR} cells mechanically disrupt the interaction between T_{FH} cells and B cells by strongly binding to the B cells and eliciting a "stop signal", or occupying the B cells for longer thus preventing T_{FH} interactions (Sage and Sharpe, 2015a). More recently, a study by Sage et al., 2016 demonstrated that T_{FR} cells have a repressive effect on germinal centre B cells (Sage et al., 2016). It is evident that we are only beginning to understand this germinal centre regulatory T cell subset, and a deeper understanding has the potential to reveal strategies for controlling the immune response and may help us elucidate therapeutic targets. Furthermore, whilst T_{FR} cells have been reported in humans, our understanding of these cells is mainly based on the role of these cells in mice.

1.4.3 Bcl6: the master regulator of the germinal centre reaction

B cell lymphoma 6 (Bcl-6) is an important master transcriptional repressor that is upregulated during initiation of the germinal centre reaction in germinal centre B cells, T_{FH} and T_{FR} cells. Bcl-6 expression is required for activated B cells to enter the follicle and form germinal centres. Importantly, Bcl-6 down-regulates G-coupled receptor 183, more commonly known as Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI-2), which with CXCR5 and CXCR7 expression, directs B cells towards the centre of the follicle. Bcl-6 also represses Blimp-1, which is important for plasma cell differentiation and the downregulation of BCR and CD40 signalling, thus preventing plasma cell differentiation allowing optimal affinity maturation. By silencing Bcl-2, Bcl-6 keeps cells in a pro-apoptotic state, and by inhibiting the DNA damage response silencing pro-apoptotic genes such as p53 and ATR, it enables them to incur frequent somatic mutations.

1.4.4 Important molecules in germinal centre interactions

As mentioned earlier, B cell activation by T cells (thymus dependent) requires the sequential integration of at least two signals. The first signal is generated by BCR cross-linking with antigen, and the second signal is provided by CD40L and cytokines produced by the T helper cells. CD40L is expressed on activated helper $CD4^+$ T cells and engages with CD40 expressed on the surface of APCs and naïve B cells, providing necessary costimulatory signals. When CD40L engages with CD40 on B cells, it provides the B cell with essential growth, survival, switching and differentiation signals that are critical for the B cells to differentiate into germinal centre B cells and undergo class switching. Specifically, this interaction rescues B cells from FAS (CD95) or IgM cross-linking induced apoptosis (Cleary et al., 1995, Garrone et

al., 1995, Lederman et al., 1994, Tsubata et al., 1993, Valentine and Licciardi, 1992), upregulates CD80 (B7-1) and CD86 (B7-2) which interact with CD28 and CTLA-4 (CD152) (Goldstein et al., 1996, Kaneko et al., 1996, Yellin et al., 1994), and increases the expression of other cell surface activation molecules. Once the germinal centre is established, T_{FH} cells continue to provide selection, survival, proliferation and differentiation signals to the germinal centre B cells, through CD40-CD40L signalling and cytokines, specifically IL-10, IL-21, and IL-4. Furthermore, CD40-CD40L interactions are also important for the maintenance of the germinal centre cycle and the cycling of cells between the light and dark zones (Liu et al., 2015a).

CD80 (B7-1) and CD86 (B7-2) are markers of B cell activation and provide important costimulatory signals through interactions with CD28 and CTLA-4 expressed on the surface of T cells, which are increased following T cell stimulation (Linsley et al., 1992, Metzler et al., 1999, Linsley et al., 1993, Linsley and Ledbetter, 1993). These interactions have been shown to be important for T cell activation, inhibition, proliferation and cytokine production, as well as for B cell antigen presentation and antibody production.

The interaction between ICOS and ICOSL also plays important and well-studied costimulatory roles during T:B cognate interactions. Mice lacking ICOS or ICOSL possess reduced numbers and sizes of germinal centres, demonstrating that ICOS signalling is important for the generation of optimal germinal centres and long-lived antibodies in thymus dependent immune responses (Good-Jacobson and Shlomchik, 2010). It has also been suggested that ICOS may regulate affinity maturation following the onset of germinal centres, with B cells promoting T_{FH} cell maturation in part through their delivery of ICOSL (Nurieva et al., 2008,

Goenka et al., 2011). Furthermore, ICOS-ICOSL interactions between T cells and bystander non-cognate B cells has been shown to facilitate migration of T cells into the germinal centre (Xu et al., 2013). B cell-derived ICOSL signals to cognate T cells has been shown to be critical for sustaining the interaction between T_{FH} and B cells within the germinal centre, via upregulating CD40L and increasing the duration of their contact (Liu et al., 2015b). In humans, T_{FH}-derived dopamine induces upregulation of ICOSL on germinal centre B cells, also leading to increased CD40L expression by T_{FH} cells and enhanced synaptic interactions between the two cell types (Papa et al., 2017).

1.4.5 PD-1-PD-L1 signalling in the germinal centre reaction

An additional ligand pair that plays an important role in T:B cognate interactions within the germinal centre is the interaction between programmed cell death protein 1 (PD-1) and its ligand PD-L1. The inhibitory receptor PD-1 is a B7-family molecule that is expressed on many different cell types, including B cells and T cells. The ligands for PD-1, PD-L1 (B7-H1) and PD-L2 (B7-DC), are expressed on B cells and dendritic cells (Good-Jacobson et al., 2010a, Haynes et al., 2007). On $CD8^+$ T cells, expression of PD-1 is associated with functional exhaustion (Barber et al., 2006, Wherry and Kurachi, 2015), however the role of high PD-1 expression on T_{FH} and T_{FR} cells is poorly understood. Several studies have suggested the roles of PD-1-PD-L1 signalling in regulating the humoral immune response, however the literature reveals contradictory results with some studies finding that the humoral response is supressed (Good-Jacobson et al., 2010a, Hamel et al., 2010a, Kawamoto et al., 2012), and others finding that it is augmented (Hams et al., 2011a, Velu et al., 2009, Butler et al., 2011).

Studies that support the theory that PD-1/PD-L1 signalling supresses the humoral response revealed that blocking PD-1 during simian immunodeficiency virus (SIV) infection (Velu et al., 2009) and knocking PD-L1 out in mice (Hams et al., 2011a) results in elevation of antigen-specific immunoglobulin responses and an increase in T_{FH} cells in response to helminth-derived antigens. A recent study investigated the effects of blocking PD-1 on humoral immunity, and showed that blocking PD-1 enhanced antibody production, and increased GCB cells and plasma cells (Zhang et al., 2018).

Other studies however report a role of PD-1 signals in promoting the humoral response, for example an absence of PD-1 signalling was associated with reduced germinal centre B cell survival and affinity maturation, and fewer long-lived plasma cells (Good-Jacobson et al., 2010a). Another study reported that PD-1 deficiency results in an increase in T_{FH} cells with skewed cytokine profiles (the T_{FH} cells produced less IL-21 and more IFN-$\boldsymbol{\gamma}$) associated with dysregulation in the selection of IgA precursor cells, leading to a dampened immune response (Kawamoto et al., 2012).

Other studies have demonstrated that PD-1-PD-L1 interactions play important roles in the generation of T_{FR} cells: mice deficient in PD-1 and PD-L1 were shown to have a greater abundance of T_{FR} cells with enhanced suppressive ability, suggesting that PD-1-PD-L1 signalling inhibits the differentiation and suppressive function of T_{FR} cells (Sage et al., 2013).

More recently, a study by Sage et al., 2018 demonstrated using PD-L1 conditional knockout mice, that PD-L1 expressed on dendritic cells limits self-reactive T cells during infection and inhibits the differentiation of T_{FH} and T_{FR} cells, and that PD-1 inhibits T_{FR} cell differentiation

and effector function (Sage et al., 2018). PD-1 was also recently found to be required for optimal germinal centre localisation of T_{FH} cells and optimal T_{FH} IL-21 production by supressing PI3K activity downstream of CXCR5 and inhibiting T cell recruitment into the follicle, a process mediated by PD-L1 expression on follicular B cells (Shi et al., 2018). PD-1 has also been shown to upregulate CXCR3 on T_{FH} cells, concentrating these cells towards the germinal centre region, and to increase the overall stringency of selection in the germinal centre through PD-L1/PD-1 interactions (Shi et al., 2018). Finally, elevated PD-L1 expression on B cells has been associated with a population of regulatory B cells that act as suppressors of T_{FH} cell differentiation and function and antibody responses (Khan et al., 2015). These PD-$L1^{hi}$ regulatory B cells have been shown to supress humoral responses through attenuation of T cell activation and antibody production, and have the ability to supress autoimmunity in a PD-L1 dependent manner that is independent of IL-10 (Khan et al., 2015). It is therefore evident that the role of PD-1-PD-L1/2 signalling in germinal centre responses and its consequence on antibody production has not yet been elucidated clearly.

1.4.6 The role of cytokines in the germinal centre niche

When an antigen presenting cell and an antigen-receptor expressing immune cell interact, they come in close contact and release molecules such as cytokines across the immunological synapse into the synaptic cleft (Dustin, 2014). The immunological synapse is a nano-scale gap between T cells and antigen presenting cells including B cells, that allows focused delivery of cytokines and other molecules between T_{FH} cells and B cells. Cytokines that are secreted across an immunological synapse can be more potent due to increased local concentration

within the confined synapse space (Huse et al., 2006), and are more specific as they can be targeted towards a particular cell.

Cytokines play important roles in the germinal centre reaction, and are produced by the B cells, the T_{FH} cells, the antigen presenting cells and the stromal cells. Cytokines produced by T_{FH} cells, specifically IL-21, IL-4 and human IL-10, play critical roles in the production of a robust humoral immune response and affinity matured antibodies. IL-21 is the main cytokine secreted by T_{FH} cells within the germinal centre, and is important for promoting B cell proliferation and plasma cell differentiation, antibody production, and switching to IgG1 in the context of thymus-dependent responses (Avery et al., 2008, Ettinger et al., 2005, Kasaian et al., 2002, Kuchen et al., 2007, Ozaki et al., 2004, Pene et al., 2004, Spolski et al., 2008, Bryant et al., 2007). The expression of IL-4 by T_{FH} is regulated by the transcription factor Basic Leucine Zipper ATF-Like Transcription Factor (BATF) (Sahoo et al., 2015) and has been shown to has been shown to be a regulator of Ig class switch recombination and promote IgE production (Pene et al., 2004, Pene et al., 2006). IL-4 has also been shown to play a role in the early stages of germinal centre B cell differentiation (Toellner, 2014) and the magnitude of the germinal centre response (Andoh et al., 2000).

Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine that plays a crucial role in the immune system, most notably in promoting the survival, proliferation and differentiation of B cells (Conti et al., 2003a, Conti et al., 2003b, Go et al., 1990, Wakkach et al., 2000). IL-10 promotes IgG class switching and secretion (Malisan et al., 1996, Shparago et al., 1996), and when produced by T_{FR} cells, it modulates and promotes the germinal centre response through modulating T_{FH} and germinal centre development (Laidlaw et al., 2017).

IFNγ is physiologically expressed at low levels or even absent in germinal centre T_{FH} cells (Yu et al., 2009), however substantial production can occur upon infection or immune stimulation (Yusuf et al., 2010). Excessive production of IFNγ however causes an increased number of pathogenic T_{FH} cells and autoimmune antibody responses (Lee et al., 2012b), as well as spontaneous germinal centre formation via cell-intrinsic induction of Bcl-6 (Lee et al., 2012b, Onuora, 2016, Domeier et al., 2017), and thus has been implicated in autoimmune pathogenesis.

Another molecule that is important in the germinal centre, specifically playing a role between follicular dendritic cell and B cell interactions, is B cell activating factor of the TNF-family (BAFF), an anti-apoptotic survival factor for B cells. BAFF is a cytokine that belongs to the TNF ligand family and is expressed by monocytes, macrophages, dendritic cells, bone marrow stroma cells and T cells (Moisini and Davidson, 2009), and its expression increases under pro-inflammatory conditions. The binding of BAFF to BAFFR activates a number of downstream pathways and regulates the survival of B cells. For example, mice deficient in BAFF or BAFFR are able to form relatively normal germinal centres, however they are unable to be sustained with the germinal centre response attenuating within a few days (Rahman et al., 2003, Shulga-Morskaya et al., 2004, Vora et al., 2003). BAFF also signals through TACI and BCMA, however mice deficient in these receptors possess normal germinal centre formation (von Bulow et al., 2001, Xu and Lam, 2001), and thus are unlikely to play essential roles in germinal centre maintenance. Finally, limiting BAFF is crucial to prevent survival of self-reactive anergic B cells, which have greater dependence of BAFFR signals, and their recruitment into immune responses (Lesley et al., 2004).

1.4.7 Antibody diversity: a double-edged sword

The combination of VDJ recombination during the early phases of B cell development and somatic hypermutation upon antigen encounter means that an infinite amount of possible antigen can be recognised, with some receptors inevitably recognising self-antigen (autoreactive). Whilst there are many tolerance checkpoints to prevent these autoreactive cells from proliferating and being released into the periphery, failures in these tolerance checkpoints can occur, and this can result in autoimmunity (Goodnow et al., 2005). The production of high-affinity antibodies can therefore be considered a double-edged sword; crucial to providing protection from pathogens, but dangerous due to the potential emergence of self-reactive cells that can persist for years and mount a response against self-antigen. There are many autoimmune diseases that are associated with the development of autoantibodies, and autoantibodies are often detected in the serum of patients with autoimmunity (Vinuesa et al., 2009). Furthermore, many mice models that develop autoimmunity, such as the *sanroque* mouse or NOD (non-obese diabetic) mouse, have spontaneous germinal centre formation in the spleen in the absence of any stimulation such as infection, as well as high titres of autoantibodies in their serum (Luzina et al., 2001). Autoimmunity that is caused by defects in the germinal centre reaction imposes many challenges in terms of treatment, as the generation of these autoreactive long-lived plasma and memory cells has already occurred, and treatments that disrupt the germinal centre reaction are potentially harmful and not always successful. It is therefore important that we understand the mechanisms involved in the germinal centre reaction in order to produce targeted therapeutic agents to prevent the production of autoreactive B cells in patients with autoimmunity.

1.5 Autoimmune diseases are caused by a break down in tolerance

1.5.1 Immunological tolerance and autoimmune diseases

In the late 1940's it was suggested by Owenthat immune tolerance to self-antigens occurs early during development when the immune system is exposed to self-antigens (Owen, 1945), and this was further supported by Medawar's findings during the 1950's that a mouse would become immunologically tolerant to foreign tissues if exposed to them during embryonic development (Billingham et al., 1953). It was Burnet's clonal selection theory, first put forward in the 1950s, which proposed that each lymphocyte is specific for one antigen, and a lymphocyte that was self-reactive would be deleted before it matures if it encountered antigen during early development (Mackay et al., 1957, Burnet, 1976). This was later modified by Lederberg, who suggested that it was the stage of development of the lymphocyte, as opposed to the stage in development of the animal (Lederberg, 1959) that determined the ability to be tolerised. In 1960, Burnet and Medawar won the Nobel Prize in Physiology and Medicine for the discovery of acquired immunological tolerance, defining principles which underpin our current understanding of the immunological basis of tolerance to self-antigens.

The immune system has many mechanisms to achieve efficient immunity against foreign pathogens without mounting immune responses against self-antigens, and many of these tolerance mechanisms are deliberately redundant. In the thymus, thymic epithelial cells present self-antigen to the T cells, and T cells which possess a high affinity for this self-antigen will be signalled to undergo apoptosis (known as clonal deletion). A small selection of these cells will also become regulatory T cells (T_{regs}). In a similar manner, as mentioned in the previous section, B cells expressing a BCR that binds self-antigen in the bone marrow will also

rearrange their receptor, undergo apoptosis or become anergic. These mechanisms of tolerance are known as central tolerance or negative selection, as they eliminate *developing* self-reactive B cells and T cells before they leave the primary lymphoid organs (the thymus and the bone marrow) (Murphy and Weaver, 2016).

Central tolerance is not completely effective and some self-reactive cells will escape into the periphery, so peripheral tolerance exists as a secondary mechanism to limit these mature cells from becoming activated once in the periphery. It is initiated in the secondary lymphoid organs when a lymphocyte interacts with its cognate antigen and is not activated. When this occurs, the T cell will be signalled to undergo induced apoptosis (clonal deletion) or become functionally inactivated (by becoming ignorant, exhausted or anergic). In the presence of tolerised T cells, even if the cognate self-reactive B cell sees self-antigen in the periphery, it will no longer be able to activate without the help of the T cell, and will instead become anergic and undergo apoptosis within a few days (Murphy and Weaver, 2016, Xing and Hogquist, 2012 , Walker and Abbas, 2002).

The challenges in both achieving and maintaining tolerance are obvious by the prevalence of autoimmune diseases as well as organ rejection. There is evidently a fine balance between protecting the body from foreign pathogens and cancer whilst still preventing autoimmune diseases. Broken or underdeveloped mechanisms of immunological tolerance can lead to the development of autoimmunity, a state where the body mounts an immune response against self-tissues (Mueller and Jenkins, 1997). Autoimmune diseases are often chronic and incurable diseases that, according to the Australasian Society of Clinical Immunology and

Allergy, affect 1 in 20 Australians. The precise aetiology of many autoimmune diseases remains unknown.

1.5.2 Defects in immune regulation lead to autoimmune diseases

The immune system is like triggering an alarm; it must be able to rapidly respond to a threat, but then have the ability to turn off when the threat is gone. This rapid induction and the efficient resolution of inflammatory responses are tightly regulated at the cellular level. A powerful example is the role that T_{regs} play in supressing T cell proliferation after an infection has been cleared, which is largely achieved through the production of cytokines.

As mentioned previously, cytokines are signalling molecules that are imperative for immune homeostasis. Under physiological conditions, inflammation will occur in a self-limiting manner through cytokine production, and homeostasis is restored through the interplay between immune cells and the functionally opposing cytokine milieu. When a breakdown in immune tolerance and autoimmunity occurs, this cytokine milieu equilibrium is disrupted, shifting the environment towards an inflammatory state which results in tissue damage. Whilst the precise triggers for this breakdown of self-tolerance and the events which lead to autoimmunity are not fully understood, it is clear that cytokines play pivotal roles in the pathogenesis of autoimmune diseases and contribute to the initiation and propagation of autoimmune inflammation. For example, in patients with psoriasis, the persistent production of TNFα causes macrophages and keratinocytes to produce chemokines that recruit immune cells to the lesion sites. These immune cells then produce proinflammatory cytokines, chemokines and antimicrobial peptides, which recruit more immune cells, amplifying the

local inflammatory response and causing a cytokine storm. Furthermore, disease susceptibility is influenced by genetic predisposition, and disease susceptibility genes encoding cytokines and cytokine receptors or their molecular pathways have been associated with autoimmune diseases, such as the JAK-STAT pathway and the nuclear factor of activated T-cell (NFAT) pathway. In some autoimmune diseases such as rheumatoid arthritis, specific myeloid-derived proinflammatory cytokines such as TNF and IL-1β have been shown to be largely responsible for the effector mechanisms of immune damage. Thus, targeting cytokines and their receptors with monoclonal antibodies (e.g. infliximab and adalimumab which are anti-TNFα therapies) has become a common therapeutic approach for some autoimmune diseases (Perosa et al., 2001).

Immune regulation can also occur at the transcriptional and post-transcriptional levels, for example through modifications that affect mRNA stability and translation (Ivanov and Anderson, 2013). This regulation is partly achieved by a plethora of RNA-binding proteins (RBPs) that regulate gene expression by modulating the stability and translation of target mRNAs, or through regulating processes such as gene splicing (Kafasla et al., 2014). Defects in RBP function have been associated with a number of severe neurodegenerative and neuromuscular diseases (Rees, 2004), as well as autoimmune diseases (Jiang et al., 2015, Kafasla et al., 2014). For example, alternative splicing of the sixth exon of the Fas receptor by an RPB leads to the production of a membrane bound form of Fas that promotes apoptosis or a soluble form that inhibits apoptosis. Variants that disrupt this process have been shown to cause autoimmunity in mice (Corrionero et al., 2011). Another example is Roquin, an RBP that plays a role in the maintenance of tolerance through promoting RNA decay of transcripts such as IL-6, TNF and ICOS (Yu et al., 2007, Schlundt et al., 2016, Heissmeyer and Vogel, 2013,

Drees et al., 2017). A single point variant in the gene that encodes for the Roquin protein in mice causes autoimmunity (Vinuesa et al., 2005a).

1.5.3 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic, debilitating and highly heterogeneous systemic autoimmune disease that can affect many organs in the body (Figure 1.5). The term *lupus*, Latin for *wolf,* was given due to the destructive cutaneous lesions that were reminiscent of wolf bites. It is believed that lupus was first described by Hippocrates, for a group of patients with cutaneous ulcerations (*herpes esthiomenos* – gnawing dermatosis) (Lahita, 1987), however the first clear description of lupus was by Laurent Biett (Smith and Cyr, 1988), whose student Pierre Louis Cazenave coined the term *lupus erythematosus* and described it as a skin condition primarily affecting the face of young women (Cazenave et al., 1852). The term SLE was first coined in 1894 when Sir William Osler observed the cardiac, pulmonary and renal disease that accompany these cutaneous manifestations (Osler, 1976). These diverse manifestations were unified by the American College of Rheumatology in 1982, allowing for a formal diagnosis of this disease based on eleven clinical and laboratory criteria, of which four are necessary for diagnosis (Table 1.1). Virtually any organ system may be affected by SLE, ranging from skin lesions, as a result of immune complex deposits between the dermal and epidermal layers of skin, to organ damage such as nephritis and arthritis (Tsokos, 2011). The most common manifestations are cutaneous lesions, arthritis and blood or serological abnormalities (Cerovec et al., 2012), however kidney disease (Manger et al., 2002), neurological and cardiac disease (Bujan et al., 2003) are the most potent risk factors for death.

Table 1.1. 1997 update of the American College of Rheumatology revised criteria for classification of systemic lupus erythematosus.

Criteria	Description
Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds.
Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions.
Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation.
Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
Non-erosive arthritis	Involving two or more peripheral joints, characterised by tenderness, swelling, or effusion.
Pleuritis or pericarditis	1. Pleuritis - convincing history of pleuritis pain or rubbing heard by a physical or evidence of pleural effusion. *OR* 2. Pericarditis - documented by electrocardiogram or rub or evidence of pericardial effusion.
Renal disorder	1. Persistent proteinuria > 0.5 grams per day or > than 3+ if quantitation not performed. *OR* 2. Cellular casts - may be red cell, haemoglobin, granular, tubular, or mixed.
Neurologic disorder	1. Seizures - in the absence of offending drugs or known metabolic derangements; e.g. uraemia, ketoacidosis, or electrolyte imbalance. *OR* 2. Psychosis-in the absence of offending drugs or known metabolic derangements e.g. uraemia, ketoacidosis, or electrolyte imbalance.
Hematologic disorder	1. Haemolytic anaemia - with reticulocytosis. *OR* 2. Leukopenia - <4000/mm^3 on ≥ 2 occasions. *OR* 3. Lymphopenia - <1500/mm^3 on ≥ 2 occasions. *OR* 4. Thrombocytopenia - <100 000/mm^3 in the absence of offending drugs.
Immunologic disorder	1. Anti-DNA: antibody to native DNA in abnormal titre. *OR* 2. Anti-Sm: presence of antibody to Sm nuclear antigen. *OR* 3. Positive finding of antiphospholipid antibodies on: 1. An abnormal serum level of IgG or IgG anti-cardiolipin antibodies. 2. A positive test result for lupus anticoagulant using a standard method. 3. A false-positive test result for at least six months confirmed by Treponema pallidum immobilisation or fluorescent treponema antibody absorption test.
Positive antinuclear antibody	An abnormal titre of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs.

The clinical heterogeneity and rarity of this disease means that diagnosis is often delayed in patients, and broad-spectrum untargeted treatments such as immunosuppressive agents and corticosteroids are typically prescribed (Petri, 2005). Furthermore, a diagnosis can become complicated when patients have manifestations that are characteristic of multiple diseases (e.g. patients with lupus, rheumatoid arthritis (RA) and Sjogren's syndrome (SS) can all experience joint pain, swelling and stiffness), or when patients have a combination of manifestations that can't be ascribed to any one autoimmune disease. We also often see families with different individuals affected by different autoimmune diseases, suggesting that there is a shared underlying predisposition that has caused different manifestations.

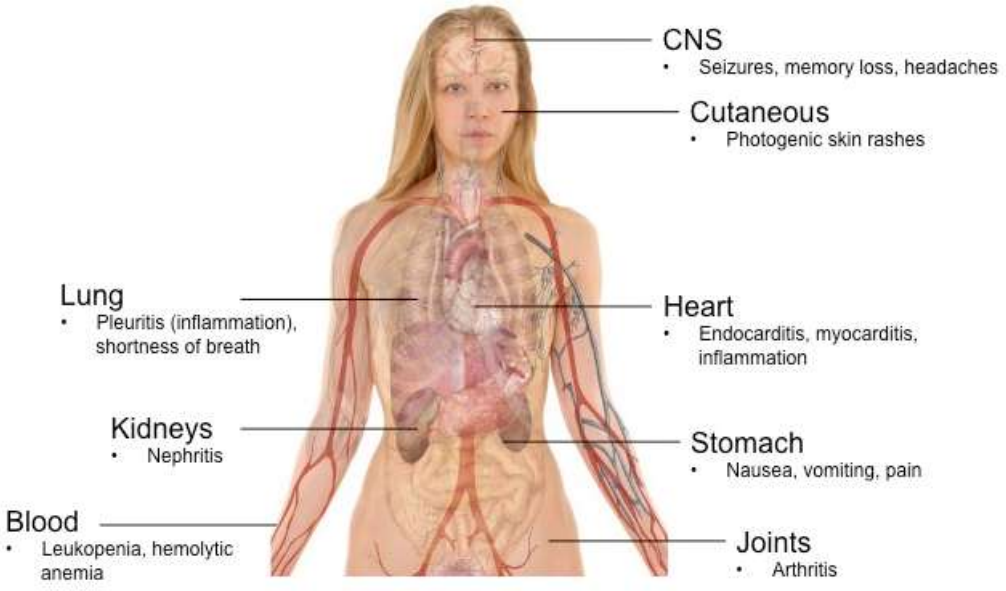


Figure 1.5. Organs affected by systemic lupus erythematosus (adapted from (Kaul et al., 2016)).

Lupus affects 1 in 700 Australians, and annual incidence rates worldwide range from 1 to 10 per 100, 000 (Jarukitsopa et al., 2015) with nine out of every ten people with lupus being women between the ages of 20 and 50 years. Race and gender have been shown to be factors that predispose to SLE and influence disease severity (Vilar and Sato, 2002, McCarty et al., 2003). Historically, the five year survival rate was below 50%, however advances in diagnosis and treatment have improved the survival rate to 95% (Borchers et al., 2004), although countries with lower socio-economic status have lower survival rates, likely due to poorer health services, education and resources (Burgos et al., 2011, Ugarte-Gil et al., 2016). Furthermore, American patients of African descent with lupus have 3 times higher mortality, and patients of Asian descent have a 1.8-2.7 times higher mortality, compared with Americans of European descent, demonstrating a higher mortality risk observed between races that cannot be accounted by access to health services (Kaslow, 1982).

Since the American College of Rheumatology's criteria were developed, there have been significant advances in the treatment of SLE. Anti-proliferative immunosuppressants and immunomodulators such as azathioprine, mycophenolate and hydroxychloroquine have led to significant improvements in mortality, however these immunosuppressive agents are non-specific and often cause serious side-effects. Furthermore, monoclonal antibodies directed against specific epitopes, such as NAFF, CD20 or TNF, promised greater precision in therapy, however the most promising agents in murine trials have been found to not be as effective in human trials. This is likely due to both the heterogeneity of disease between patients, and due to the complex pathophysiology within a patient. Therefore, it is possible that monoclonal antibodies that are directed against specific epitopes may be more effective if we

categorise patients in terms of the molecular pathways that are dysregulated, and then target these pathways.

1.5.4 Pathophysiology of systemic lupus erythematosus

SLE has classically been considered a disease characterised by a breakdown of B cell self-tolerance and the production of auto-antibodies, however almost all components of the innate and adaptive immune systems have been implicated in the development of SLE. Abnormalities in B cell and T cell biology, the complement system, the clearance of apoptotic debris, phagocytosis, autophagy, NETosis, DNA repair, post-translational modifications, human leukocyte antigen (HLA), and cytokine signalling have all been attributed to SLE pathogenesis, however many of these factors may ultimately lead to the break down in B cell tolerance.

The complement system has been implicated in SLE pathogenesis, in both preventing and exacerbating the disease (Walport, 2002a, Leffler et al., 2014). On one hand, hereditary homozygous deficiency of the initiator of the classical pathway, C1q, has been shown to strongly predispose to SLE, and deficiencies or variants in the other components of the classical pathway (C1r, C1s, C4 and C4) also increase the risk, suggesting that complement is protective against SLE. On the other hand, the characteristic finding of colocalization of IgG, IgA and IgM with C1q, C4 and C3 in the glomeruli of patients with lupus nephritis (known as the “full-house” pattern) of immune complex deposition is a characteristic finding in SLE nephritis and is the consequence of complement activation. Furthermore, complement has been shown to play important roles in thrombotic complications associated with SLE, with

antiphospholipid autoantibody-containing immune complexes binding to platelets which activate the classical complement pathway.

The characteristic feature of SLE is the production of auto-antibodies against nuclear complexes, and this may be due to abnormal B cell activation and/or a break down in B cell tolerance. As mentioned previously, B cells are subjected to several tolerance checkpoints, both central tolerance checkpoints during B cell development, and peripheral tolerance checkpoints after B cell development, and defects in any of these checkpoints could predispose to autoimmunity. In SLE, it is unclear what leads to this breakdown in tolerance checkpoints, however there is strong evidence that supports the role of both BCR and TLR signalling. For example, genetic disruption in BCR signalling genes such as *PTPN22* and *LYN* leads to an expansion of autoreactive B cells, and many of the genes that have been associated with SLE by GWAS are B cell genes, such as BANK1 and BLK (Deng and Tsao, 2010). Furthermore, SLE patients possess abnormalities in B cell populations, such as expanded plasmablasts which are capable of producing anti-DNA antibodies, and that circulating plasma cells in patients with active disease express highly mutated Ig genes with variants consistent with GC hypermutation (Grammer and Lipsky, 2003).

SLE has been classically been considered a B cell mediated disease, however there is growing evidence that implicated T cells in disease pathogenesis by amplifying inflammation through pro-inflammatory cytokine secretion and helping B cells generate autoantibodies, and the production of autoreactive memory T cells. There are a number of mouse models that have demonstrated an important role for T cells or T cell-derived molecules in SLE pathogenesis, for example in the NZB/BXSB F1 mice which develop anti—phospholipid syndrome (APL) and

proliferative glomerulonephritis, depletion of $CD4^+$ T cells ameliorates disease (Adachi et al., 1998). Furthermore, T cell abnormalities in human patients with SLE have been well described, however it is hard to conclude whether they are a cause or consequence of the disease. Patients with SLE demonstrate abnormalities in effector, memory and regulatory T cell subtypes. For example, CD8 T cells in SLE have dampened cytotoxic function that can lead to an increased risk of infection, which may also trigger autoimmunity (Gravano and Hoyer, 2013). Double negative (DN) T cells, which are TCR$\alpha\beta^+CD4^-CD8^-$, are thought to result from inactivation or exhaustion of autoreactive or continuously stimulated CD8 T cells, and are related to pathogenesis and kidney disease in SLE patients, where they accumulate and produce IL-17 (Crispin et al., 2008). Accumulation in extra-follicular T helper cells has been described in the peripheral blood of SLE patients, which may explain increased B cell activation and antibody production (Wang et al., 2014b, Choi et al., 2015, Xu et al., 2015, Zhang et al., 2015), and an accumulation of effector/memory T cells has been described in patients with SLE which are resistant to activation-induced cell death. Studies have also demonstrated a dysregulation in immune response control by T_{regs} in SLE patients, with reports demonstrating that patients with active disease have reduced numbers of T_{regs} compared to healthy individuals (Valencia et al., 2007, Lyssuk et al., 2007, Barath et al., 2007) with impaired function (Yates et al., 2008, Venigalla et al., 2008).

Several cytokines have been strongly associated with SLE, including type 1 interferon, IL-1, IL-2, IL-6, IL-17, IL-21, IL-23, TNF, B cell activating factor (BAFF) and IFNγ. Type 1 interferons include IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω, which are capable of binding the common receptor IFNAR (Platanias, 2005). IFN-α is considered to be a key IFN in lupus pathogenesis and is often elevated in the serum of patients with SLE (Banchereau and Pascual, 2006). It is

produced by many different leukocytes but it is plasmacytoid dendritic cells (pDCs) that produce it in large quantities (Honda et al., 2005). IFN$\boldsymbol{\gamma}$, a type II interferon, has also been found in increased levels in SLE patients, and is associated with disease activity (Frieri, 2012). It may be involved in lupus pathogenesis through its induction of BAFF production (Davis et al., 2011, Iwata et al., 2011). Absence of IL-2 in *IL-2*$^{-/-}$ mice results in lymphoid hyperplasia and inflammatory bowel disease, and ex-vivo activated T cells in patients with SLE produce decreased amounts of IL-2 (Linker-Israeli et al., 1983). IL-6 is important for stimulating B cell proliferation and differentiation into plasma cells, escalating antibody production from plasma cells, and also promoting T cell differentiation including T_{reg} differentiation (Yap and Lai, 2013, Davis et al., 2011). IL-6 has also been strongly associated with B cell hyperactivity and immunopathology, with SLE patients producing more IL-6 and the amount of IL-6 correlating with disease activity (Jones et al., 1999). Additionally, IL-10 has also been found to be elevated in human and murine SLE, and is also related to disease activity, stimulating B cell proliferation and plasma cell differentiation, and decreasing apoptosis of autoreactive B cells in the germinal centre (Davis et al., 2011, Karageorgas et al., 2011, Vincent et al., 2014). Finally, IL-21 which is mainly produced by T_{FH} cells is also present at increased levels in SLE patients and is thought to trigger antibody production in auto-reactive B cells.

SLE is characterised by the production of autoantibodies directed against nuclear antigens, particularly DNA, with anti-DNA antibodies correlating with disease activity in patients (Arbuckle et al., 2003, ter Borg et al., 1990). It is thought that this results in disease, at least in part through deposition of antigen-antibody complexes in tissue, which leads to activation of complement and inflammatory lymphocytes, and subsequent inflammation and organ damage. Antibodies forming immune complexes can also activate pDCs to produce type I IFN,

which when in excess cause inflammation and induce further activation and differentiation (Ronnblom and Pascual, 2008, Ronnblom et al., 2006). Polymorphisms in DNA processing genes such as *TREX1* results in monogenic lupus, with excessive DNA and type I IFN production, overwhelming tolerance mechanisms in the immune system (Ellyard et al., 2014).

It is evident that SLE is considerably heterogenous in terms of both clinical symptoms and pathophysiology, and that a number of mechanisms have been implicated. It is believed that anti-nuclear antibody (ANA) to self-DNA or RNA, produced by self-reactive B cells, and activation of complement leads to immune complex deposition and pro-inflammatory cytokine production, which causes the severe organ damage we see in SLE patients (Yu et al., 2014). The exact mechanisms that lead to this loss of self-tolerance are unknown, however it is believed that a combination of genetic, epigenetic, environmental, hormonal and immunoregulatory factors (Tsokos, 2011) causes defects in B cells (Fields et al., 2005), T cells (Perl et al., 2009), complement activation (Walport, 2002b) and/or cytokine signalling (Lourenco and La Cava, 2009), and DNA processing. Out of all of these factors, genetics is considered to play a substantial role in lupus pathogenesis and severity.

1.5.4 The genetics of systemic lupus erythematosus and next generation sequencing

The human genome is based on sequences of DNA purines, *guanine (G)* and *cytosine (C)*, and pyrimidines, *adenine (A)* and *thymine (T),* which is replaced by *uracil (U)* in ribonucleic acid (RNA), and are bound to a backbone of alternating phosphate and sugar residues. These strands are paired through complementary base paring, with adenines binding to thymine (or uracil) and guanines binding to cytosines through hydrogen bonds, making up over three

billion bases of DNA encoding 19,000 genes in the human genome (Ezkurdia et al., 2014). The human genome is divided into two major components: coding (exons) and non-coding (introns and intergenic regions). Non coding regions make up 98.5% of the human genome yet remain poorly understood and are believed to contain regulatory regions such as gene promoters and enhancers. The remaining 1.5% of the human genome consists of exons, which encodes a part of the final mature RNA of a gene, including untranslated regions of mRNA, after introns have been removed by RNA splicing.

The chemical structure of DNA makes it prone to the occurrence of variants, which can range from chromosome segment deletions, inversions or translocations (Jacobs et al., 1992), to copy number variations (CNVs), which are insertions or deletions of genomic material greater than 1000 base pairs, to single nucleotide variants (SNVs). SNVs are single base-pair changes in DNA sequence that can occur anywhere within the genome. When a SNV occurs within an exon, if it is non-synonymous (the base change alters the amino acid) it can have a profound effect on the structure and function of a protein. SNVs typically have two possible allele variants (there are two commonly occurring base-pair possibilities at a genetic position, one in each chromosome) and the frequency of a SNV is described as *minor allele frequency (MAF),* or the frequency of the less common allele. For example, a MAF of 0.4 estimates that 40% of the population carries one nucleotide, and 60% of the population carries the other base type (known as the major allele). It is estimated that there are over 10 million common (minor allele frequency >1%) SNVs in the genome (Akey et al., 2004), however only a small number of these result in phenotypic differences despite non-synonymous variation accounting for approximately half of the known genetic lesions responsible for inherited human diseases (Krawczak et al., 2000).

Monozygotic twin studies have shown a 24-75% concordance rate of lupus (Farh et al., 2015), suggesting that genetic susceptibility alone is sufficient to trigger disease in some individuals. Identifying susceptibility genes has proven difficult, however it is becoming increasingly easier with advances in technology, and there is now substantial evidence to support the role of genetics in the pathogenesis of autoimmune diseases (Ceccarelli et al., 2016, Mariani, 2004, Ramos et al., 2015). Genome wide association studies (GWAS) are a method for identifying genes involved in disease, searching the genome for SNVs that occur more frequently in people with particular diseases. These advances have provided substantial evidence to support the role of genetics in the pathogenesis of autoimmune diseases (Ceccarelli et al., 2016, Mariani, 2004, Ramos et al., 2015), and have identified a number of lupus susceptibility genes including complement genes (International Consortium for Systemic Lupus Erythematosus et al., 2008, Hom et al., 2008a, Nath et al., 2008), TLR genes (Wang et al., 2014a) and genes involved in B cell signalling such as BANK1 (Chang et al., 2009). GWAS identify common loci and variants (frequency greater than 5%) and environmental factors that are involved in disease risk on a global scale (McCarthy and Hirschhorn, 2008). Whilst this technology has rapidly identified SNVs related to several complex conditions and has advanced our understanding of genetically complex diseases, a large fraction of genetic heritability remains unaccounted for in most complex diseases as the SNVs only account for a small proportion of the genetic component to disease as they are mostly non-coding, and often do not alter protein structure or protein function. It is estimated that in lupus these GWAS susceptibility genes and loci only account for approximately 15% heritability (Thompson et al., 2005, International Consortium for Systemic Lupus Erythematosus et al., 2008).

Some explanations that may explain this missing heritability include the inability to detect gene-gene interactions, insertions, deletions and copy number variations such as inversions and translocations (Manolio et al., 2009). Genome wide association studies (GWAS) are based on the polygenic hypothesis (many variants with weak effects cause disease), however increasing evidence also suggests that this missing heritability may in great part be explained by rare or novel gene variants (frequency less than 1%) (Figure 1.6) (Gunther et al., 2009), specifically one (monogenic) or a few (oligogenic) rare and novel damaging variants contribute to disease pathogenesis (Belot and Cimaz, 2012, Lo, 2016b). There has been an increasing number of monogenic cases of lupus being described in literature, examples include: C1q (Bryan and Wu, 2014), TREX1 (Ellyard et al., 2014, Rice et al., 2007), ACP5 (Briggs et al., 2011, An et al., 2017), DNASE1 (Napirei et al., 2000), DNASE1L3 (Al-Mayouf et al., 2011), PKCD (Belot et al., 2013). Table 1.2, extracted from a review article written by Mindy S. Lo, 2016 (Lo, 2016a), lists all the monogenic conditions that are known to cause lupus and lupus-like diseases.

Whole exome sequencing (WES) and whole genome sequencing (WGS) are relatively recent advances in sequencing techniques that have allowed us to identify these novel and rare gene variants through greater resolution and examination of the human genome. Furthermore, rare, novel and de novo variants that are highly deleterious have already been shown to contribute to many common and/or complex diseases such as epilepsy, autism, schizophrenia, mental retardation and amyotrophic lateral sclerosis (ALS) (Camacho-Garcia et al., 2013, Vance et al., 2009, Girard et al., 2015). These studies demonstrate the power and effectiveness of whole exome and genome sequencing and supports the hypothesis that a

small number of rare and highly deleterious variants can contribute to complex immune phenotypes and pathogenic pathways. WGS has now been used in the identification of rare variants causing or contributing to SLE, and it has led to effective diagnoses. Our team identified TREX1 as causative of childhood-onset SLE with stroke (Ellyard et al., 2014), and is now being utilised to identify other putative monogenic SLE causes (Pullabhatla et al., 2018)

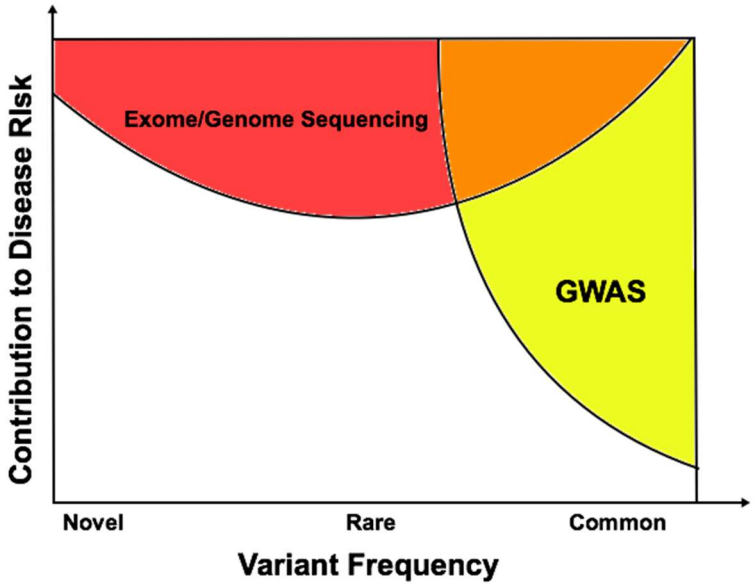


Figure 1.6. Identifying the genetic cause of disease. Genome Wide Association Studies (GWAS) have primarily identified common variants that may contribute to disease risk. Recent developments in exome and genome sequencing have enabled the identification of rare and novel variants that have strong effects and contributions to disease risk. Adapted from Singleton et al., 2010 (Singleton et al., 2010).

With the costs of sequencing whole exomes and genomes declining, WES and WGS will continue to become an attractive alternative to GWAS, however in comparison with the issues of inadequate detection faced by GWAS, WES and WGS will face the inverse problem of generating vast amounts of genomic data that needs to be analysed. This analysis requires several methods and computational programs to predict whether a SNV may affect protein function, most of which rely on conservation or chemical change to predict the effect of the

variant. Commonly used programs include Sorting Intolerant from Tolerant (SIFT), which relies heavily on if a specific type of amino acid (e.g. hydrophobic) residue is conserved and sequence conservation, Polymorphism Phenotyping2 (Polyphen2), which is also dependent on sequence conservation but takes into account domains, function and 3D structure, and Combined Annotation Dependent Depletion (CADD) tools which integrates multiple annotations including conservation and functional information. These tools however have significant limitations, especially as they are based on known pathogenic variants and thus are subject to ascertainment biases (Grimm et al., 2016), and that they only deal with coding variants and do not consider the majority (>99%) of genetic variation (Flanagan et al., 2010). Furthermore, these tools are less effective at predicting gain-of-function variants, with SIFT and polyphen2 having poor sensitivity (69% and 68% respectively), and poor specificity (13% and 16% respectively). Whilst CADD was thought to bring significant improvements, it was also shown to be a poor predictor of variant effect (Miosge et al., 2015).

Table 1.2. Established genes implicated in SLE (adapted from Ramos et al., 2011) (Ramos et al., 2010).

Gene	Gene Location	Protein	Pathway	Phenotype
ACP5	19p13.2	TRAP	Nucleic acid sensing and degradation, Type I IFN	SPENCD, SLE
ADAR1	1q21.3	ADAR1	Type I IFN	AGS, SLE
C1Q	1p36.12	C1q	Complement	Complement deficiencies SLE
C1R	12p13.31	C1r	Complement	Complement deficiencies SLE
C1S	12p13.31	C1s	Complement	Complement deficiencies SLE
C2	6p21.33	C2	Complement	Complement deficiencies SLE
C4A	6p21.33	C4	Complement	Complement deficiencies SLE
C4B	6p21.33	C4	Complement	Complement deficiencies SLE
CYBB	Xp21.1-p11.4	NADPH oxidase 2	Phagocytosis	Chronic granulomatous disease
DNASE1	16p13.3	DNASE1	Nucleic acid sensing and degradation	SLE
DNASE1L3	3p14.3	DNASE1L3	Nucleic acid sensing and degradation	SLE
FAS or RNFRSF6	10q23.31	FAS	Apoptosis	ALPS
FASL	1q24.3	FASL	Apoptosis	ALPS
HLADR2	6p21.32	HLADR2		
IFIH1	2q24.2	IFIH1	Type I IFN	AGS, SLE
ISG15	1p36.33	ISG15	Type I IFN	AGS
KRAS	12p12.1	KRAS	RAS-MAPK signalling	Noonan syndrome
LAMAN or MAN2B1	19p13.13	LAMAN	Metabolism of carbohydrates	Alpha-mannosidosis
PEPD	19q13.11	PEPD	Aminopeptidase activity	Prolidase deficiency
PRKCD	3p21.1	PRKCD	Self-tolerance	SLE
PSMA3	14q23.1	PSMA3	Proteasome	CANDLE
PSMB4	1q21.3	PSMB4	Proteasome	CANDLE
PSMB8	6p21.32	PSMB8	Proteasome	CANDLE
PTPN11	12q24.13	PTPN11	RAS-MAPK signalling	Noonan syndrome
RAG2	11p12	RAG2	Self-tolerance	SLE
RNASEH2A	19p13.13	RNASEH2A	Nucleic acid sensing and degradation	AGS
RNASEH2B	13q14.3	RNASEH2B	Nucleic acid sensing and degradation	AGS
RNASEH2C	11q13.1	RNASEH2C	Nucleic acid sensing and degradation	AGS
SAMHD1	20q11.23	SAMHD1	Type I IFN	AGS, SLE, FCL
SHOC2	10q25.2	SHOC2	RAS-MAPK signalling	Noonan syndrome
SLC7A7	14q11.2	SLC7A7	Amino acid transporter	Lysinuric protein intolerance
TMEM173	5q31.2	STING	Type I IFN	SAVI
TREX1	3p21.31	TREX1	Nucleic acid sensing and degradation	AGS, FCL

The monogenic versus polygenic hypothesis has led to lively debates in literature between strongly held viewpoints. As lupus is a complex disease with no clear pattern of Mendelian inheritance, it is plausible that lupus could be explained by monogenic, oligogenic and polygenic causes in different individuals/families. In the cases that are monogenic or oligogenic, we can use whole genome sequencing to identify rare or novel deleterious variants, enabling us to identify the mechanisms leading to the breakdown in tolerance and development of autoimmunity.

We can validate these possible disease-causing candidate genes using DNA editing tools such as CRISPR/Cas9 (Platt et al., 2014). This is a sophisticated way to analyse disease heterogeneity and discover the genetic causes of disease. Using this method, we can categorise patients based on the underlying pathways involved in disease pathogenesis, with the aim of testing or designing therapies that are targeted to this underlying pathway. This is necessary, as the therapies we currently have to treat these autoimmune diseases are untargeted and are based on broad immune-suppression, and many do not succeed in clinical trials and fail to treat the most severe disease manifestations. This means that patients often require a trial-and-error method of evaluating the benefit of these drugs that in the long term may be of more harm than benefit.

Our lab is testing a reverse approach, by which we uncover possible disease-causing gene variants and then introduce them into mice using CRISPR/Cas9 technology to determine if and how the variant causes disease. We can then use these mice to test or develop therapies that are targeted to the underlying causative pathway of disease in these patients, or even allow for prevention or early detection of disease in other family members. Using this

approach, we have recently uncovered numerous rare and low frequency coding variants in *BLK* and *BANK1* that occur in a significant fraction of SLE patients and are likely to contribute to lupus pathogenesis through impaired repression of IRF5 and type I IFN (Jiang et al., 2019, Nature Communications – In press).

1.6 Hypothesis and aims of this thesis

Cytokines are essential communication molecules in the immune system, playing a role in the recruitment, function, survival, proliferation and suppression of leukocytes, and their role in the pathogenesis of autoimmune diseases has been extensively studied (Falcone and Sarvetnick, 1999, Santamaria, 2003). The common thread of this thesis is the potential regulatory and pathogenic roles of human T cell cytokines IL-32 and IL-2 respectively in the control or pathogenesis of B cell responses and autoimmunity.

The first project concerns a multiplex family affected by SLE. Whole genome sequencing identified a heterozygous SNV in an RNA binding protein called ZFR2, whose role in the immune system has not been identified, although paralogs of this protein have been shown to regulate the gene expression of multiple cytokines. The aim of this study was to elucidate the role of ZFR2 in the immune system, and how single nucleotide variants identified in this gene could be contributing to autoimmune disease pathogenesis. Paralogs of ZFR2 play important roles in regulating expression of IL-2 and SLE-promoting cytokines such as IL-6 and IL-10. We therefore we hypothesised that ZFR2 plays an important role in the immune system, regulating gene expression of proinflammatory cytokines, and that variants in ZFR2 may lead to a dysregulation in proinflammatory cytokine gene expression and production.

The second project investigates the role of interleukin-32 (IL-32), a recently descrived cytokine that we found to be highly expressed in a subset of human germinal centre T cells that appear to be related to T_{FR} cells. The function of human T_{FR} cells remains poorly understood, and thus the aim of this project was to elucidate the role of T_{FR}-derived human IL-32 in B cell responses. We hypothesise that this cytokine plays an important role in the germinal centre reaction, controlling germinal centre derived output and autoreactivity.

Chapter Two: Materials and Methods

2.1 Identification of candidate gene variants

2.1.1 APOSLE samples and DNA sequencing

Written informed consent was obtained as part of the Australian Point Variant in Systemic Lupus Erythematosus (APOSLE) study and the Centre for Personalised Immunology (CPI) study, which recruit patients with autoimmune disorders within Australia and internationally. The diagnosis of lupus for these patients was made by the treating physician (Professor David Fulcher for Family A). The study was approved by the Australian National University and the ACT Health Human Ethics Committees. Saliva was collected in Oragene^{TM} DNA self-collection kits and purified using PrepIT^{TM} DNA purification kits (Oragene) and treated with RNAse out (Ribonuclease A, Qiagen Catalogue Number 19101). DNA samples were enriched with Human SureSelect ST2 All Exon V4 Kit and sequenced by Illumina HiSeq 2000 (Illumina, Inc.) with 90 base pair (bp) paired-end reads. Raw image files were processed by Illumina base calling Software 1.7. Bioinformatics analysis was performed by Dr Thomas Daniel (Dan) Andrews, Dr Matthew Field, and Dr Vicky (Eun Chung) Cho, members of the Genome Informatics group at JCSMR, ANU. Raw sequence reads were aligned to the reference genome (Hg19) and SNVs, insertions and deletions were called using SAMTools. SNVs of interest were confirmed by PCR (using the Phusion Hot Start DNA Polymerase Ii (Finnzymes) according to the manufacturer's instructions) and Sanger sequencing (performed at the ACRF Biomolecular Resource Facility, The John Curtin School of Medical Research, Australian National University).

2.1.2 Identification of variants

To identify candidate gene variants that may be contributing to disease, the variants identified by whole exome/genome sequencing were analysed using a filtering scoring system

devised by the Vinuesa laboratory. The aim of this strategy was to isolate rare and deleterious variants predicted to be playing a role in disease pathogenesis in the affected individuals. Moderate or high frequency variants (frequency greater than 2%) and the synonymous variants (silent variants) were excluded, leaving the low frequency non-synonymous, nonsense or splice site disrupting variants (frequency less than 2%). Refer to Figure 2.1 for an overview of the scoring system. Bioinformatic resources used for this analysis are outlined in Table 2.2.

One point was given if the gene variant was uncommon (dbSNP 131) and one point was given if the variant was rare (dbSNP 131). If the gene variant was novel (dbSNP 131), a total of two points were given. One point was given if the SIFT was less than 0.05 or the PolyPhen2 was greater than 0.7 (Table 2.1), and then another point was given if the CADD score was greater than 10 (see table). One point was given if the gene ontology terms (obtained from databases on gene and protein characterisation (UniProt, Ensembl and PhosphoSitePlus) and human phenotypes (Omim and PubMed)) contained immune keywords (e.g. dendritic, myeloid, macrophage), and from here variants with two points or less were eliminated, leaving variants with three points or more. Two points were then awarded if the mouse knockout phenotype (from the Mouse Genome Informatics) displayed an immune phenotype, or one point was awarded if the phenotype had not yet been investigated. Gene variants with four points or less were eliminated, leaving variants with five points or more.

Variants predicted to be damaging were individually evaluated for their expression in immunological tissues (using ImmGen and BioGPS), with those not expressed in immunological tissues given lower priority. Homozygous gene variants, nonsense variants,

splice site variants, insertions and deletions, and variants previously associated with autoimmune disorders were individually selected from the data and analysed separately. Homozygous and nonsense Splice site variants were analysed using Alternative Splice Site Predictor (ASSP) software (Wang and Marin, 2006), to predict the effect of the variant.

Gene variants that were present in three out of four and four out of four of the affected individuals were selected for further analysis. From here, the bioinformatic alignments were analysed using Integrative Genomics Viewer (IGV) (version 2.3.40) (Broad Institute) to investigate coverage of the region and ensure variants were not missed due to poor coverage. Finally, each of the candidate variants were run through a collation of exome and genome data from healthy controls and patients with a variety of immune disorders including SLE (Centre of Personalised Immunology, JCSMR. 2015) to investigate whether variants in those candidate genes were present in a larger cohort.

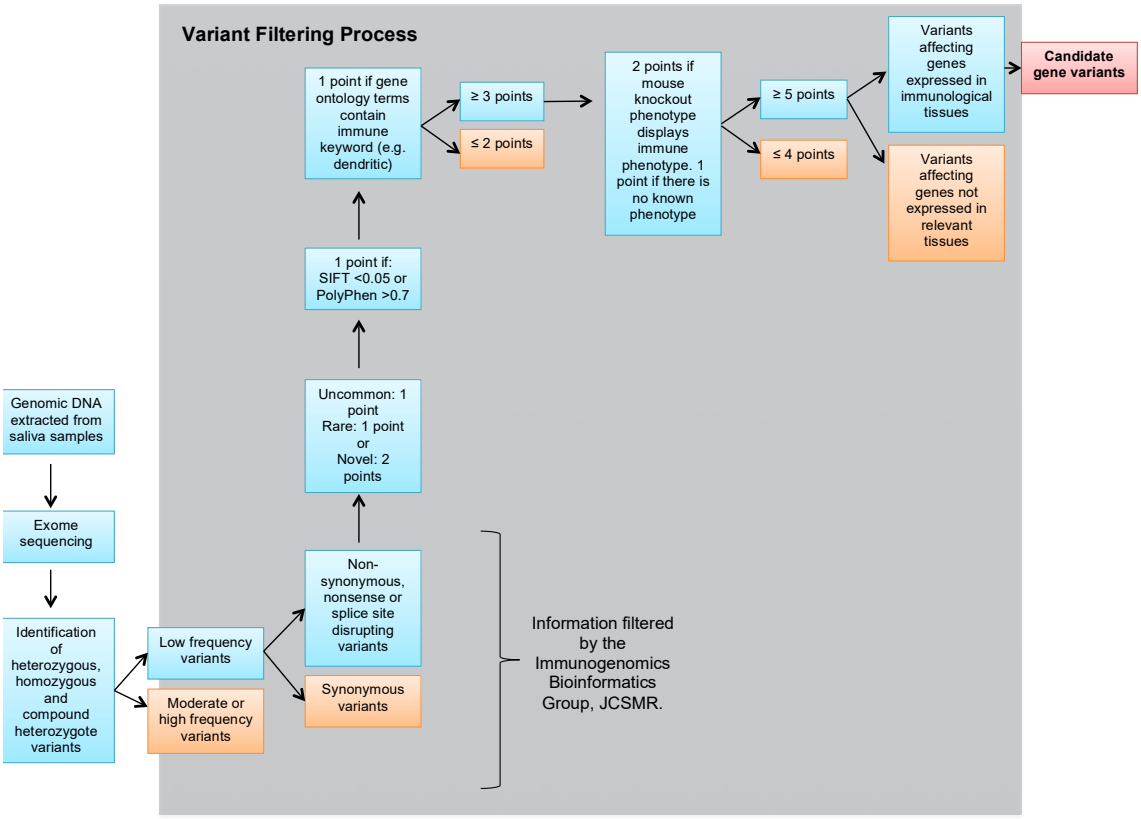
Table 2.1. Summary of the tools used to predict the effect of a variant

PolyPhen-2	Predicts the effect of an amino acid substitution based on the structure and function of a protein using sequence homology, protein family annotations, three-dimensional structures and other databases and tools. Scores greater than 0.7 are predicted to be damaging.
SIFT	Prediction of whether an amino acid substitution is likely to affect protein function based on sequence homology and the physiochemical similarity between the alternate amino acids. Scores less than 0.05 are predicted to be damaging.
CADD	Integrates information from many annotations, including SIFT and PolyPhen2, and condenses this information into a single score.

Table 2.2. Bioinformatic resources used in analysis of exome sequencing data

Database of Single Nucleotide Polymorphisms (dbSNP)	http://www.ncbi.nlm.nih.gov/SNP/
Ensemble Genome Browser	http://www.ensembl.org/index.html
ImmGen	http://www.immgen.org/
BioGPS	http://biogps.org/#goto=welcome
The Exome Aggregation Consortium (ExAC)	http://exac.broadinstitute.org/
Gene Ontology Consortium (GOC)	http://geneontology.org/
Mouse Genome Informatics (MGI)	http://www.informatics.jax.org/
PolyPhen-2 (version 2.2.2)	http://genetics.bwh.harvard.edu/pph2/
SIFT	http://sift.jcvi.org/
CADD	https://cadd.gs.washington.edu/
Online Mendelian Inheritance in Man (OMIM)	http://www.omim.org/
Universal Protein Resource (UniProt)	http://www.uniprot.org/
BioGrid	https://www.biogrid.org.au/
IntAct	http://www.ebi.ac.uk/intact/
Search Tool for the Retrieval of Interacting Genes/Proteins (STRING)	http://string-db.org/

Figure 2.1. Variant filtering scoring system



2.2 Cell isolation, culture and stimulation

2.2.1 Purification of PBMCs and isolation of plasma

Peripheral blood mononuclear cells (PMBCs) (lymphocytes and monocytes) were purified by density gradient centrifugation using Ficoll-Hypaque Plus (GE Healthcare, Life Sciences). Cells were resuspended in FBS 10% DMSO to yield a concentration of 4 million cells/mL. PBMCs and plasma were stored in CryoTubes (Thermo Fisher Scientific) at -80^{o}C.

2.2.2 Human tonsil collections

Human tonsils were obtained from children undergoing routine tonsillectomy at The Canberra Hospital and Calvary John James Hospital. A single cell suspension was obtained by mechanical disruption of the tissue followed by cell separation using Ficoll-Hypaque (GE

Healthcare Life Sciences) gradient. Informed consent was obtained from all subjects. All experiments with human samples were approved by the Australian National University's Human Experimentation Ethics Committee and the University Hospitals Institutional Review Board. All tissue samples used for histology were retrieved from the paraffin archives of ACT Pathology, The Canberra Hospital, and were used according to the ACT Health Human Research Ethics Committee's Low Risk Sub-Committee (LRSC) guidelines.

2.3 Flow cytometry analyses and cell sorting

2.3.1 Flow cytometry and cell sorting

Human tonsillar lymphocytes, PBMCs or mouse lymphocytes (following red blood cell lysis) were stained with the antibodies listed in Table 2.3. All surface stains were performed in the presence of Human Fc Block (cat. 564219, BD Bioscience) for human samples, or Mouse TruStain FcX (cat. 101320, BioLegend) for mouse samples and BD Horizon Brilliant Stain Buffer (563794, BD Biosciences) if brilliant violet conjugated antibodies were used in the stain. Intracellular staining was performed using the FOXP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions for intracellular transcription factors, or BD Cytofix/Cytoperm (BD Biosciences) for intracellular cytokine staining. Cells were stained with antibodies for 30 minutes at 4^{o}C. Data was collected on BD LSRFortessa^{TM} X-20 (BD Biosciences) and analysed with FlowJo software (TreeStar), or cells were sorted on BD FACS Aria I or II (BD Biosciences). 7-AAD (Invitrogen), Zombie Aqua (BioLegend), LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Life Technologies) or Fixable Viability Dye eFluor^{TM} 780 (eBioscience) to exclude dead cells in sorting or analysis.

2.3.2 Flow cytometry antibodies

Table 2.3. Anti-human antibodies used for flow cytometry and cell sorting

Antibody	Clone	Conjugation	Source
CD14	MϕP9	PerCP	BD Biosciences
CD56	NCAM16.2	PECy7	BD Biosciences
CXCR5	RF8B2	Alexa Fluor 647	BD Biosciences
IgG	G18-145	PECy7	BD Biosciences
CD21	B-LY4	APC	BD Biosciences
CD4	RPA-T4	PerCpCy5.5	BD Biosciences
CD123	7G3	PE	BD Biosciences
CD11c	B-LY6	APC	BD Biosciences
CD16	3G8	APC-H7	BD Biosciences
CD38	HIT2	PerCpCy5.5	BD Biosciences
CD3	UCHT1	Pacific Blue	BD Biosciences
CD10	HI10a	PE-CF594	BD Biosciences
CD45RA	HI100	PeCy7	eBioscience
CD8	SK1	APC-eFluor 780	eBioscience
CD27	O323	APC-eFluor 780	eBioscience
IgM	SA-DA4	eFluor 450	eBioscience
CD279 (PD-L1)	J105	Biotin	eBioscience
Streptavidin	-	APC-eFluor 780	eBioscience
CCR7	G043H7	Alexa Fluor 488	BioLegend
CCR6	G034E3	Brilliant Violet 510	BioLegend

CXCR3	G025H7	PE	BioLegend
CD127	AO19D5	Brilliant Violet 650	BioLegend
CD25	BC96	Brilliant Violet 650	BioLegend
HLA-DR	L243	Brilliant Violet 510	BioLegend
IgD	IA6-2	Brilliant Violet 510	BioLegend
CD24	MLS	Brilliant Violet 605	BioLegend
CD19	HIB19	Brilliant Violet 605	BioLegend
CD57	HNK-1	PE/Dazzle	BioLegend
IgA	IS11-8E10	PE	Miltenyi Biotec
CD38	LS198-4-3	APC	Beckman Coulter
CD3	OKT3	Alexa Fluor 700	eBioscience
CD19	HIB19	Brilliant Violet 605	BioLegend
CD4	GK1.5	APCCy7	BioLegend
CD57	NK-1	PE-CF594	BD Biosciences
Granzyme B	NGZB	PE	eBioscience
IFN-γ	4S.B3	PECy7	BioLegend
IL-2	JES6-5H4	FITC	eBioscience
CD4	RPA-T4	APCCy7	BD Biosciences
CD8	RPA-T8	FITC	BD Biosciences
CD8	SK1	PE	BD Biosciences
CD19	SJ23C1	FITC	BioLegend
CD19	SJ25C1	PECy7	BD Biosciences
CXCR5	RF8B2	Alexa Fluor 488	BD Biosciences
CXCR5	J252D4	PerCPCy5.5	BD Biosciences

CD45RA	H100	PE	BioLegend
CD45RO	UCHL1	PE	BioLegend
CTLA-4	BNI3	PE	BD Biosciences
CTLA-4	L3D10	PECy7	BioLegend
PD-1	MIH4	PE	eBioscience
PD-1	EH12.2H7	BV605	BioLegend
PD-1	J105	BV421	eBioscience
CD127	11-1278	FITC	eBioscience
CD127	A019D5	BV421	BioLegend
CD25	BC96	Biotin	BioLegend
CD25	BC96	PECy7	BioLegend
CD25	2A3	APC	BD Biosciences
CD127	A019D5	FITC	eBioscience
CD127	A019D5	BV421	BioLegend
CD127	HIL-7R-M21	BV510	BD Biosciences
FoxP3	A259D	Alexa 647	eBioscience
CD40L	24-31	Pacific Blue	BioLegend
BCL6	K112-91	Alexa Fluor 647	BD Biosciences
BCL6	K112-91	PECy7	BD Biosciences
IL-10	JES3-19F1	PE	BioLegend
IL-21	3A3-N21	PE	BD Biosciences
CD3	UCHT1	FITC	BD Biosciences
CD3	UCHT1	Alexa Fluor 700	BD Biosciences
CD27	N-T271	FITC	BD Biosciences

CD27	N-T271	APC	BD Biosciences
CD38	HIT2	FITC	BD Biosciences
CD38	HB7	PE	BD Biosciences
LAG3	2319-L3-050	PE	R&D Systems
LAG3	2319-L3-050	FITC	R&D Systems
PD-L1	10F.9G2	BV605	BioLegend
PD-L1	10F.9G2	APC	BioLegend
PD-L1	10F.9G2	Biotin	BioLegend
PD-L1	29E.2AE	BV605	BioLegend

2.3.3 Analysis of cytokine secretion

Cytokine secretion in human plasma, mouse serum or culture supernatants following stimulation were analysed by BD™ Cytometric Bead Array (CBA), using either the Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences), the Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences) or the Human Inflammatory Cytokine Kit (BD Biosciences) respectively, according to the manufacturer's recommendations (and at time points indicated if relevant). Analysis was performed using the BD™ Cytometric Bead Array (CBA) FCAP Software v3.0, and data was presented as either total MFI or relative to unstimulated. Each assay was performed with a control sample to determine the limit of detection.

2.3.4 Lymphocyte culture and stimulation

Indicated lymphocyte subsets were cultured in complete RPMI medium at indicated cell concentrations, for indicated time points, in 96-well plates at 37°C, 5% CO_2 with the indicated stimuli (listed below).

Conjugation of recombinant cytokines (IL-32γ and BAFF) was performed using the Alexa Fluor™ 647 Microscale Protein Labeling Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Protein concentrations following conjugation were determined using a Nanodrop Spectrophotometer.

Table 2.4. Stimuli for cell cultures.

Stimuli	Form	Final concentration	Source
LEAF™ purified anti-human CD40 antibody (HB14)	Soluble	5 µg/mL	BioLegend
Goat anti-human IgA + IgG + IgM (H+L)	Soluble	10 µg/mL	Jackson ImmunoResearch
Recombinant human IL-32 gamma protein, CF	Soluble	Indicated concentrations	R&D Systems
Recombinant human IL-32 alpha protein, CF	Soluble	Indicated concentrations	R&D Systems
Recombinant human IL-32 beta protein, CF	Soluble	Indicated concentrations	R&D Systems
Recombinant human IL-21	Soluble	50 ng/mL	PreproTech
T cell activation/expansion kit human	Soluble	1:1 bead to cell	Miltenyi Biotec
Phorbol 12-myrostate 13-acetate (PMA)	Soluble	1 ng/mL	Sigma Aldrich
Ionomycin	Soluble	1.3 ng/mL	Sigma Aldrich
Brefeldin A	Soluble	5 µg/mL	Sigma Aldrich
Recombinant human BAFF	Soluble	1 µg/mL	Sino Biological

2.3.5 Mouse serum preparation for serological investigations.

200 µL blood samples were collected from 10 and 24-week-old mice via retro-orbital bleeding. The blood samples were centrifuged at 4^{o}C for 15 minutes at 790 x g. Approximately 100 µL of serum supernatant was aliquoted into PCR strip tubes and the pelleted blood was discarded. Serum was stored at -20^{o}C before screening for the presence of anti-nuclear antibodies and anti-dsDNA antibodies using ELISA and HEp-2 indirect immunofluorescence ANA tests (IF-ANA). In all assays assessing serological phenotypes, serum from RAG$^{-/-}$ and MRL$^{lpr/lpr}$ mice were included as negative and positive controls respectively to validate assay function and accuracy.

2.3.6 Immunofluorescence ANA test

The IF-ANA test detects ANAs that adhere to HEp-2 cells fixed on a microscope slide. These ANAs are bound by a secondary anti-mouse IgG antibody conjugated to a fluorophore which fluoresces upon excitation by a specific wavelength of light during imaging of samples. The test output requires visual interpretation of fluorescence intensity.

HEp-2 (human epithelial cell) substrate slides (INOVA Diagnostics) were hydrated by submersion in PBS for 10 minutes at room temperature. Mouse serum prepared as per Section 2.2.8. was diluted 1:40 in PBS. 20 µL of diluted serum was added to wells on the slides and incubated in a humid chamber for 30 minutes. Slides were washed three times by submersion in PBS before addition of 20 µL of anti-mouse IgG Alexa-488 (Cat. No.1030-30) secondary antibody (1:400) to each well. Slides were incubated in a humid chamber for 30 minutes and the wash steps were repeated to remove unbound secondary antibodies. 10 µL

of Vectashield anti-fade mounting media (Cat. No. H-1000, Vector Laboratories, Novosibirsk, Russia) was added to each well. A coverslip was placed over the slide to prevent evaporation of samples.

Slide wells were imaged at 20X magnification Olympus IX70 fluorescence microscope and fluorescence was scored blindly (i.e. sample ID was unknown to the scorer). ANA scores were determined by comparing samples to several controls: a PBS control (score of 0-nil control), serum from a healthy $RAG1^{-/-}$ mouse which cannot generate antibodies (score of 1-negative control), and serum from a $MRL^{lpr/lpr}$ mouse which exhibits lupus-like autoimmunity and has elevated autoantibodies (score of 4-positive control). ANAs were scored between 0 and 4 based on comparisons to these controls.

2.3.7 ELISA for quantification of anti-dsDNA autoantibodies in serum

Poly-L-Lysine solution (0.1% weight/volume, Cat. No. P8920, Sigma-Aldrich) was diluted 1:50 in ELISA Coating Buffer (see Table 2.2). 100 µL of the dilute poly-L-Lysine solution was added to each well of a 96-well flat bottom plate (Santa Cruz, Cat. No. SANTSC-204463) and incubated in a humid chamber at room temperature for 5 hours. Calf-thymus derived dsDNA (Cat. No. D8661, Sigma-Aldrich) was resuspended in ELISA Coating Buffer at 50 ng/µL during the incubation period. Residual Poly-L-Lysine solution was removed and 50 µL of the dsDNA resuspension was added to each well. Plates were incubated overnight at 4^{o}C. Following the incubation, residual solution was removed from wells by flicking. 150 µL of blocking buffer was added to the wells and incubated at room temperature for 2 hours. Mice serum that was prepared as described in Section 2.2.8 was diluted 1:40 parts in blocking buffer. Plates were

washed three times by adding 150 µL of a solution containing 0.05% Tween 20 in phosphate buffered saline to all wells followed by removal via flicking. 50 µL of the pre-diluted serum was added to the wells and plates were incubated overnight at 4°C. The wash steps were repeated following incubation. 50 µL goat anti-mouse IgG-AP (Cat. No. 1030-04, Southern Biotech) diluted 1:1000 in blocking buffer was added to the wells and incubated for 1 hour at 37°C. Plates were washed five times before addition of 50 µL phosphate substrate (1 mg/mL) (Cat. No. S0942, Sigma-Aldrich) to the wells and left to develop in the humid chamber at 37 °C. Absorbance of the reaction solutions were measured at 405 nanometers using a microplate reader (Infinite 200 PRO NanoQuant, Tecan) at hourly intervals for 4 hours or until samples approached saturation (>4 units).

2.4 Mouse strains and Immunisations

2.4.1 CRISPR-Cas9 mediated genome-editing of mouse zygotes

C57BL/6 mice were housed under specific pathogen-free conditions at the Australian Phenomics Facility (APF). All mouse procedures were approved by the Australian National University Animal Experimentation Ethics Committee (AEEC A2018/38) under the NHMRC Australian code of practice. gRNA from ZFR2< DHX58 and Cas9 protein was obtained from PNABio. Oligomers and ssOligomers were purchased from IDT. C57BL/6Ncrl mice (4-5 weeks old) were super ovulated with Pregnant Mare Serum Gonadotrophin (PMSG) 5UI on day 1 and Human Chorionic Gonadotrophin hormone (HCG) 5UI on day 3. After detection of a vaginal plug in these females, indicating mating has occurred, mouse zygotes were harvested from the ampullae and placed in KSOM media (Sigma).

Cas9 protein (100 ng/µL) was co-injected with a mixture of sgRNA (50 ng/µL) and ssOligomer (100 ng/µL) into the cytoplasm of the fertilised egg into M2 medium (Sigma). The zygotes were then incubated overnight at 37^{o}C, 5% CO_2. Embryos that had processed through to a two-cell stage (that had undergone one division) were surgically transferred unto the uterus of a pseudo- pregnant CD1 recipient female at 2.5 days post coitum (dpc).

The offspring were then punched three weeks after birth for genotyping. DNA was extracted, and Sanger Sequencing was performed to confirm presence of variant. Selection and testing of gRNAs, mouse zygote preparations and micro-injections was carried out at the Australian Phenomics Facility, Australian National University by a team led by Stuart Read and Dr Gaétan Burgio, with microinjections performed by Nikki Ross. The sequencing was carried out at the Australian Cancer Research Foundation (ACRF) Biomolecular Resource Facility (BRF) and Genome Discovery Unit, Australian National University.

2.4.2 LCMV injections

At Day 0, ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice were infected with chronic (Clone 13) LCMV docile infection (2 x 10^6 pfu). Mice were bled at Day 8 and Day 18, and then were sacrificed at day 28 post chronic LCMV infection for splenic analysis. Infection and flow cytometric analysis was performed by Dr Sarah Gabriel from the Walter and Eliza Hall Institute of Medical Research, Melbourne University.

2.5 Molecular and biochemical studies

2.5.1 Expression vectors and plasmids

Table 2.5. Expression vectors and plasmids

Plasmid	Gene	Tag	Source
pCMV6-Entry	ZFR2	FLAG	OriGene Technologies
pCMV6-Entry	ILF2	Untagged	OriGene Technologies
pCMV6-Entry	ILF3	Untagged	OriGene Technologies
pCMV6-Entry	IL2	Untagged	OriGene Technologies
pcDNA3.1	RG6	dsRed/GFP	Gift from Dr. Thomas Cooper (Baylor College of Medicine). Addgene plasmid #80167
pcDNA3.1	ETR-3	FLAG	Gift from Dr. Thomas Cooper (Baylor College of Medicine). Addgene plasmid #96900
pcDNA3.1	MBNL3	FLAG	Gift from Dr. Thomas Cooper (Baylor College of Medicine). Addgene plasmid #96901)
MSCV IRES GFP	-	IRES-EGFP	Gift from Dario Vignali (St Jude Children's Hospital). Addgene plasmid #52107
MSCV IRES mCherry	-	IRES-mCherry	Gift from Dario Vignali (St Jude Children's Research Hospital). Addgene plasmid #52114

pT2cfosGW	NFκB	EGFP	Fist from John Rawls (University of North Carolina). Addgene plasmid #44922
pGL3-basic	3xAP-1	Luciferase	Gift from Alexander Dent (The Walther Oncology Centre). Addgene plasmid #40342
pGL3	Human IFN-Beta promoter	Luciferase	Gift from Nicolas Manel (Labex Research Institute). Addgene plasmid #102597
pGL3	3xNFAT	Luciferase	Gift from Jerry Crabtree (Howard Hughes Medical Institute). Addgene plasmid #17870
PMSCVpuro	Dead Cas9 with 3X NLS	3x NLS	Gift from Stanley Qi (University of California). Addgene plasmid #44246
pU6	Guide RNA	Mouse U6	Gift from Stanley Qi (University of California). Addgene plasmid #44248

2.5.2 Lipofectamine® 2000 Transfections

Lipofectamine® 2000 protocol (Invitrogen) was used for all lipid-based transfections. HEK 293 cells cultured and seeded at the appropriate densities (according to the manufacturer's protocol). The indicated plasmids were then transfected according to the manufacturer's protocol. Transfected cells were then cultured for 48 hours at 37^{o}C, 5% CO_2.

2.5.3 NEON® Transfection System

The NEON® Transfection System (Thermo Fisher Scientific) was used for all electroporation-based transfections. Jurkat cells were cultured, and were transfected with the indicated plasmids according to the manufacturer's protocol. Each transfection consisted of 1 million

Jurkat cells. Transfected cells were then cultured in pre-warmed complete RPMI-1640 overnight at 37ºC, 5% CO_2. Cells were sorted on eGFP expression to enrich for those which had taken up the plasmids.

2.5.4 Dual luciferase assays

HEK293 cells were co-transfected with indicated firefly luciferase reporter (100 ng, Stratagene), the Reinlla luciferase control reporter pRL-TK (5 ng, Promega), and indicated plasmids. Cells were incubated for 48 hours at 37ºC, 5% CO_2. Following incubation, dual luciferase assay was performed according to the Luc-Pair™ Duo-Luciferase HS Assay protocol (GeneCopoeia).

2.5.5 Bichromatic fluorescent reporter for screening alternative splicing

HEK 293 cells were seeded into a 24 well plate, and the following day were transfected using Lipofectamine 2000 (Life Technologies) with the indicated plasmids, according to the published protocol (Orengo et al., 2006). 48 hours after transfection, cells were washed and resuspended in FACS wash. Data was collected on BD LSRFortessa™ X-20 (BD Biosciences) and analysed with FlowJo software (TreeStar).

2.5.6 Retroviral transfections

HEK 293 cells were seeded into a 24-well plate. Indicated retroviral plasmids were transfected the following day were transfected using Lipofectamine 2000 (Life Technologies). 48 hours after transfection, cells were washed and resuspended in FACS wash. Data was collected on BD LSRFortessa™ X-20 (BD Biosciences) and analysed with FlowJo software (TreeStar).

2.5.7 CRISPR interference (CRISPRi) for ZFR2 knockdown

CRISPRi was performed according to the published protocol, using the indicated plasmids and primers.

2.5.8 RNA sequencing

RNA sequencing (RNAseq) was performed by Pablo Fernandez De Canete Nieto. Human follicular helper T cells, follicular regulatory T cells and naive T cells were FACS purified from 3 fresh tonsil donors. mRNA was extracted and sent to the ACRF Biomolecular Resource Facility, The John Curtin School of Medical Research, Australian National University for library construction using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina). Library samples were sequenced on a HiSeq2000 with a coverage of 25 million reads. The data was then sent to the Genome Discovery Unit (ANU Bioinformatics Consulting Unit, JCSMR, ANU) for analysis. Initial quality control checks with FastQC revealed that none of the samples were problematic. All reads were alighted to the *H. sapiens* genome reference sequence using TopHat version 2.0.13 with default parameters. Read counts were then generated for each gene in each sample using featureCounts version 1.4.6-p1 by using annotated gene locations. Differential expression analysis was performed using the edgeR package version 3.10. Read counts per gene were normalized by trimmed mean of M-values (TMM). As edgeR uses the negative binomial distribution as its basic model for differential expression data, dispersion estimates were obtained using the quantile-adjusted conditional maximum likelihood (qCML) method for single factor experiments. Then, the qCML-based exact test for the negative binomial distribution was performed to test for differentially expressed genes in our groups of samples.

We used a Benjamini-Hochberg adjusted p-value threshold of 0.05 to identify significantly differentially regulated genes. NCBI Sequence read archive accession number (SRP072739).

2.5.9 RNA isolation, cDNA synthesis and real-time PCR

Total RNA was isolated from freshly sorted T cell subsets using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA quality was determined using a Bioanalyzer (Agilent). Transcription into cDNA was performed using the miRNA Reverse Transcription Kit: miScript II RT Kit (Qiagen) according to the manufacturers protocol, on 1 µg of RNA. Quantitative RT-PCR was performed using QuantiTect® SYBR® Green PCR Kit (Qiagen) and the ABI Prism 7700 (Applied Biosystem). cDNA was then diluted 1:5 in RNAse free-water and used as a template for qPCR to assess IL-32 (Qiagen QuantiTect Primer Assay) with beta-2 microglobulin (Qiagen QuantiTect Primer Assay) used as a housekeeping gene.

2.5.10 Mass spectrometry

Dr Edmond Linossi from the Walter and Eliza Hall Institute of Medical Research, Melbourne University, used a glutathione-S-transferase (GST) pull-down assay to elucidate interactions between GST tagged $ZFR2^{WT}$/$ZFR2^{A771T}$ and unknown targets *in vitro.* The GST tagged $ZFR2^{WT}$/$ZFR2^{A771T}$ protein (known as the "bait" protein) was immobilised on glutathione-agarose beads, and then (unstimulated) Jurkat cell lysate (known as the "prey" proteins) was incubated on the beads. The protein complexes were then eluted from the beads, resolved by SDS-PAGE and stained with Comassie Silver. Finally, the protein bands were excised, digested with trypsin and then analysed by mass spectroscopy.

2.5.11 ZFR2 protein expression and purification

ZFR2 protein expression, purification and crystallography was performed by Jeff Babon and team, Walter and Eliza Hall Institute of Medical Research, Melbourne University. ZFR2 and ILF2 constructs were cloned using standard methodologies. ZFR2 (residues 584-902) was cloned into a modified PGEX-4T vector that contains a TEV protease cleavage site using Asc1 and EcoR. ILF2 (residues 29-362) was cloned into pACYC-DUET (using EcoR1 and Xho1 restriction sites). The two plasmids were co-transformed into E. coli BL21(DE3) competent cells (Millipore) and glycerol stocks were frozen for later expression.

Glycerol stock was inoculated into a 5mL Superbroth starter with ampicillin and chloramphenicol and incubated overnight at 37°C. Starter culture was added to 1L Superbroth with antibiotics and incubated at 37°C until A600 = 0.8-1.0. Expression was induced with 1mM IPTG, cells were incubated at 18°C overnight, harvested by centrifugation, snap frozen on liquid nitrogen, and stored at -30°C for later purification.

All following steps for this purification were performed at 4°C. Cell pellets were thawed and resuspended in 5mL Lysis Buffer (TBS, 2mM TCEP) for each 100mL original cell culture. Cells were lysed by sonication and insoluble material was pelleted by centrifugation at 40,000g for 30min. Supernatant was passed over 1mL packed glutathione affinity beads (GE Health), and beads were washed with 50mL lysis Buffer. Beads were resuspended in 20mL lysis Buffer with 0.5mg TEV protease and allowed to incubate overnight. The ZFR2/ILF2 complex was separated from beads using a gravity flow column, concentrated to a volume of 3mL, and loaded onto a Superdex 200 16/600 size exclusion chromatography (GE Health), pre-equilibrated with lysis Buffer. Fractions containing pure complex were pooled, buffer

exchanged into 20mM Tris pH 7.5, 50mM NaCl, 2mM TCEP, concentrated to 10 mg/mL, snap frozen on liquid nitrogen, and used to set up crystallisation screens.

3.5.12 X-ray crystallography

Samples were initially screened at the Collaborative Crystallisation Centre (CSIRO, Melbourne) by vapour diffusion. The protein complex crystallised in crystallisation conditions 18-20% PEG 3350, 200mM sodium sulphate, 100mM Bis-Tris pH 6.5. Crystals were cryo-protected in mother liquor + 17% glycerol.

3.5.13 X-ray crystallography data collection and processing

Data were collected at the Australian Synchrotron MX2 beamline at a wavelength of 0.9537Å. Data were integrated using XDS and scaled using XSCALE. Molecular replacement was performed in Phaser with the ILF2/ILF3 structure as a search model (PDB ID 4AT7). Refinement was performed using PHENIX and model building was performed in COOT.

3.5.14 Primers

Forward Primer (5'->3')	Reverse Primer (3'->5')	Purpose
CGAGTCCCTGGCCACCCTCCGTCATGC	GCATGACGGAGGGTGGCCAGGGACTCG	ZFR2 SDM
GGCGAATTCATGTACAGGATGCAACTCC	GGCCTCGAGTATTAAATAGTTTTACTAACCAATC	Cloning IL-2 into pMIG II
GGCGAATTCATGTACAGGATGCAACTCC	GGCCTCGAGTCAAGTCAGTGTTGAGATGATGC	Cloning IL-2 Δ3'UTR into pMIG II
GGCGAATTCATGGCGACGAGTCAGTATTTC	GGCAGATCTTCACACGAGCCCCTCTCC	Cloning ZFR2 into pMIG II
TTGG TATTTCGACTTCGCGCA G TTTTAGAGCTAGAAATAGCAAG TTAAAATAAGGCTAGTCCGTTAT CAACTTGAAAAAGTGGCACCGA GTCGGTGCTTTTTTTC	TCGAGAAAAAAAGCACCGACTCG GTGCCACTTTTTCAAGTTGATAAC GGACTAGCCTTATTTTAACTTGCT ATTTCTAGCTCTAAAACTGCGCGA AGTCGAAATA CCAACAAG	Primer for ZFR2 KD
TTGG CGAGCGGCACGCGGCTC G GTTTTAGAGCTAGAAATAGCA AGTTAAAATAAGGCTAGTCCGT TATCAACTTGAAAAAGTGGCAC CGAGTCGGTGCTTTTTTTC	TCGAGAAAAAAAGCACCGACTCG GTGCCACTTTTTCAAGTTGATAAC GGACTAGCCTTATTTTAACTTGCT ATTTCTAGCTCTAAAACCGAGCCG CGTGCCGCTCG CCAACAAG	Primer for ZFR2 KD

2.6 Microscopy

2.6.1 Transfections and Immunofluorescence

HEK 293 cells were seeded onto coverslips, and the follow day was transfected using Lipofectamine 2000 (Life Technologies) with the relevant plasmids ($ZFR2^{WT}$-FLAG, $ZFR2^{A771T}$-FLAG or NF-κB-EGFP) as per the manufacturer's recommendation to a total of 4 µg of DNA per well (balancing with pcDNA3.1 if required). Cells were incubated for 24 hours at 37°C, 5% CO_2. Cells were the fixed with 3.7% paraformaldehyde and blocked using 3% BSA, and then stained with the relevant primary antibody (overnight 4°C) and secondary antibodies (1 hour RT in the dark) (see Table 2.6 for immunofluorescence antibodies). Stained cells were

mounted using Vectashield with DAPI mounting media (Vector Laboratories) and images were collected with an Olympus 1X71 microscope with DP Controller software (Olympus) and combined using Adobe Photoshop (Adobe Systems).

Table 2.6. Antibodies used for immunofluorescence

Antibody	Catalogue Number	Source
Rabbit anti-ZFR2	ab204415	Abcam
SC35	ab11826	Abcam
Donkey anti-rabbit IgG Alexa Fluor 594	A-11037	ThermoFisher Scientific
Donkey anti-goat IgG Alexa Fluor 488	A-11055	ThermoFisher Scientific
Donkey anti-mouse IgG Alexa Fluor 244	A-21202	ThermoFisher Scientific
Monoclonal Anti-FLAG (Clone M2)	F3165-5MG	Sigma Aldrich

2.6.2 Immunohistochemistry

Using immunohistochemistry, the presence and localisation of IL-32 positive cells was evaluated in human tonsils. Immunohistochemistry was performed by Jane Dahlstrom and Amanda Bullman, ACT Pathology, The Canberra Hospital.

Table 2.7. Antibodies used for immunohistochemistry

Antibody	Clone	Conjugation	Source
IL-32αβγδ	KU32-09	Purified	BioLegend
IL-32αβγδ	KU32-52	Biotin	BioLegend
IL-32αβγδ	KU32-56	Purified	BioLegend

Immunohistochemistry was performed on the Ventana BenchMark ULTRA. 3µm tissue sections from archival material (kept in the Department of Anatomical Pathology, ACT Pathology, The Canberra Hospital) was used. A 1/200 dilution of anti-IL32 (Mouse Monoclonal-KU32-09, BioLegend) was used following heat retrieval for 64 mins at 95 degrees in CC1 buffer. The IL32 antibody was incubated for 36 min at 36 degrees. The Ventana Ultraview Universal DAB detection kit was used to amplify and visualise the signals. Hematoxylin counterstaining allowed visualization of cell nuclei.

For the dual staining a 1/40 dilution of anti-CD4 (Mouse Monoclonal- 4B12); Leica (NCL-L-CD4-368) was used following heat retrieval for 64min at 95 degrees in CC1 buffer. The CD4 antibody was incubated for 36 min at 36 degrees. The Ventana Ultraview universal alkaline phosphatase red detection kit was used to amplify and visualise the CD4 signals. A 1/200 dilution of anti-IL32 antibody was incubated for 36 min at 36 degrees. The Ventana Ultraview universal DAB detection kit was used to amplify and visualise the IL 32 signals. Hematoxylin counterstaining allowed visualization of cell nuclei. Omitting the primary antibody was used as a negative control while tonsils (CD4) and a cell block of cells (known to express IL32) were used as a positives control.

2.7 Statistical Analysis

All human and mouse immunophenotyping was analysed using a two-tailed non-parametric Mann-Whitney test (U test). Where possible, *in vitro* overexpression assays were analysed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test to determine statistical significance between multiple groups, unless otherwise stated. All *in*

vitro stimulation experiments were analysed using a two-tailed unpaired Student's t-test or a paired t-test, as stated in the figure legends. Statistical tests with appropriate underlying assumptions on data distribution and variance characteristics were used. Statistical tests were performed using R and GraphPad Prism Version 7.0. Statistically significant differences are induced as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 and ns = not significant.

Chapter Three: Investigating the role of ZFR2 in the regulation of T cell-derived IL-2 and lupus pathogenesis

3.1 Preamble

This chapter addresses the first project of this thesis, concerning a multiplex family affected by SLE (called Family A). The family of interest consists of four affected females (II.1, II.6, II.4, III.3) and an affected male (I.1) who is deceased (Figure 3.1). Table 3.1 outlines a brief clinical history of the patients. Interestingly, the patients presented with manifestations around the same time, suggesting exposure to an environmental trigger such as an infection.

Figure 3.1. Pedigree of multiplex family with SLE (Family A)

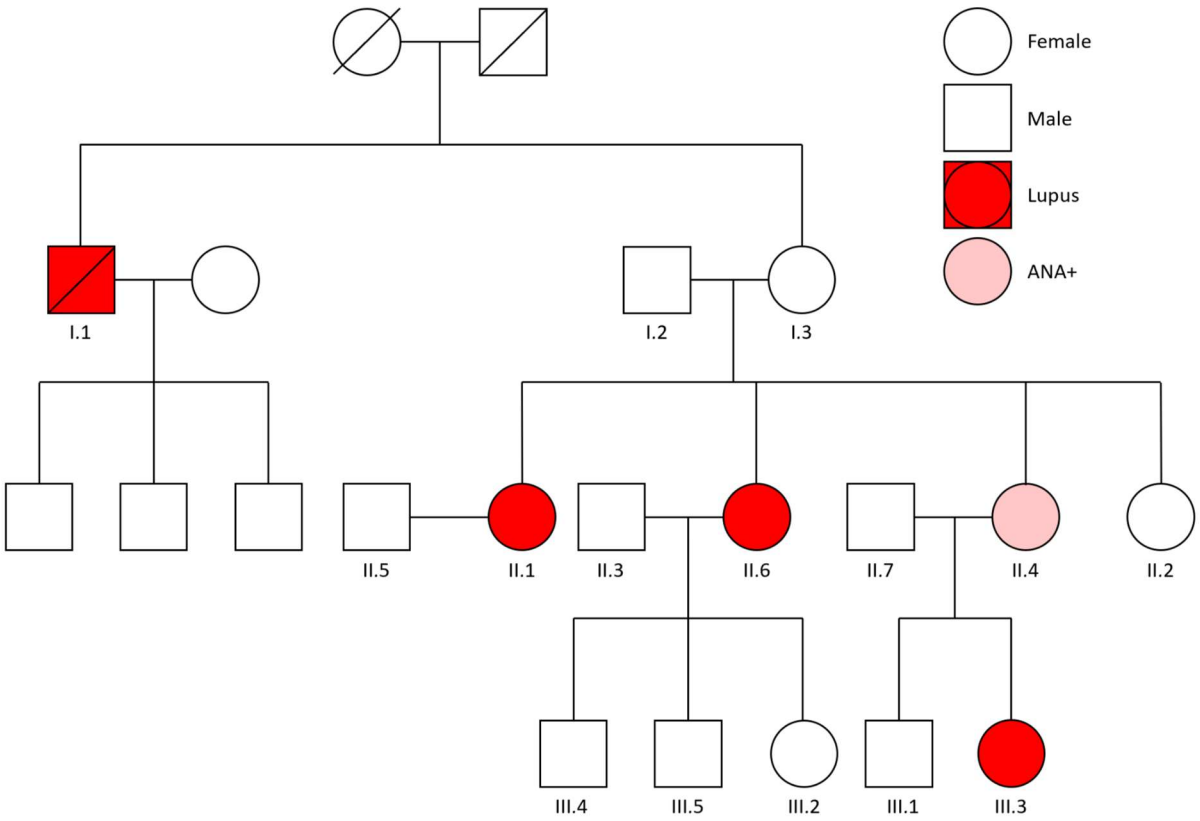


Table 3.1. Clinical history of patients (Family A)

Individual	**Date of Birth**	**Clinical History**
I.1	Unknown	Deceased. Polyarthralgia, nephritis. Full blood count normal.
1.3	Unknown	Healthy
II.1	11/10/1964	+ Anti-dsDNA, polyarthralgia. Symptoms currently controlled on Plaquenil. Normal complement levels. Full blood count normal.
II.3	1/11/1972	+ Anti-dsDNA, + anti-SSA, polyarthralgia, central nervous system involvement. Symptoms currently controlled on mycophenolate mofetil, Plaquenil and cyclophosphamide injections. Normal complement levels. Full blood count normal.
II.4	04/10/1974	+ ANA. Normal complement levels. No medications. Full blood count normal.
III.3	31/01/1998	Polyarthralgia. Symptoms currently controlled on Plaquenil and Arcoxia. Full blood count normal.

Whole genome sequencing identified a heterozygous SNV in the DZF (domain associated with zinc fingers) domain of an RNA binding protein called zinc finger RNA binding protein 2 (ZFR2) in the four affected females (and I.3, the sister of the deceased male who also presented with SLE) in Family A. Analysis of the Centre for Personalised Immunology Variant Database (Centre for Personalised Immunology) revealed another male lupus patient from a different family (Family B) carrying a different mutation within the DZF domain of ZFR2, inherited from the patient's unaffected father. Whilst the *in vitro* and *in vivo* effects of this second mutation were not addressed, this individual was included in cellular and plasma immunophenotyping when possible to identify shared trends.

A role of ZFR2 in the immune system has not been reported, although paralogs of this protein have been shown to regulate the gene expression of multiple cytokines (further described in Section 3.2). In this chapter, we demonstrate that the patients carrying these *ZFR2* variants in Family A and Family B possess similar and unique cellular and molecular phenotypes, specifically an increase in plasma IL-2, an increase in T_{regs} and an expanded $CD4^+$ $CD57^+$

cytotoxic T cell population. We go on to identify a number of immune pathways ZFR2 is involved in, such as regulation of cytokine production, specifically enhancement of IL-2 transcription via NFAT and AP-1, as well as regulation of interferon-β activity and mRNA splicing. Furthermore, we demonstrate that the variant in Family A is a gain-of-function, further increasing NFAT and AP-1 activity, as well as IL-2 production. Finally, we demonstrate that, whilst the mice carrying the orthologous variant did not develop overt autoimmunity, they did develop some cellular phenotypes observed in the patients, including an increase in T_{regs}. Therefore, we hypothesise that ZFR2 plays a role in the immune system in regulating the gene expression of cytokines, and the *ZFR2* variant in these patients is leading to a dysregulation in cytokine gene expression and production, contributing to disease pathogenesis in both families.

3.2 Identification of possible disease-causing variants

Whole exome sequencing (WES), which identifies all the single nucleotide variants (SNVs) in the protein-coding regions of the genome (the exons), was performed on DNA purified from saliva from patients I.3, II.1, II.4, II.6, II.7 and III.1. WES data was assembled against a reference genome using the Raijin supercomputer at the National Computation Infrastructure, Australian National University (ANU). Bioinformaticians (Dan Andrews, Matthew Field and Vicky Cho) at the JCSMR collated data from several public databases to produce a report on the identified SNVs identified in this family. This report included the SNVs position (chromosome, coordinate, amino acid change and exon), frequency (dbSNP, GMAF and EXAC), *in silico* predictions (PolyPhen2, SIFT and CADD), gene expression, association with human and mouse diseases and Gene Ontology (GO) function. These SNVs were filtered to focus on those that segregated with disease in the family.

As the hypothesis of this study was to explore rare and novel variants that may contribute to disease pathogenesis, a scoring system was devised to prioritise rare and novel variants using minor allelic frequency (MAF), initially prioritising those with a frequency of less than 2% but assigning higher scores to novel and rare (MAF <0.005) variants. The scoring algorithm also included weighting for variants with a PolyPhen2, SIFT or CADD score that suggested deleteriousness; expression in immunological tissues; a known role in the immune system (gene ontology (GO) terms); and immune human (OMIM) or mouse phenotypes (mouse KO database). This scoring system therefore prioritised the novel and rare variants that were predicted to be damaging and had a known role in the immune system over gene variants that were predicted to be benign or genes with well-established roles remote from the immune system. These variants were ranked and scored, and variants in the top 3 ranks (≥6

out of 8) were reviewed. Homozygous variants, X-linked variants and variants associated with autoimmune disorders were investigated individually. This analysis resulted in 11 candidate gene variants (Table 3.2).

Following identification of the candidate gene variants, a strategy for proof of causation was devised. To conclude any candidate variant is likely to contribute to disease it needed fulfil the following criteria:

1. The candidate gene variant segregates with autoimmunity in the family;
2. The candidate gene variant alters gene or protein function;
3. The consequence of this altered function results in a change in cellular physiology;
4. This change in cellular physiology alters a biological system in a manner that may lead to autoimmunity.

3.2.1 Rare variants in ZFR2, DHX58 and SMG7 identified in multiplex lupus family

The exomes from the five individuals sequenced were collated, and variants that were present in all five individuals were chosen and scored. Only 11 variants scored six or above. Each of these 11 gene variants were analysed individually, and the two variants that attained the highest score (score 7, Figure 3.2), a novel heterozygous missense variant in ZFR2 encoding an alanine to tyrosine substitution at amino acid position 771 and a rare heterozygous missense variant in DHX58 encoding an arginine to cysteine substitution at position 334, were chosen as candidate gene variants for this study. Upon subsequent reanalysis, a rare missense variant in SMG7 was added as a third candidate gene variant due to its recent association with lupus by GWAS (Deng et al., 2016), but the functional

consequences of this SNV could not be addressed in this study. Each of these three genes however have known roles in the immune system.

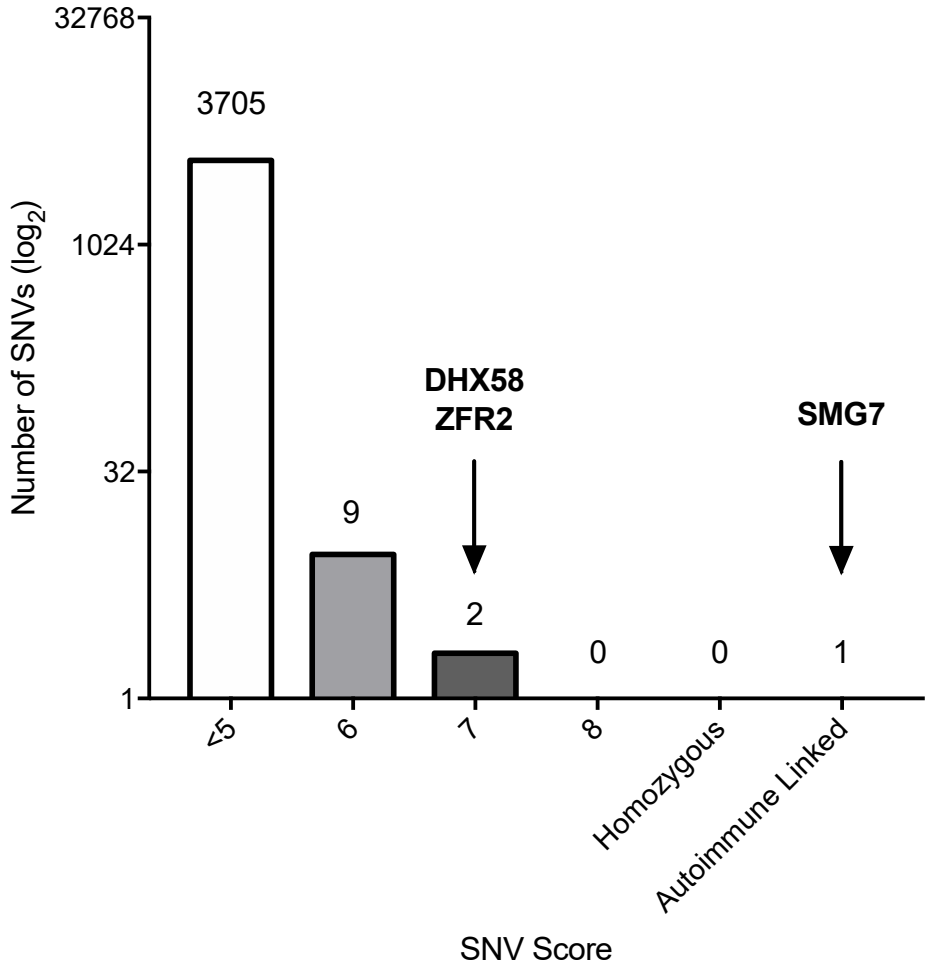


Figure 3.2. Numbers of non-synonymous SNV variants identified in SLE family by score.

Scoring was awarded for allele frequency, bioinformatic prediction of damage, association with immune function and association with immunological disease. A high score indicates the strongest correlation. DHX58 and ZFR2 were the top candidate SNVs that segregated with disease, and SMG7 was the only SNV that was linked with immunological disease and segregated with disease in the family.

Table 3.2. Candidate gene variants identified in multiples SLE family.

Gene	Variant	MAF	SIFT	PolyPhen2	CADD	Mouse Phenotype	Function	Score
DHX58	R334C Missense	0.0055	0	0.39	22.5	Knockout allele-altered susceptibility to infection	Antiviral signalling. Involved in the innate immune responses to RNA and DNA viruses. Part of the RIG-I signalling pathway.	7
ZFHX4	N306S Missense	Novel	NA	0.486	22	NA	DNA binding, neuronal differentiation, knockdown inhibits cancer cell migration and invasion, associated with ptosis, neutral and muscle differentiation, transcription.	6
ROR2	A748P Missense	Novel	0.04	0.028	17.02	Knockout lethal-respiratory and cardiovascular abnormalities, and skeletal defects	Early cartilage and growth plate development. Variants are associated with a skeletal disorder called brachydactyly type B and Robinow syndrome, characterised by skeletal dysplasia. Involved in transferase activity and protein tyrosine kinase activity. It is a tyrosine-protein kinase receptor.	6
FUK	P143L Missense	Novel	0	0.995	21	N/A	Kinase that catalyses the phosphorylation of L-fucose to form beta-l-fucose-1-phosphate, which is important in cell-cell interactions.	6
ZFR2	A771T Missense	Novel	0	0.929	28.1	N/A	RNA binding protein. Role in immune system (IL-2 mRNA stability and transcription).	6
DAPK3	V257I Missense	Novel	0.27	0.148	22.5	N/A	Plays a role in induction of apoptosis, autophagy, transcription and translation, actin cytoskeleton reorganization,	6

							muscle contraction. Regulation of cell cycle and cell proliferation. Associated with recessive dystrophic epidermolysis bullosa.	
MAPK8IP2	G282R Missense	Novel	0	1	21.1	Knockout small and have infertility, behavioural and cerebellar transmission deficits.	Scaffold protein involved in JNK signalling. Associated with cervical verrucous carcinoma and ameloblastic carcinoma. Involved in ERK and Akt signalling.	6
IGFN1	A1600T Missense	0.0667	0.01	0.996	21.7	N/A	Structural support to skeletal muscle.	6
MMD2	Splice3	Novel	NA	NA	NA	N/A	Associated with Miyoshi muscular dystrophy and Moyamoya disease 1. Transmembrane protein. Localises to the Golgi apparatus to modulate Ras signalling.	6
FRG1B	E180G Missense	0.0027	0.11	0.002	17.86	N/A	Pseudogene. Function unknown.	6
SMG7	D518Y Missense	0.000008	0	0.855	28.8	N/A	Nonsense mediated decay and mRNA export from nucleus. Variants associated with lupus.	Autoimmune Linked

3.2.2 ZFR2 paralogs are DNA and RNA binding proteins and regulate cytokine production

ZFR2 is a largely uncharacterised protein that is expressed ubiquitously in most organs of the body including lymphoid organs, however it is highly expressed in the human brain, retina, testis and adrenal gland (Uhlen et al., 2015). ZFR2 is also expressed at moderate levels in most immune cells, however its role in the immune system has not been fully characterised (Uhlen et al., 2015). Expression data from our collaborator Dr Jarny Choi at The University of Melbourne shows high expression of ZFR2 in human mast cells, human T cells and OP9 cells, a mouse bone marrow stromal cell line (Figure 3.3). ZFR2 is predicted to be important for cancer development and organ and system development, specifically brain development, including shuttling and transportation of ribonuclear particles in the brain (Rippey et al., 2013a, Rubicz et al., 2016), axon guidance and dendritic branching (Rippey et al., 2013b).

ZFR2 comprises of three N-terminal C2H2 zinc finger motifs (Figure 3.4), which likely engage RNA targets (Wolkowicz and Cook, 2012), and are conserved from *Caenorhabditis elegans* to humans indicating strong selective pressure to maintain specific interactions. It also possesses a C-terminal domain, called a 'domain associated with zinc fingers' or DZF domain, which is the domain where the candidate gene variant in Family A lies (Figure 3.4).

This DZF domain is an important dimerisation domain that is highly conserved amongst species (from *Homo sapiens* to *Danio rerio*) and between the four other paralogs that possess this domain, namely ILF2, ILF3, ZFR and STRBP. This DZF domain possesses the same overall structure as a nucleotidyltransferase (an enzyme that transfers nucleotides to other proteins), and no other proteins (besides ZFR2 and its family members) possess this domain. ZFR2, ZFR,

ILF2, ILF3 and STRBP have lost the residues that make this domain catalytically active, however they still possess the ability to bind nucleotides, although the reason for this is unknown. As elucidated by our collaborator Jeff Babon, who managed to crystallise this domain, the A771T variant lies within the nucleotide-binding region of the DZF domain, approximately 3.8 angstroms from the bound nucleotide, and could therefore interfere with nucleotide binding. As threonine can be phosphorylated, this variant could cause a phosphorylation site that would block nucleotide binding, or steric hindrance caused by the threonine (as it is larger than the alanine) could also prevent nucleotide binding. Another possibility is that this variant may form a hydrogen bond (via the –OH group on the threonine), which could enhance nucleotide binding.

ILF2, ILF3 and STRBP, a testes-specific paralogue of ILF3, have a different domain organisation, with the DZF domain located at the N-terminus. In ILF3 and STRBP, a testes-specific paralogue of ILF3, the DZF domain is followed by two double stranded RNA binding domains and a low-complexity region at the C-terminus which can be alternatively spliced to produce variation. ILF2, the partner of ILF3, is much smaller than all the other proteins in the family, with the DZF domain being the only domain present in the protein (Figure 3.4). Interestingly, ZFR2, ZFR and ILF3 interact with ILF2 (in humans and in mice), forming complexes with each other (Wan et al., 2015). STRBP is not known to interact with any of these proteins. ZFR and ZFR2 are structurally similar because a gene duplication event occurred in *Danio rerio* (zebrafish), which is why ZFR2 is not present in more primitive species (Figure 3.5). The alanine that is mutated in the DZF domain of these patients is conserved throughout all species which possess ZFR2, including *Danio rerio,* suggesting that this alanine is critical for the function of

this domain and protein. Interestingly, the gene duplication event that gave rise to ILF3 and the testes specific paralog STRBP included the substitution of this same alanine to become a serine, with ILF2, ZFR and ZFR2 still possessing the alanine. This serine to alanine substitution consisted of a simple change to the carboxyl group, forming an -OH group at the -H position.

ZFR, like ZFR2, is a highly conserved gene that is essential in mice and flies and has a similar domain organization to ZFR2 (Figure 3.5). It is also expressed in similar organs as ZFR2 (Uhlen et al., 2015) and plays an important role in gastrulation and embryonic development (Meagher and Braun, 2001), brain development (Meagher and Braun, 2001), RNA transport (Elvira et al., 2006), and tumorigenesis (Zhao et al., 2016), however its physiological and biochemical functions are mostly unknown. Variants in human ZFR have been shown to cause a neurological disorder called hereditary spastic paraplegia (Lo Giudice et al., 2014). In terms of the immune system, ZFR has only recently been shown to play a role in the repression of the interferon response by regulating alternative pre-mRNA splicing, with interferon stimulated genes becoming constitutively activated in the absence of ZFR (Haque et al., 2018).

The roles of ILF2 (NF45) and ILF3 (NF90) have been better characterised both in the immune system and in other systems, functioning together as a heterodimer as well as independently of each other (Wolkowicz and Cook, 2012). ILF2 and ILF3 play important roles in transcriptional (Kiesler et al., 2010, Rigo et al., 2012) and post-transcriptional (Masuda et al., 2013, Jayachandran et al., 2016, Rigo et al., 2012) mRNA regulation and processing, DNA repair (Shamanna et al., 2011), viral regulation (Li and Belshan, 2016) and translation (Merrill and Gromeier, 2006). Their roles in RNA gene regulation has been described in processes such

as splicing (Rigo et al., 2012), export (Pei et al., 2008) and stability (Pei et al., 2008). More specifically in the immune system, ILF2 and ILF3 play essential roles in the regulation and expression of cytokine mRNAs, the most characterised being *interleukin-2 (IL-2)* (Zhu et al., 2010), an important growth and survival factor produced by activated T cells that can stimulate B cell differentiation and immunoglobulin secretion, enhance monocyte cytotoxicity, increase phagocytic functions of macrophages and stimulate immune cells to proliferate (Smith, 1988). Other cytokines and chemokines that ILF2/ILF3 regulate include IL-6 (Tominaga-Yamanaka et al., 2012), IL-8 (Tominaga-Yamanaka et al., 2012), monocyte chemoattractant protein-1 (MCP-1) (Tominaga-Yamanaka et al., 2012), IL-13 (Kiesler et al., 2010) and type 1 interferons (IFNs) (Krasnoselskaya-Riz et al., 2002).

ILF2 (NF45) and ILF3 (NF90) have been shown to interact with nuclear factor of activated T cells (NFAT), a transcription factor that was first identified as an inducible nuclear factor that binds to the *IL-2* promoter in activated T cells (Shi et al., 2007a). The complex, in cooperation with AP-1, acts at the antigen receptor response element-2 (ARRE-2) sequence/NFAT target DNA sequence in the *IL-2* promoter to regulate *IL-2* expression, as well as the expression of other cytokines (Shi et al., 2007a). Studies by a group at Stanford University Medical Centre demonstrated that Jurkat cells overexpressing NF45 have a 120-fold increase in *IL-2* promoter and NFAT/ARRE luciferase activity following activation, and a two-fold increase in IL-2 production (Zhao et al., 2005). In a separate study they also showed that NF90$^{-/-}$ RAG chimeric mice have impairments in the transcriptional activation of *IL-2* and IL-2 expression, as well as severe T cell lymphopenia (Shi et al., 2007a). Furthermore, activation of NFAT is essential for T cells to carry out effector functions, with T cells deficient in NFAT having impaired

production of IL-2, IL-4, IL-10, IFNγ, TNF, IL-5, CD40L, CD95L and granulocyte macrophage colony stimulating factor (GM-CSF) (Macian, 2005). AP-1 proteins are the main transcriptional partners of NFAT during T cell activation, with their cooperation integrating two signalling pathways, namely calcium signalling which activates NFAT proteins and the RAS-MAPK pathway inducing expression of the Fos and Jun transcription factors. Together they play a role in processes such as growth, differentiation and apoptosis, and are key regulators of many proinflammatory and immunoregulatory cytokines. AP-1 proteins are a heterodimer of c-Fos and c-Jun (Macian et al., 2001), which is interesting as ILF2 and ILF3 are able to enhance the transcriptional induction of c-Fos through its promoter region (Nakadai et al., 2015).

ILF2 and ILF3 also have the ability to regulate the stability of *IL-2* mRNA transcripts, demonstrating a second level of control that these proteins have on IL-2 production (Shim et al., 2002a). These proteins bind to the AU-rich elements (AREs) in the 3'UTR and slow down the degradation of the transcript by preventing destabilising proteins from binding to the 3'UTR (Shim et al., 2002a). In the study mentioned above, the NF90$^{-/-}$ RAG chimeric mice also had impaired stabilisation of *IL-2* mRNA (Shi et al., 2007a). This binding also facilitates the export of the transcript from the nucleus to the cytoplasm, as the stabilisation of the transcript depends on its localisation to the cytoplasm (Shi et al., 2007a). All these studies mentioned above clearly demonstrate that ILF2 and ILF3 are important multifunctional coactivators that coordinate gene expression and can facilitate the rapid induction and/or suppression of genes, a process that is very important during an immune response.

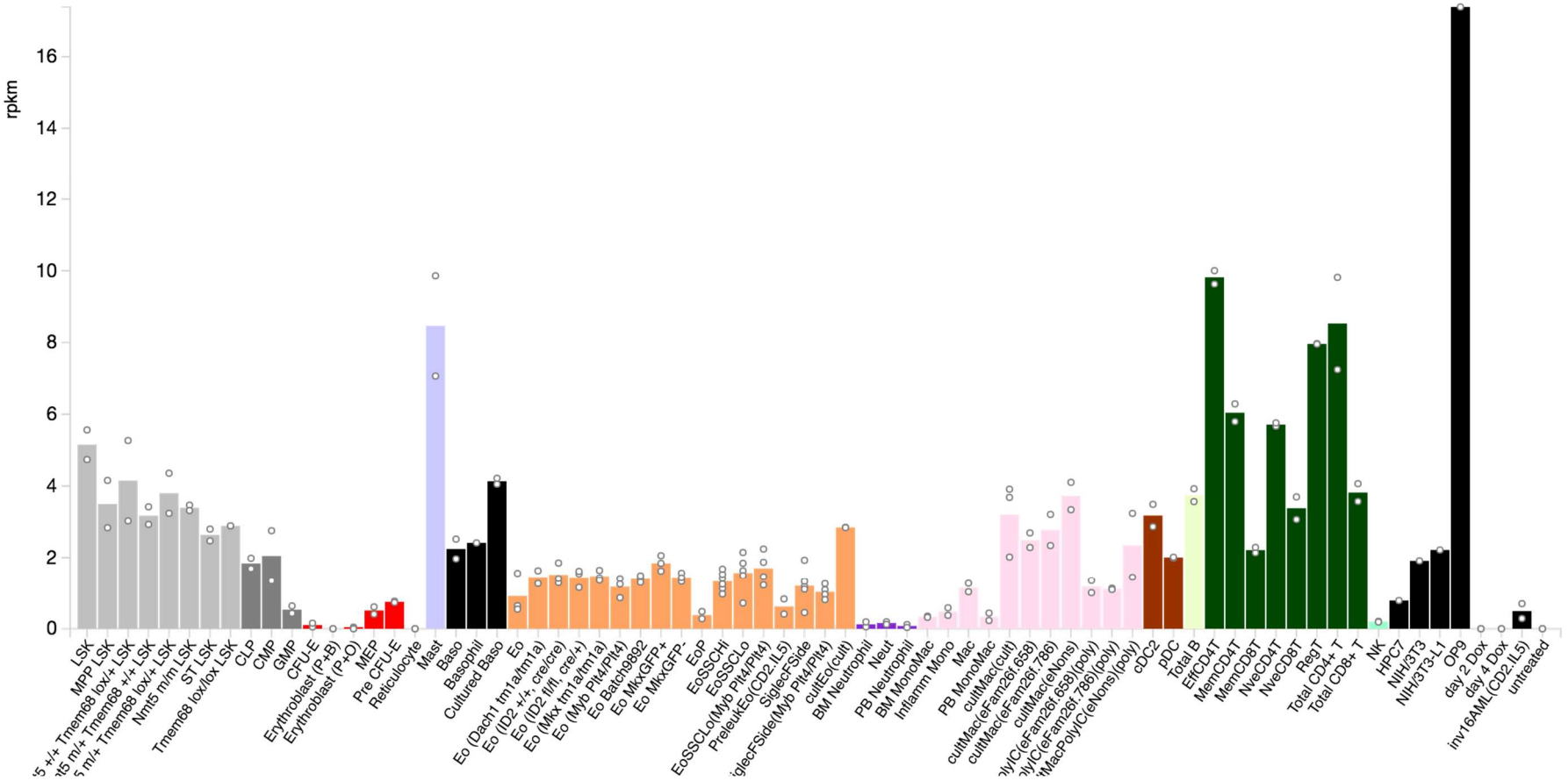


Figure 3.3. RNA-sequencing data for ZFR2 in lymphocytes and lymphocyte-derived cell lines.

RNA-sequencing (RNAseq) expression data demonstrating ZDR2 is highly expressed in human mast cells, human T cells and OP9 cells. Data provided by Dr Jarny Choi, University of Melbourne.

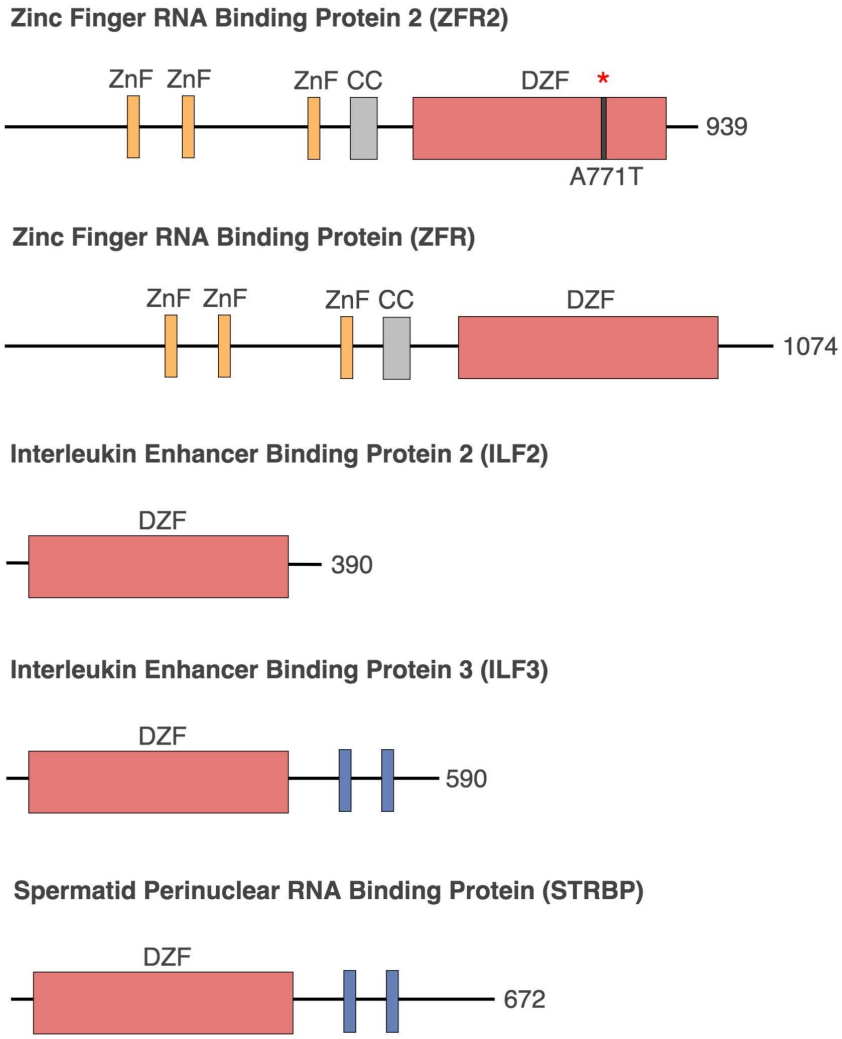


Figure 3.4. Two-dimensional schematic of ZFR2 and its paralogs.

Two-dimensional schematic of ZFR2, ZFR, ILF2, ILF3 and STRBP, demonstrating their domains and location of A771T variant in ZFR2.

A

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Danio-rerio                VIFSSKEPKMQVTISLTSPVMREDPVPNME--K-VSEKDPPDLLNRIKCLEYLAALRHAK  429
Gallus-gallus              ILNSCVEPKMQVTITLTSPVIREENMREGDVTS-GMVKDPPDVLDRQKCLDALAALRHAK  903
Mus-musculus               VISACVEPGVKVTVSATSPLMREDPSVK-QGQQ-DALSDPEDVLDRERCLETLAALRHAK  709
Rattus-norvegicus          VISACVEPGVKVTVSATSPLMREDPSVK-QELQ-DSLSDPEDILDRERCLETLAALRHAK  658
Cavia-porcellus            VISSCEEPRMQVTVAITSPLMRQDPPADPEGRE-AAQPDP-DILNPDKCLESLAALRHAK  564
Macaca-mulatta             VISSCEEPRMQVTVSITSPLMREDPSTD-PGVE-KPQADPGDVLSSEKCLESLAALRHAR  734
Homo-sapiens               VISSCEEPRMQVTISVTSPLMREDPSTD-PGVE-EPQADAGDVLSPKKCLESLAALRHAR  776
Pan-troglodytes            VISSCEEPRMQVTISVTSPLMREDPSTD-PGVE-EPQADAGDVLSPKKCLESLAALRHAR  745
Canis-lupus-familiaris     IISSCEEPRMKVIVCVTSPLMREDSSVDQEGVQ-VPQPNPDDILSPEKCLQSLAALRHAK  800
Bos-taurus                 VISSCEEPRIQVAVSITSPLMREDPSADQEGTE-VPLPDPGDVLSPEKCLQALAALRHAK  806
Sus-scrofa                 IISSCEEPKMQVTVSITSPLMREDPSTDREGTE-APLPDPGDVLSPEKCLQSLAALRRAK  778
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B

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ILF2       TVPP-------------NLRKLDPELHLDIKVLQSALAAIRHARWFEENASQS-TVKVLI 224
STRBP      LTSPLIRDELEKK-DGENVSMKDPPDLLDRQKCLNALASLRHAKWFQARANGLKSCVIVL 222
ILF3       LTSPVVREEMEKVLAGETLSVNDPPDVLDRQKCLAALASLRHAKWFQARANGLKSCVIVI 230
ZFR        LTSPIIREENMREGDVTSGMVKDPPDVLDRQKCLDALAALRHAKWFQARANGLQSCVIII 926
ZFR2       VTSPLMREDPSTDPGVEE-PQADAGDVLSPKKCLESLAALRHARWFQARASGLQPCVIVI 792
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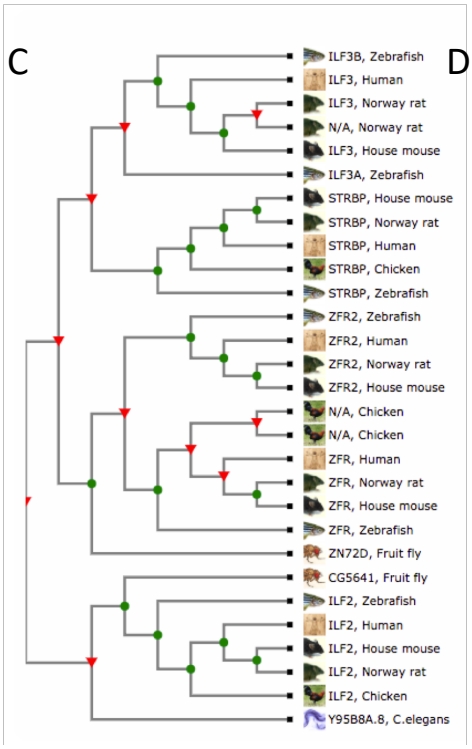
C



D

	ZFR2	ZFR	ILF2	ILF3	STRBP
ZFR2	100				
ZFR	48.18	100			
ILF2	25.98	24.78	100		
ILF3	50.70	51.39	26.91	100	
STRBP	48.03	51.69	22.61	57.82	100

Figure 3.5. Conservation of ZFR2.

(A) Conservation of ZFR2 alanine amongst species demonstrating that the alanine is evolutionarily conserved (B) Conservation of the alanine between ZFR2 and paralogs, demonstrating that ILF3 and STRBP do not possess the alanine. (C) Phylogenetic tree of ZFR2 and paralogs. (D) Sequence comparison of ZFR2 and paralogs, presented as percent identity. Sequence comparisons were performed using Clustal Omega Software (European Molecular Biology Laboratory).

3.2.3 Identification of another lupus patient carrying a rare ZFR2 variant (Family B)

A second heterozygous variant in ZFR2 (A916W) was identified in a male lupus patient from another kindred (Family B), inherited from his father. This variant was identified through our Centre for Personalised Immunology Variant Database (Centre for Personalised Immunology), and was the only other autoimmune patient from a different family that possessed a non-synonymous variant in ZFR2. No healthy controls with ZFR2 variants were found. This patient presents with arthritis, pericarditis, ANAs, anti-dsDNA, complement defects, and lymphocytopenia. This patient has no siblings, and his parents are both unaffected (Figure 3.6). The rare variant (MAF 0.08%) identified in this patient also lies within the DZF domain of ZFR2 and is predicted to be damaging based on SIFT (0.02) (Figure 3.6). Where possible, this patient was included in cellular and plasma phenotyping, however this variant was discovered towards the end of this project and thus the *in vitro* and *in vivo* effects of this variant were not investigated.

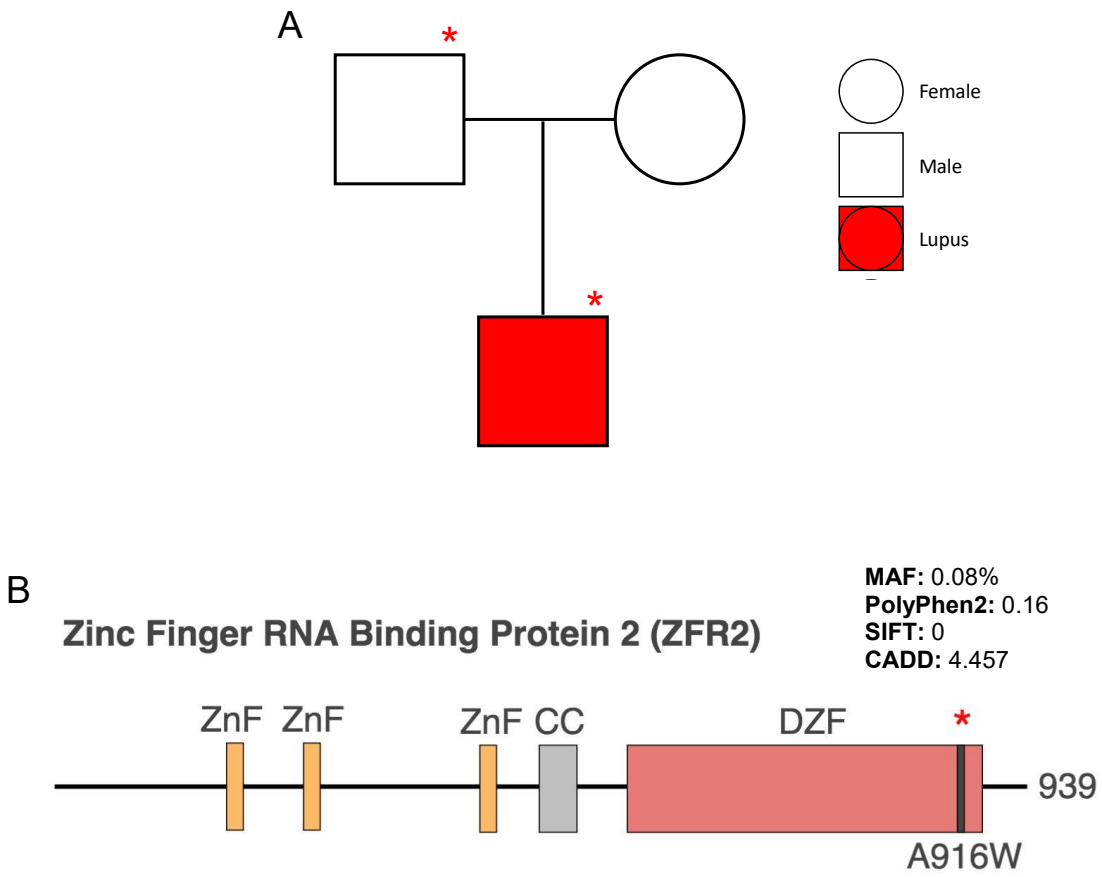


Figure 3.6. Familial segregation of the ZFR2 variant in Family B.

(A) Family tree depicting the segregation of the ZFR2 A916W variant in Family B. (B) Two-dimensional schematic of ZFR2, demonstrating its domains and the location of the A916W variant.

3.2.4 DHX58 is a viral RNA sensor that induces type I IFN and may interact with ZFR2

The second candidate rare gene variant that also segregates with disease in Family A is in a protein called LGP2 (laboratory of genetics and physiology 2), which is encoded by *DHX58 (DExH-Box Helicase 58)*. This variant lies within the helicase domain (Figure 3.7) and resides next to an predicted acetylation site. LGP2 is a RIG-1-like receptor (RLR), which is a family of DExD/H box RNA helicases which sense pathogen-associated molecular patterns (PAMPs) within viral dsRNA to initiate innate immunity and inflammation to control infection (Gu et al., 2015). These RLR proteins can detect a number of viruses and then interact with MAVS (mitochondrial antiviral-signalling protein) to activate antiviral signalling pathways (Wu and Hur, 2015). There are three proteins that are part of the RLR family that have been identified to date; LGP2, RIG-I (retinoic acid-inducible gene I) and MDA5 (melanoma differentiation associated factor 5), which are expressed in most tissues where they can initiate innate immune activation through the induction of type I-IFN pathways and NFκB signalling (Sayed et al., 2017). These proteins are not only similar in function, but also structure and sequence homologies, with each protein sharing a homologous DExH box helicase domains that bind dsRNA, suggesting them as an evolutionary conserved gene family.

Aberrant RLR signalling and defects in RLR expression have been implicated in autoimmune disorders such as type 1 diabetes, multiple sclerosis, psoriasis, selective IgA deficiency, dilated cardiomyopathy and SLE (Kato and Fujita, 2015). Interestingly, a missense variant in *Ifih1,* the gene which encodes MDA5, causes mice to develop lupus-like symptoms (nephritis and skin rash) with a type I IFN signature and aberrant IL-6 and TNFα production. Furthermore, a gain of function variant in *IFIH1* caused early onset SLE and IgA deficiency in one patient, and

Aicardi-Goutières syndrome (AGS) in another patient (Rice et al., 2014). Finally, results from the String Consortium, 2018, suggests that Zfr2 may interact with Dhx58 in mice, through a common binding partner Eif2ak2, suggesting that ZFR2 and DHX58 function in a similar pathway. Eif2ak2, or protein kinase R (PKR), is an enzyme that is activated by dsRNA and induced by interferons, and is known to play a role in the innate immune response to viral infection (Balachandran et al., 2000).

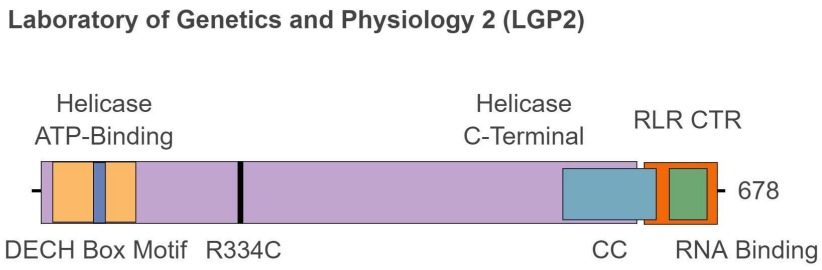


Figure 3.7. Protein schematic of LGP2.

Protein schematic of LGP2 demonstrating the identified variant (R334C) lies within the helicase domain. RLR CTR: RIG-I-like receptor C-terminal regulatory domain, CC: Coiled coil domain.

3.2.5 SMG7 regulates mutated RNA transcripts and is an SLE-risk gene

The third candidate gene is in a gene called *SMG7 (suppressor with morphological defects in genitalia 7),* with the variant residing within the tetratricopeptide-like domain (Figure 3.8). The encoded protein plays an essential role in nonsense mediated mRNA decay (NMD) (Jo et al., 2017), a pathway which functions to post-transcriptionally regulate transcriptomes. NMD removes mRNAs that arise when mRNAs are truncated, have errors in splicing, or nonsense and frameshift variants. It does this by recruiting mRNA decay enzymes, such as POP2 (Poly(A) ribonuclease), which prevent the accumulation of the truncated and mutated transcripts which are toxic and can have deleterious functions (Karousis et al., 2016). T cell receptors and immunoglobulin genes are some of the main substrates for NMD since they undergo programmed rearrangements and recombination, and Ig genes undergo somatic hypermutation, which expectedly results in premature translation-termination codons (PTCs) in TCR and Ig mRNA. These PTC containing mRNAs encode non-functional immune receptors and are therefore degraded by NMD (Wang et al., 2002).

Using GWAS, Deng et al., 2016 demonstrated an association between SNPs in *SMG7* and SLE incidence, establishing *SMG7* as a novel SLE-risk gene. Some of these risk alleles were associated with a decrease in *SMG7* transcript levels in PBMCs isolated from patients. There was also a correlation between *SMG7* mRNA levels and ANA titres, as patients with high ANA titres had lower *SMG7* transcript levels. Furthermore, inhibition of SMG7 expression in PBMCs using small interfering RNAs (siRNAs) resulted in increased ANA titres, and inhibited NMD activity resulting in enhanced expression of PTC-containing transcripts (Deng et al., 2016). Deng et al., 2016 hypothesised that the lower *SMG7* transcript levels may lead to the RNA-

protein complex accumulation and subsequent autoantibody production and high ANA titres. A team in the United States also described another role of SMG7, in the regulation of the tumour suppressor gene p53. SMG7 has the ability to bind, stabilise and activate p53 after DNA damage, as well as promote the phosphorylation of MDM2, an E3 ubiquitin ligase that can repress p53-mediated transcriptional activation and induce its degradation (Luo et al., 2016). Finally, in January 2017, a group from South Korea demonstrated that frameshift variants in SMG7 in gastric cancers and colorectal cancers may alter NMD in cancer cells, contributing to cancer pathogenesis (Jo et al., 2017).

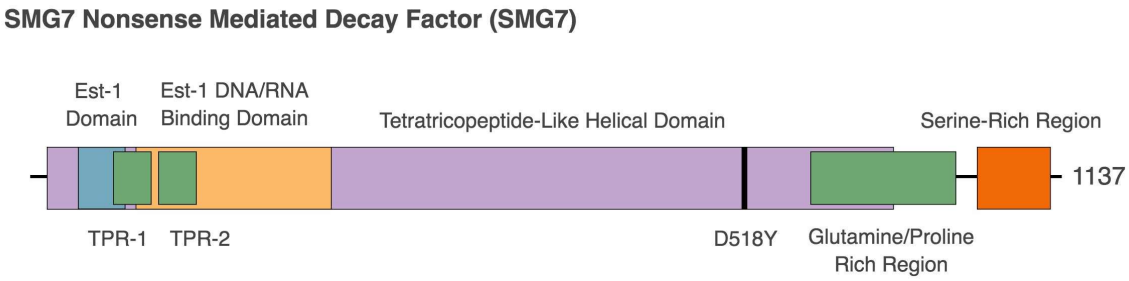


Figure 3.8. Protein schematic of SMG7.

Protein schematic of SMG7 demonstrating the SNV lies within the tetratricopeptide-like helical domain. TPR: tetracopeptide, EST: Ever shorter telomerases.

3.2.6 The three candidate variants are RNA regulators and segregate with disease

It is very intriguing that the three candidate gene variants are in proteins that regulate RNA and DNA, which suggests that these proteins may act together in similar pathways. Furthermore, dysfunction in DNA and RNA sensing and processing has been linked to SLE pathogenesis, making these candidate gene variants particularly interesting. Figure 3.9 demonstrates that these candidate gene variants segregate with disease in Family A. Unfortunately, we were unable to determine if other relatives possess these candidate gene variants due to poor DNA quality.

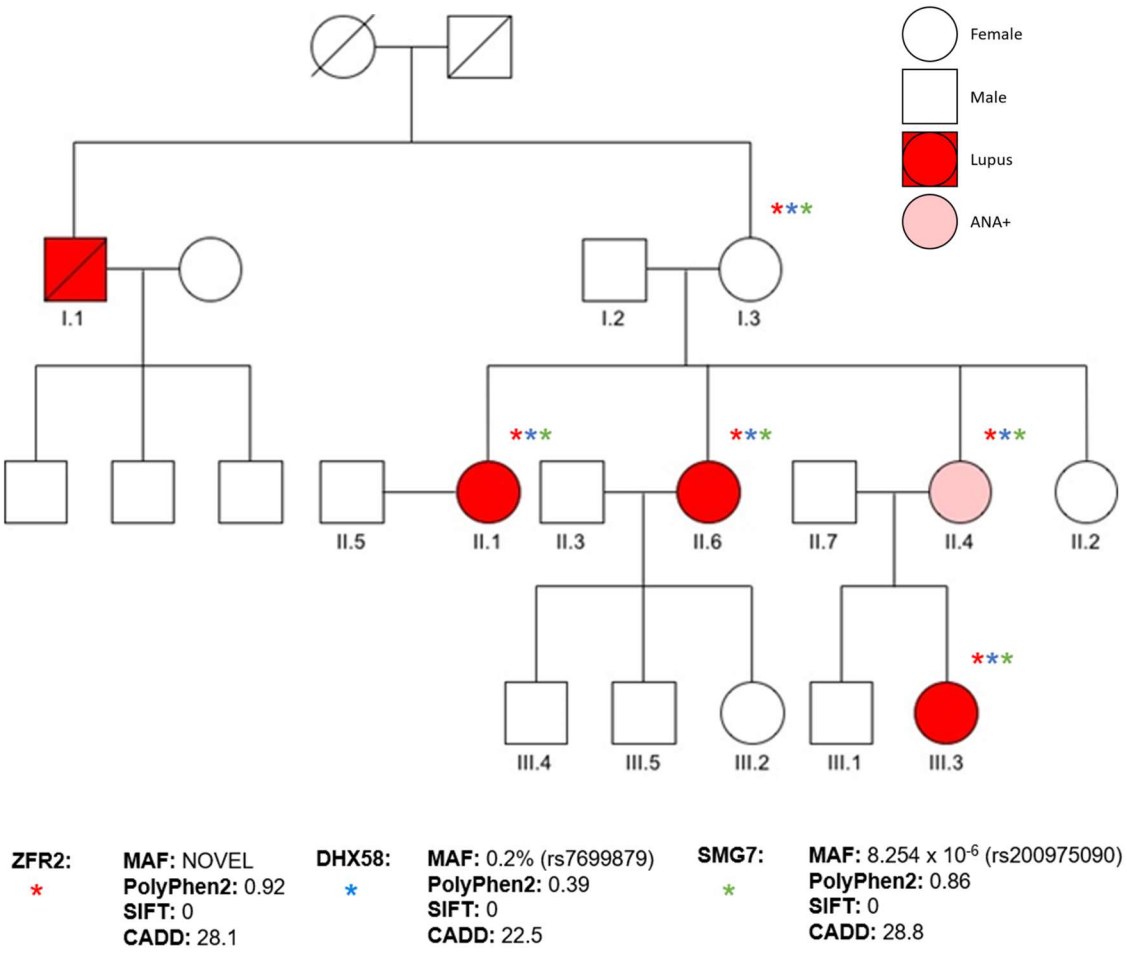


Figure 3.9. Familial segregation of the ZFR2, DHX58 and SMG7 SNVs in Family A.

Family tree depicting the segregation of the ZFR2, DHX58 and SMG7 variants.

3.3 Phenotyping of patient peripheral blood mononuclear cells and plasma

Immunophenotyping enables a heterogenous population of cells to be analysed so that the presence and proportions of different immune cells can be investigated. The Centre for Personalised Immunology (CPI) has designed and optimised an in-depth immunophenotyping panel to analyse a number of immune populations (Figure 3.10), including T cell populations, B cell populations, NK cells and monocytes. This panel has been performed on approximately 100 healthy controls, enabling cellular phenotypes in patients to be compared to a large cohort of healthy controls, so significant cellular phenotypes can be identified.

Immunophenotyping of the individuals in Family A demonstrated a significant (p=0.0025) increase in circulating effector T_{FH} cells (Figure 3.11), and a significant (p=0.00002) decrease in $CD16^+$ myeloid dendritic cells (mDC) (Figure 3.12) compared to healthy controls, however these populations were normal in the proband of Family B (data not shown). No phenotype in B cells was observed in patients from Family A or Family B compared to healthy controls. Interestingly, Figure 3.13 demonstrates that the individuals from Family A and Family B both had increased regulatory T cells (Family A p=0.0034), as well as an expanded $CD3^+$ $CD4^+$ $CD57^+$ population (Figure 3.14) compared to the healthy controls (Family A p=0.0023) or the relative from Family A that does not carry the ZFR2 variant (depicted in grey).

CD57 was first identified on a subset of NK cells, however whilst the patients in Family A has increased NK cells (p=0.0177), they did not have expanded $CD57^+$ NK cells (Figure 3.15). CD57 has subsequently been identified on neural cells, $CD4^+$ and $CD8^+$ T cells and germinal centre T_{FH} cells, and has most widely been explored as a marker for T cell cytotoxicity (Alshekaili et

al., 2018a), clonal exhaustion and proliferative senescence (Lopez-Verges et al., 2010b), and is associated with Granzyme B expression. In the healthy controls, the majority of their $CD4^+$ $CD57^+$ population expressed PD-1 (and therefore may be circulating T_{FH} cells), however in the patients less than half this population expressed PD-1 (Figure 3.16), suggesting that this population is unlikely to be circulating cytotoxic T_{FH} cells (Alshekaili et al., 2018a). Interestingly however, Figure 3.18 demonstrates that almost all of the $CD4^+$ $CD57^+$ cells express granzyme B in the patients from Family A, with the patient's $CD4^+$ $CD57^+$ cells expressing even higher amounts of granzyme B compared to the healthy controls (p=0.0480), suggesting that these cells may be a subset of cytotoxic $CD4^+$ T cells. Due to limited PBMCs, this granzyme B assay was unable to be performed on the proband of Family B. Immunophenotyping of all other patient immune cell populations (Figure 3.10) revealed frequencies within normal limits.

Finally, cytokine profiles of the patient's plasma from both Family A and Family B were determined using cytokine cytometric bead array (Figure 3.19 and Appendix A). Results from the CBA showed a significant increase in IL-2 (Family A p=0.0303) and IFN-γ (Family A p=0.0480) in the individuals carrying the ZFR2 variants from both families, with the exception of III.3 who is on Arcoxia (etoricoxib), which is an anti-inflammatory drug that reduces proinflammatory cytokine synthesis (Nadda et al., 2013). IL-4 was also increased in the patient's plasma from Family A (p=0.0177), however no changes were observed in IL-10, IL-6 or TNF (Appendix A). No differences in plasma IL-4, IL-10, IL-6 or TNF levels were observed for Family B.

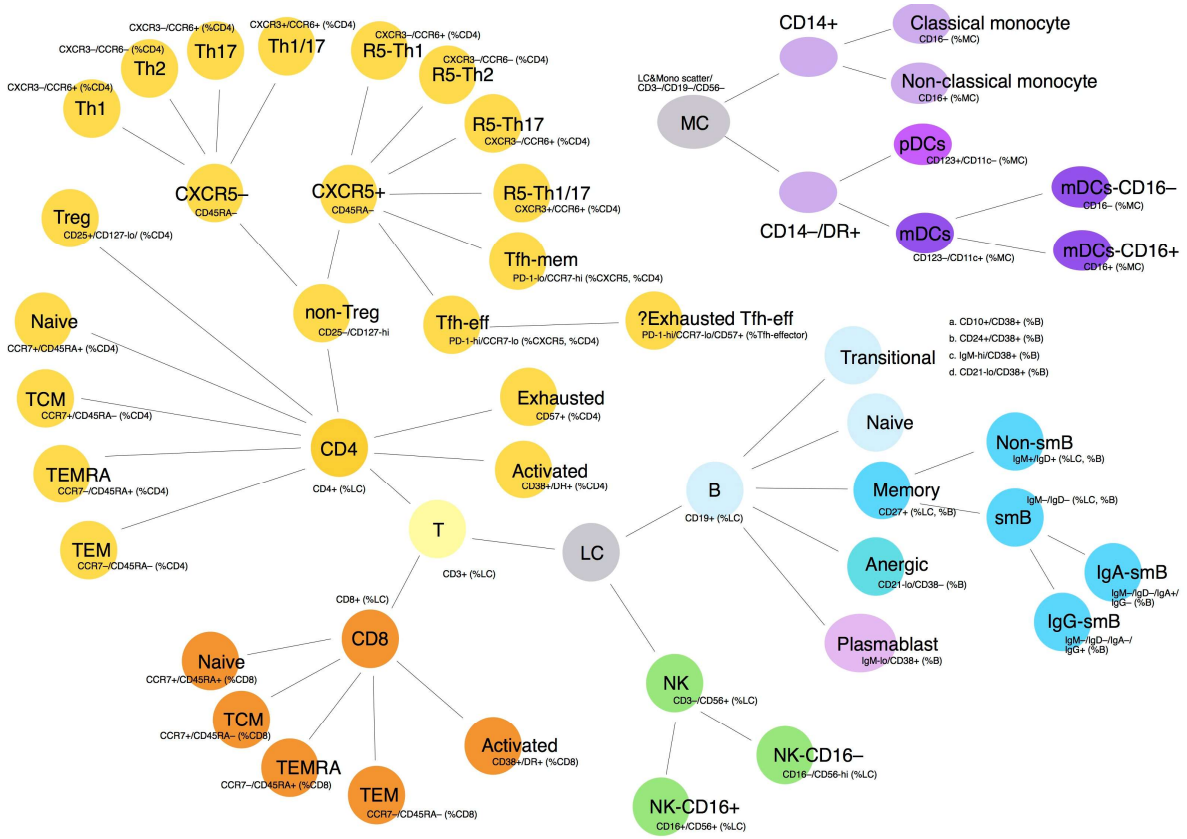


Figure 3.10. Schematic of the Centre for Personalised Immunology immunophenotyping panel.

Overview of the lymphocyte populations analysed in the Centre for Personalised Immunology (CPI) PBMC phenotyping panel.

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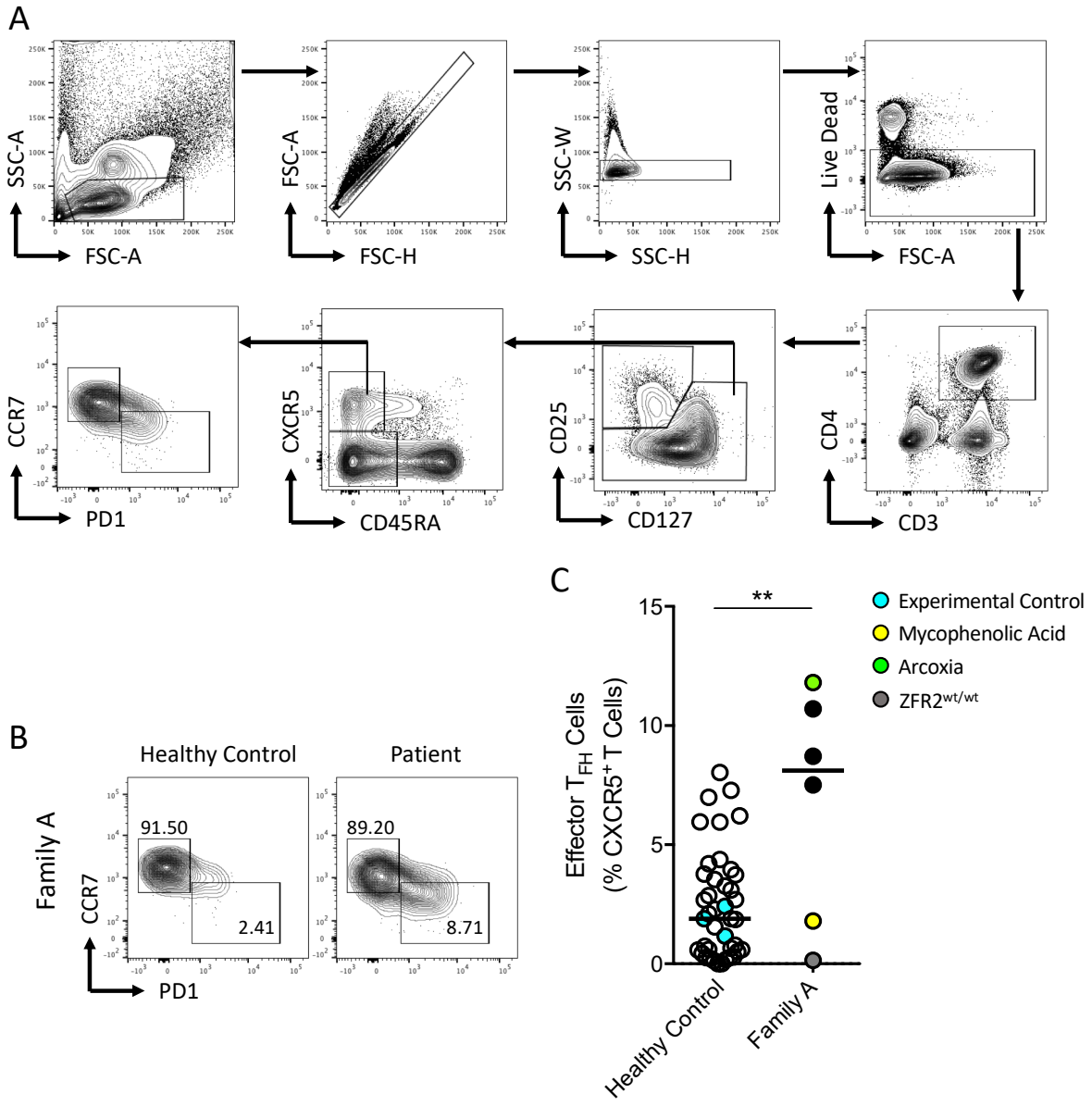


Figure 3.11. Flow cytometric analysis of effector T_{FH} cells in Family A.

(A) Gating strategy used to define T_{FH} effectors in Family A. (B) Representative plot of T_{FH} effectors in a healthy control and patient from Family A. (C) Column graph representing the median percentage of T_{FH} effectors in the individuals from Family A, compared to the cohort of CPI healthy controls. The aqua dots represent experimental control PBMCs from healthy donors that were investigated on the same day with the patient PBMCs. The yellow dot represents the patient treated with mycophenolic acid. The green dot represents the individual who is treated with Arcoxia. The grey dot represents a relative of Family A who does not carry the ZFR2 mutation. Each dot represents an individual. (**p≤0.01) Mann-Whitney U test. (is $ZFR2^{wt/wt}$).

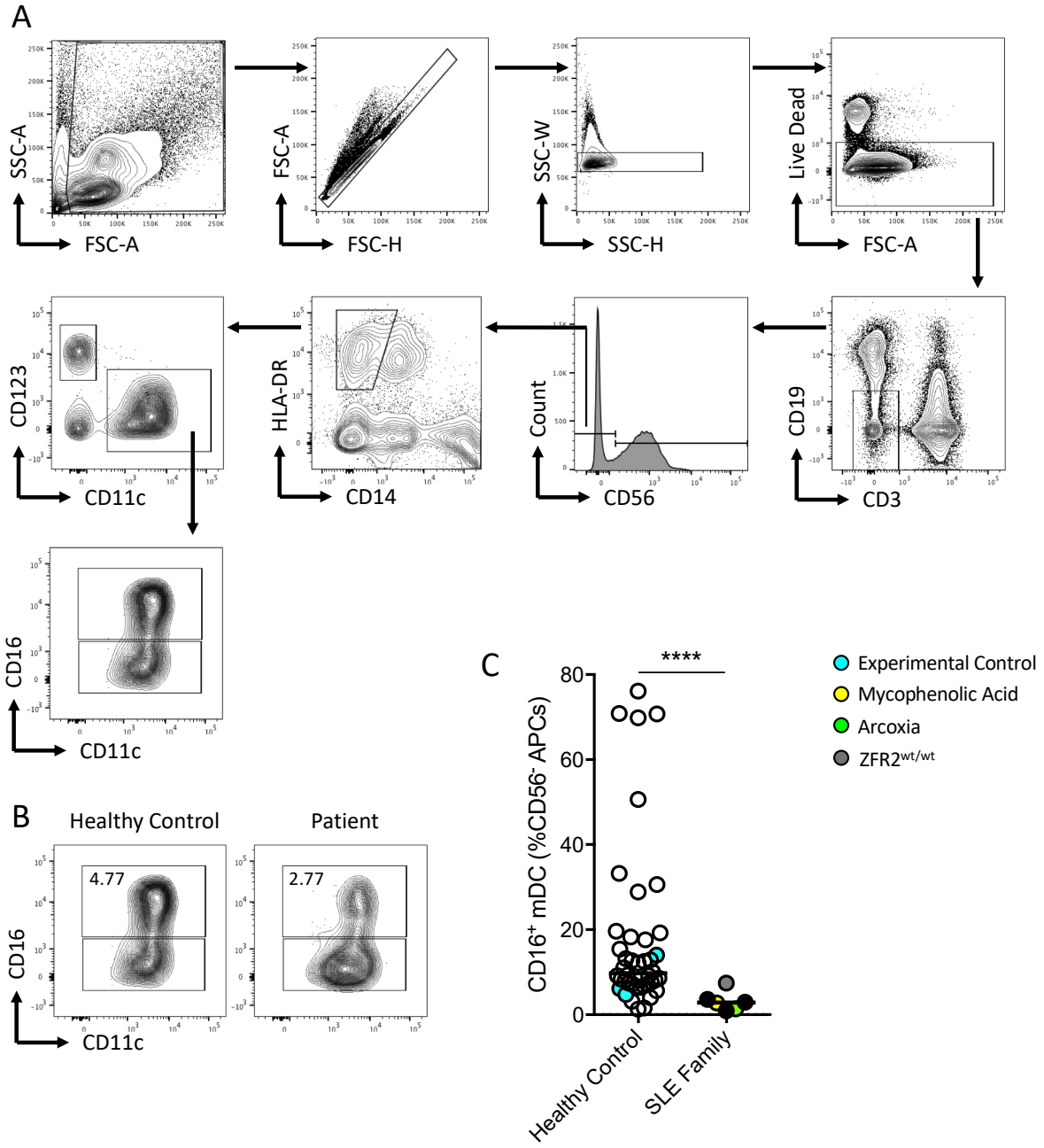


Figure 3.12. Flow cytometric analysis of CD16^{+} mDCs in Family A.

(A) Gating strategy used to define CD16^{+} mDCs in Family A. (B) Representative plot of mDCs in a healthy control and patient from Family A. (C) Column graph representing the median percentage of mDCs in the individuals from Family A, compared to the cohort of CPI healthy controls. The aqua dots represent experimental control PBMCs from healthy donors that were investigated on the same day with the patient PBMCs. The yellow dot represents the patient treated with mycophenolic acid. The green dot represents the individual who is treated with Arcoxia. The grey dot represents a relative of Family A who does not carry the ZFR2 mutation. Each dot represents an individual. (****p≤0.0001) Mann-Whitney U test.

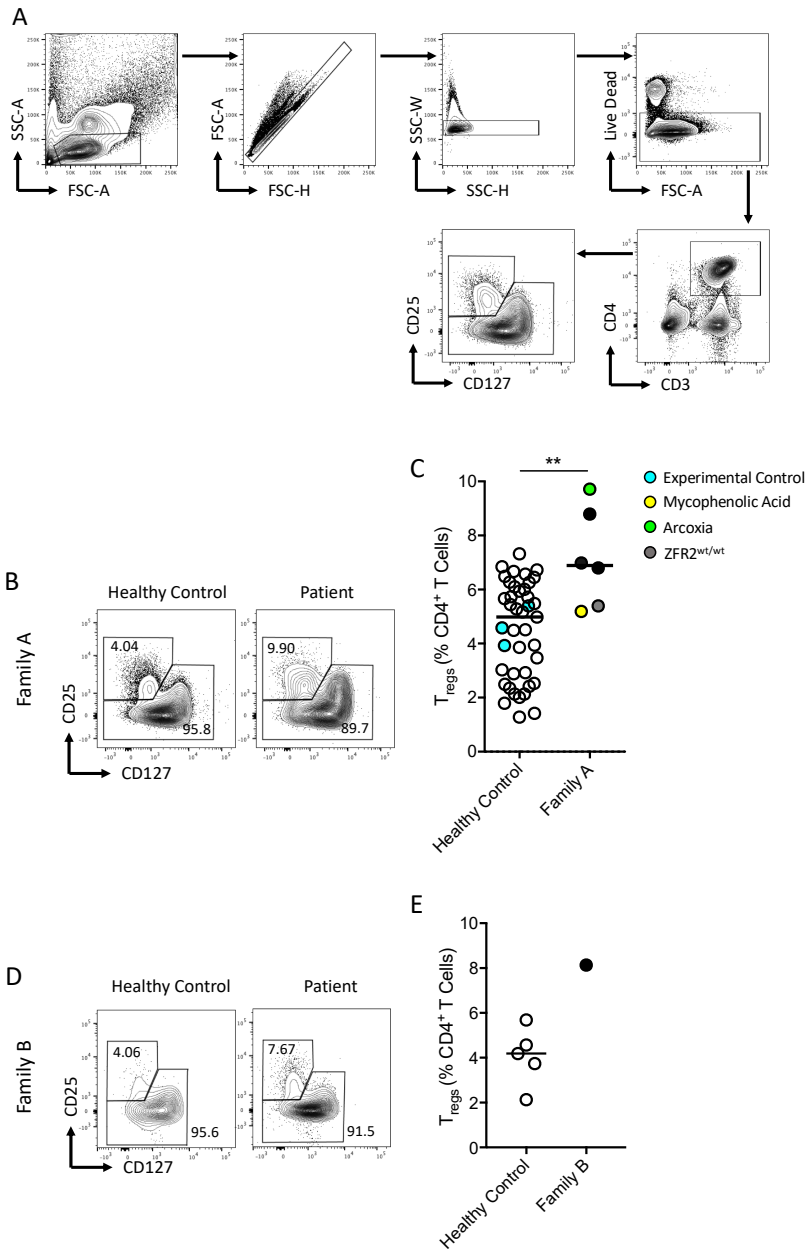


Figure 3.13. Flow cytometric analysis of T_{regs} in Family A and Family B.

(A) Gating strategy used to define T_{regs} in Family A and Family B. (B) Representative plot of T_{regs} in a healthy control and patient from Family A. (C) Column graph representing the median percentage of T_{regs} in the individuals from Family A, compared to the cohort of CPI healthy controls. (D) Representative plot of T_{regs} in a healthy control and the patient from Family B. (E) column graph representing the percentage of T_{regs} in healthy controls and the patient from Family B. The aqua dots represent experimental control PBMCs from healthy donors that were investigated on the same day with the patient PBMCs. The yellow dot represents the patient treated with mycophenolic acid. The green dot represents the individual who is treated with Arcoxia. The grey dot represents a relative of Family A who does not carry the ZFR2 mutation. Each dot represents an individual. (**p≤0.01) Mann-Whitney U test.

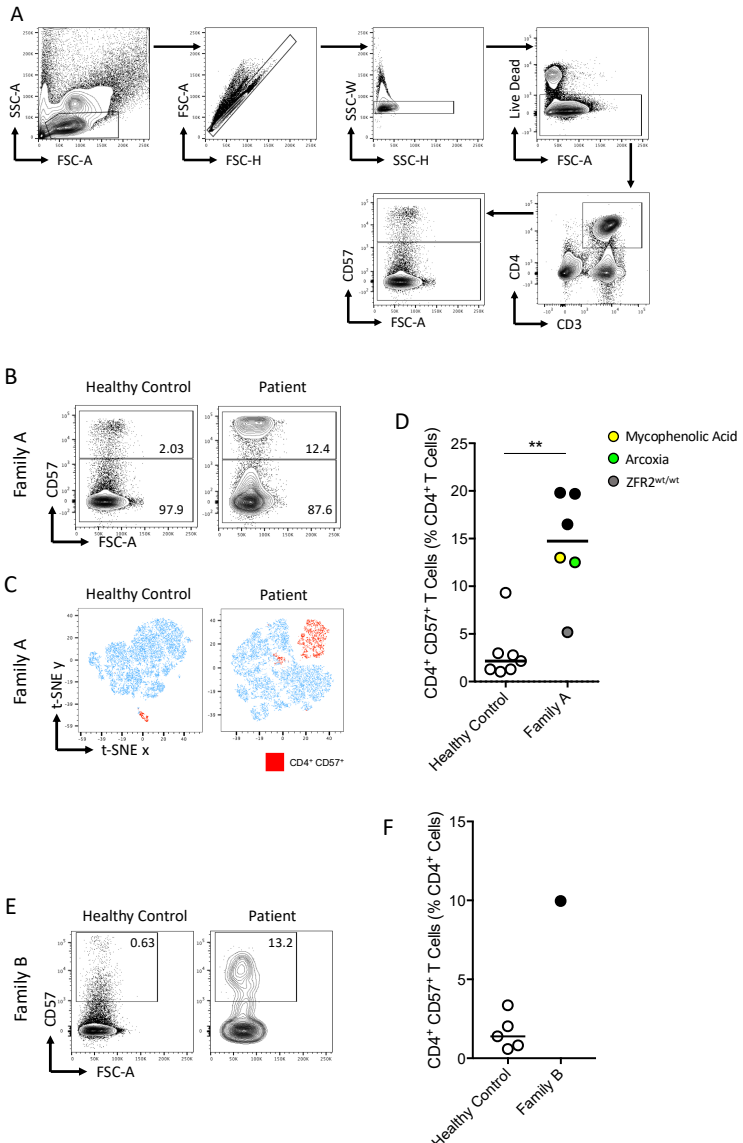


Figure 3.14. Flow cytometric analysis of CD4⁺ CD57⁺ cells in Family A and Family B.

(A) Gating strategy used to define CD4⁺ CD57⁺ cells in Family A and Family B. (B) Representative plot of CD4⁺ CD57⁺ cells in a healthy control and patient from Family A. (C) t-SNE plot demonstrating a representative plot of the CD4⁺ CD57⁺ expanded population in a healthy control and patient from Family A. (D) Column graph representing the median percentage of CD4⁺ CD57⁺ cells in the individuals from Family A, compared to the cohort of CPI healthy controls. (E) Representative plot of CD4⁺ CD57⁺ cells in a healthy control and the patient from Family B. (F) Column graph representing the percentage of CD4⁺ CD57⁺ cells in the healthy controls and patient from Family B. The aqua dots represent experimental control PBMCs from healthy donors that were investigated on the same day with the patient PBMCs. The yellow dot represents the patient treated with mycophenolic acid. The green dot represents the individual who is treated with Arcoxia. The grey dot represents a relative of Family A who does not carry the ZFR2 mutation. Each dot represents an individual. (**p≤0.01) Mann-Whitney U test.

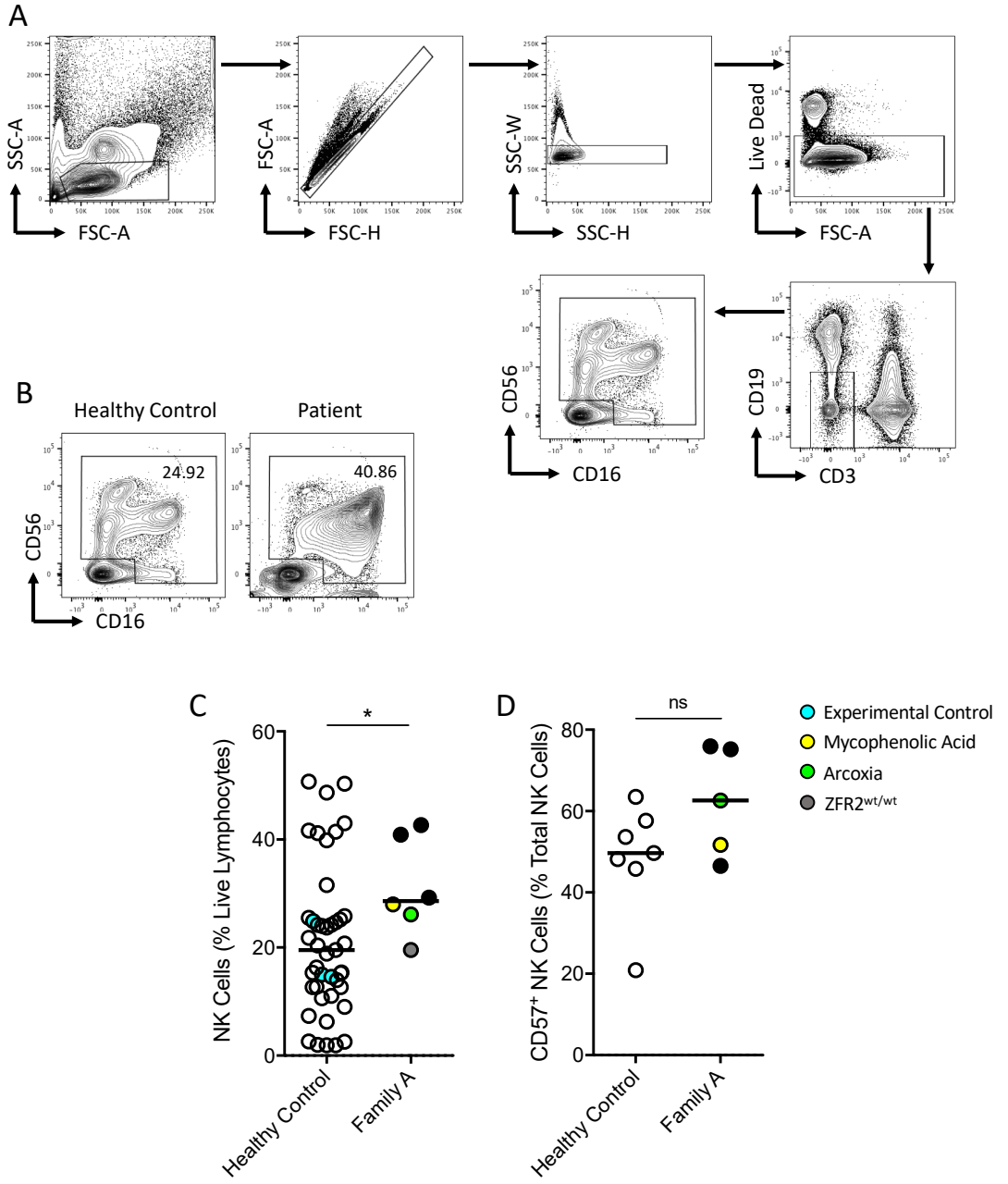


Figure 3.15. Flow cytometric analysis of NK cells in Family A.
(A) Gating strategy used to define NK cells in Family A. (B) Representative plot of NK cells in a healthy control and patient from Family A. (C) Column graph representing the median percentage of NK cells in the individuals from Family A, compared to the cohort of CPI healthy controls. The aqua dots represent experimental control PBMCs from healthy donors that were investigated on the same day with the patient PBMCs. The yellow dot represents the patient treated with mycophenolic acid. The green dot represents the individual who is treated with Arcoxia. The grey dot represents a relative of Family A who does not carry the ZFR2 mutation. Each dot represents an individual. (*p≤0.05) Mann-Whitney U test.

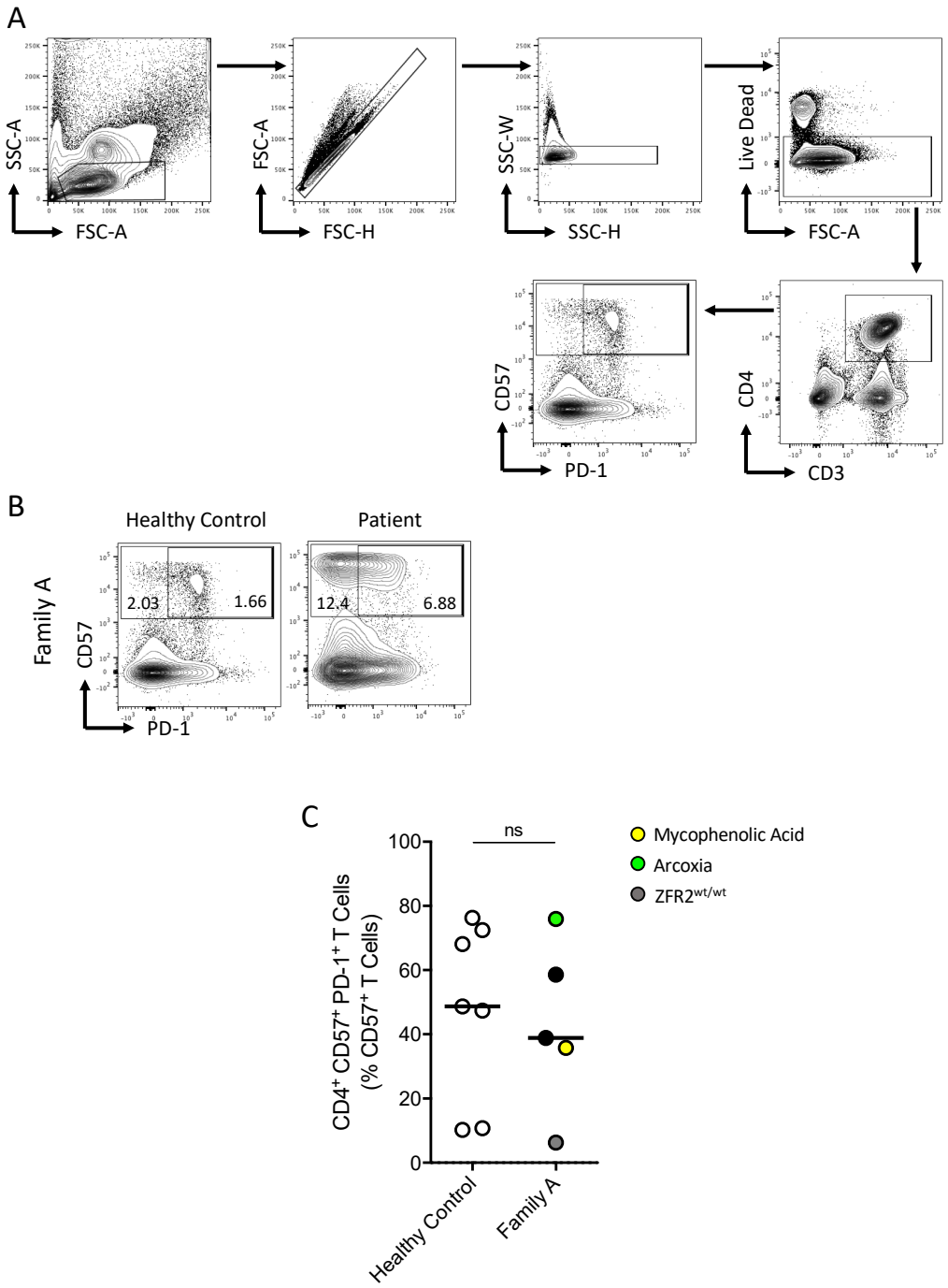


Figure 3.16. Flow cytometric analysis of PD-1 expression on CD4⁺ CD57⁺ cells in Family A.

(A) Gating strategy used to determine PD-1 expression on CD4⁺ CD57⁺ cells in Family A. (B) Representative plot of CD4⁺ CD57⁺ cells and CD4⁺ CD57⁺ PD-1⁺ in a healthy control and patient. (C) Column graph representing the median percentage of CD4⁺ CD57⁺ PD-1⁺ cells in the individuals from family A (out of CD57⁺ T cells), compared to cohort of CPI healthy controls. The yellow dot represents the patient treated with mycophenolic acid. The green dot represents the individual who is treated with Arcoxia. The grey dot represents a relative of Family A who does not carry the ZFR2 mutation. Each dot represents an individual. (**p≤0.01) (ns=not significant) Mann-Whitney U test.

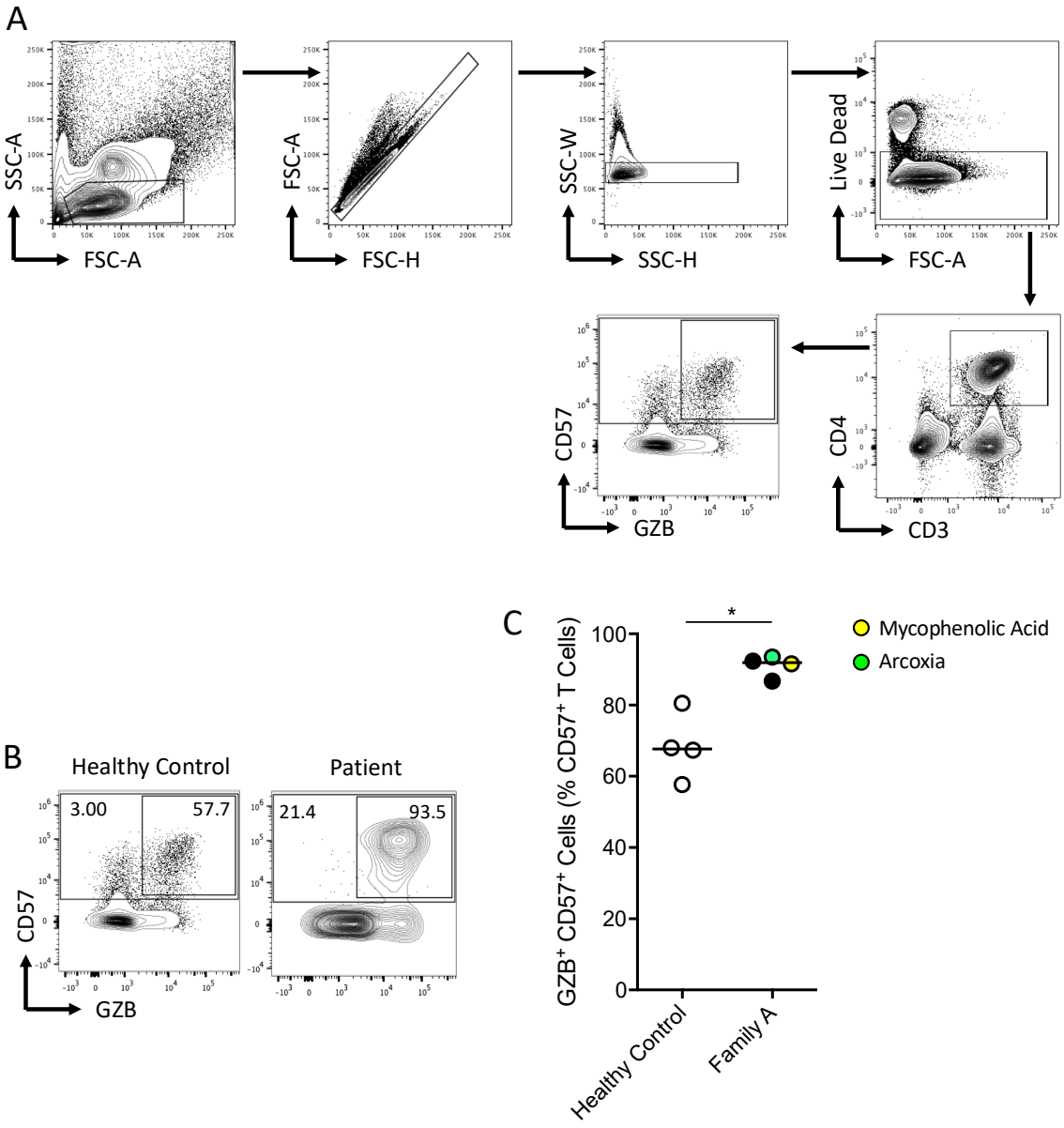


Figure 3.17. Flow cytometric analysis of GZB expression on CD4⁺ CD57⁺ cells in Family A.

(A) Gating strategy used to determine granzyme B (GZB) expression on CD4⁺ CD57⁺ cells. (B) Representative plot of GZB⁺ CD4⁺ CD57⁺ cells in a healthy control and patient. (C) Column graph representing the median percentage of GZB⁺ CD4⁺ CD57⁺ cells in the individuals from Family A compared to the cohort of CPI healthy controls. The yellow dot represents the patient treated with mycophenolic acid. The green dot represents the individual who is treated with Arcoxia. Each dot represents an individual. (*p≤0.05) Mann-Whitney U test.

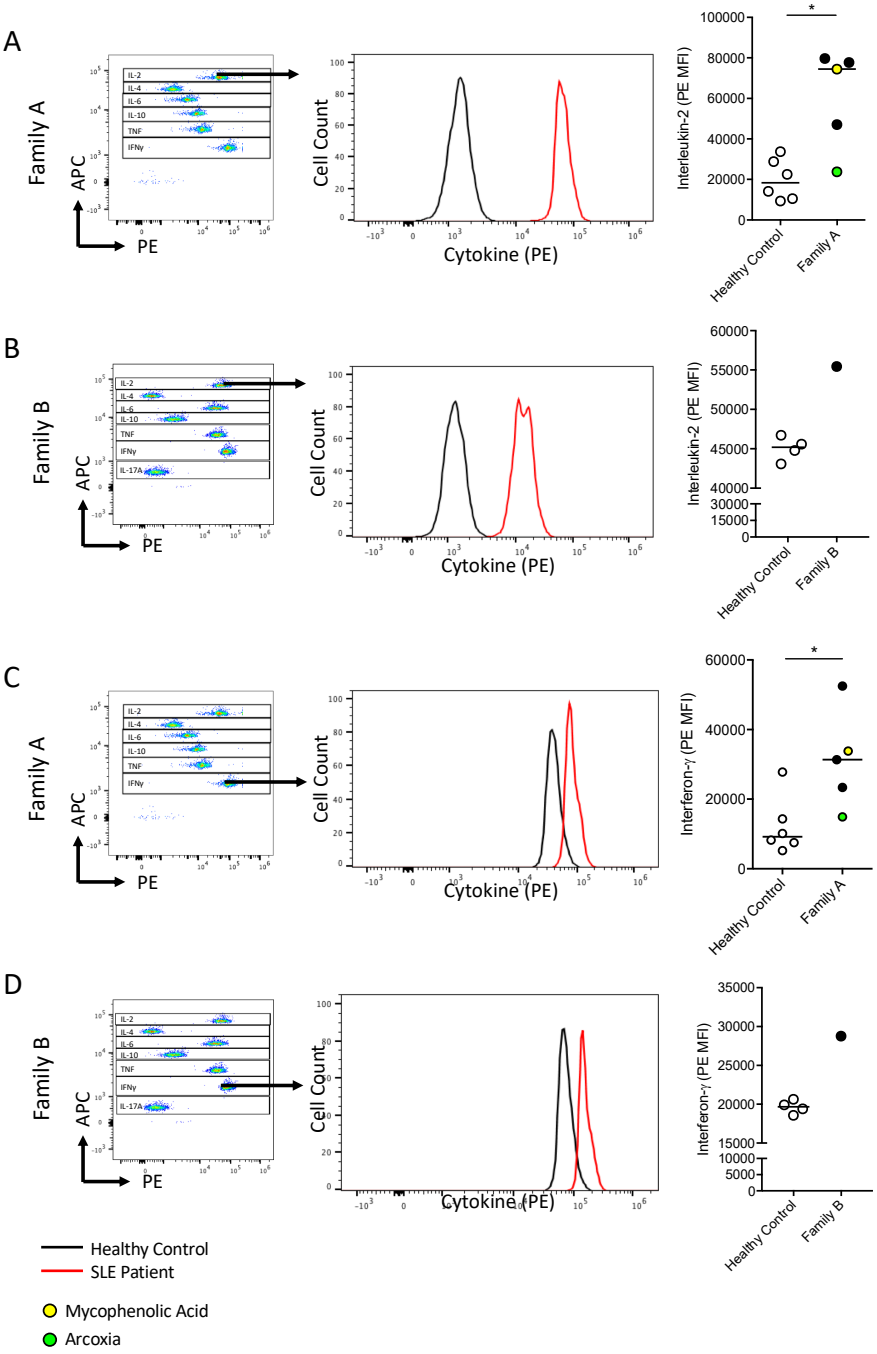


Figure 3.18. Quantification of inflammatory cytokines in patient plasma.

APC fluorescence intensity indicates the cytokine measured, and the amount of PE corresponds to the amount of analyte bound, and thus the amount of cytokine present. Quantification of (A) IL-2 in Family A, (B) IL-2 in Family B, (C) IFNγ in Family A and (D) IFNγ in Family B using the Human Th1/Th2/Th17 CBA, depicted as a representative histogram of a healthy control and patient, and as a column graph demonstrating the median PE MFI of each cytokine. The yellow dot represents the patient treated with mycophenolic acid. The green dot represents the individual who is treated with Arcoxia. Each dot represents an individual. (*p≤0.05) Mann-Whitney U test.

3.4 ZFR2 gene variant alters protein function in vitro

3.4.1 ZFR2^{A771T} does not alter protein conformation.

The A771T variant lies within the DZF domain of ZFR2, which as mentioned above is an important domain that allows the protein to dimerise with the DZF domain of the paralogs. To determine whether the mutation affects the conformation of the DZF domain, the DZF domains of ILF2 and ILF3 were first co-expressed and crystalized by Jeff Babon at the Walter and Eliza Hall Institute of Medical Research, Melbourne University. This reveals that ILF2 and ILF3 interact through their DZF domain (Figure 3.19A) and bind adenosine triphosphate (ATP) in the catalytic cleft of the DZF domain (circled), confirming the findings of Cook and Wolkowicz, 2012 (Wolkowicz and Cook, 2012). Neither wild-type or mutant ZFR2 was able to be expressed as a soluble protein or as a homodimer as the crystal structure was unstable, however both were able to be co-expressed and stablilised with ILF2 (Figure 3.19 B and C). The substitution of the threonine for the alanine did not appear to affect the conformational structure of the DZF domain, however it was determined that the mutation resides within the catalytic cleft of the DZF domain (approximately 3.8 angstroms from where nucleotides are bound). There was also no ATP bound in the catalytic cleft of ZFR2, however there was a sulphate ion bound at this site in both the wild-type and mutant proteins which may have blocked ATP. Given the sequence homology between ILF2 and ILF3, it is likely that ZFR2 also binds ATP in this catalytic cleft, as well as other nucleotides, and Jeff Babon hypothesised that the hydrogen bond formed with the threonine substitution could strengthen this nucleotide binding.

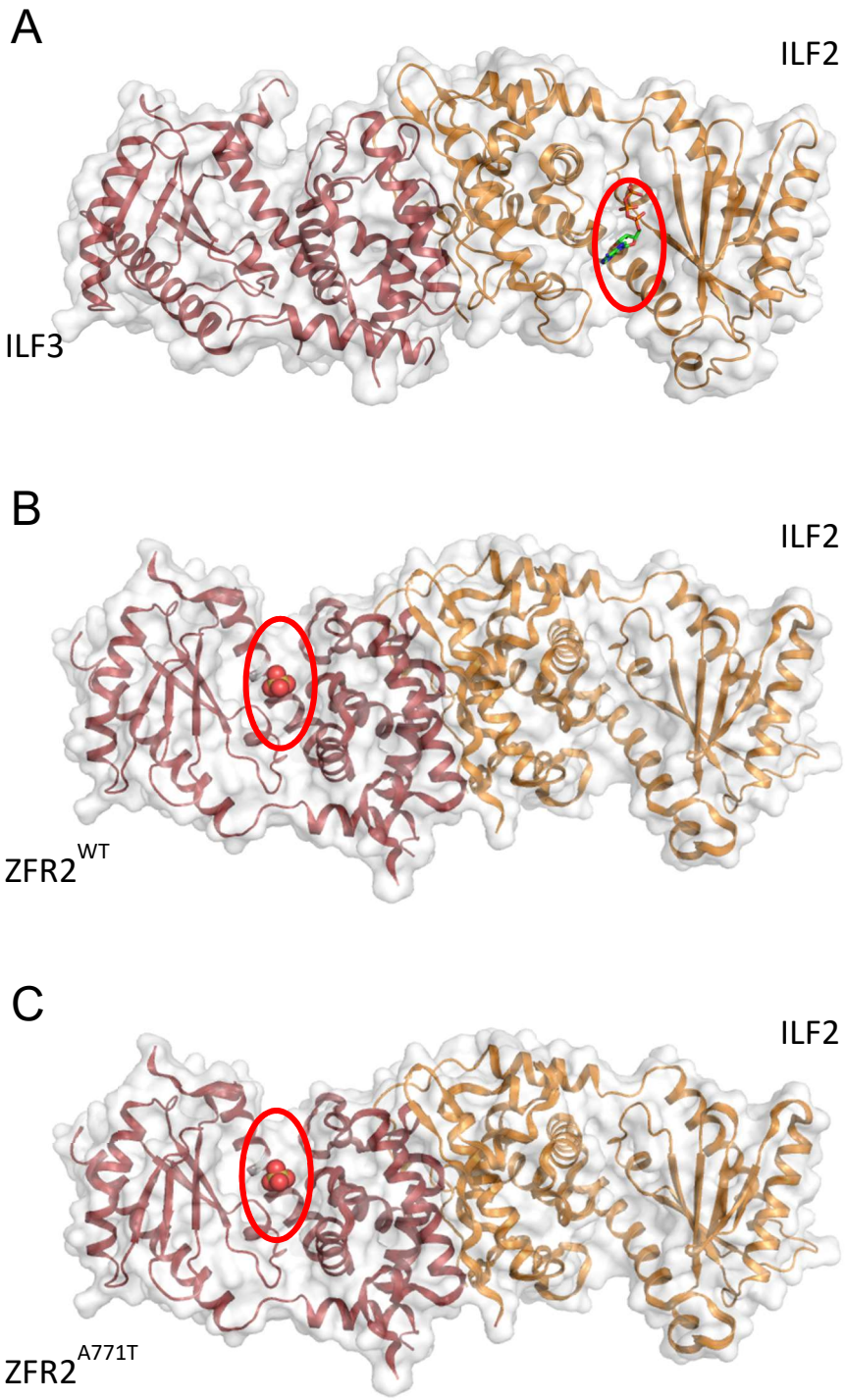


Figure 3.19. Structure of DZF domains of ZFR2 and paralogs.

(A) Structure of the ILF2 DZF domain in complex with the ILF3 DZF domain, with the catalytic cleft highlighted with the bound ATP. (B) Structure of the ZFR2^{WT} DZF domain in complex with the ILF2 DZF domain with the catalytic cleft highlighted with a bound sulphate. (C) Structure of the ZFR2^{A771T} DZF domain in complex with the ILF2 DZF domain with the catalytic cleft highlighted with a bound sulphate. Protein purification and crystalisation was performed by J. Babon and team.

3.4.2 ZFR2 protein interactions and the effect of the ZFR2^{A771T} variant on interactions

In the literature, human ZFR2 has been shown to have two confirmed protein interactions, the ZFR2 paralog ILF2 and APEX1, a protein that plays an important role in the cellular response to oxidative stress (Di Maso et al., 2015, Grindel et al., 2016, Tell et al., 2005). In an attempt to uncover additional ZFR2 interacting-proteins, and to determine whether the ZFR2^{A771T} variant alters protein-protein interactions, Dr Edmond Linossi from the Walter and Eliza Hall Institute of Medical Research, Melbourne University, used a glutathione-S-transferase (GST) pull-down assay to elucidate interactions between GST tagged ZFR2^{WT}/ZFR2^{A771T} and unknown targets *in vitro.* The GST tagged ZFR2^{WT}/ZFR2^{A771T} protein (known as the "bait" protein) was immobilised on glutathione-agarose beads, and then (unstimulated) Jurkat cell lysate (known as the "prey" proteins) was incubated on the beads. The protein complexes were then eluted from the beads, resolved by SDS-PAGE and stained with Comassie Silver. Finally, the protein bands were excised, digested with trypsin and then analysed by mass spectroscopy.

Figure 3.20 demonstrates the proteins that ZFR2^{WT} interacts with, with each dot representing a protein. The dots in red represent a ≥2-fold change in protein ratio, and the dots in green represent a ≥4-fold change in protein ratio between GST tagged ZFR2^{WT} and GST control. These protein interactions are summarised in Table 3.3, where a ≥2-fold change and a ≥4-fold change in protein ratio (between ZFR2^{WT} and GST control) correspond to a score of -/+ and --/++ respectively, with a negative sign indicating the protein is more enriched with the GST control pull-down, and a positive sign indicating the protein is more enriched in the ZFR2^{WT} pull down. Each of the proteins that ZFR2^{WT} interacts with was inputted into the STRING

Consortium 2018, to identify functional protein association networks. These networks were then summarised in Figure 3.21, with the size of the circle indicating the relevance of the protein networks to an immune related pathway (larger circles being most relevant to the immune system and smaller circles having no known role in the immune system. Information about each protein was obtained from GeneCards Human Gene Database (Weizmann Institute of Science). In summary, a number of immune-related proteins and networks that interact with ZFR2 were uncovered, including the ILF2, ILF3, NFAT, AP-1 and ETS1 network which is responsible for transcriptional activation and expression of cytokine genes in T cells, SOCS3 and CUL5 which are responsible for the negative regulation of cytokines through JAK/STAT, the ADAR family which is a group of RNA-editing enzymes important for type I interferon expression associated with interferonopathies and SLE pathogenesis (Liu et al., 2014a, Wang et al., 2014c), and the hnRNP, LSm families and EDC3 which are RNA binding proteins important for alternative splicing, mRNA stabilisation, transcription and translation. Interestingly, APEX1 was not found to interact with ZFR2 in this assay.

Figure 3.22 demonstrates the proteins that ZFR2^{A771T} interacts with, with dots in red representing ≥2-fold change in protein ratio, and dots in green representing ≥4-fold change in protein ratio between the GST tagged ZFR2^{A771T} and GST control. Each dot represents a protein that ZFR2^{A771T} interacts with. These protein interactions are summarised in Table 3.3, where a ≥2-fold change and a ≥4-fold change in protein ratio (between ZFR2^{A771T} and GST) correspond to a score of -/+ and --/++ respectively, with negative indicating the protein is more enriched with the GST control pull-down, and positive indicating the protein is more enriched in ZFR2^{A771T} pull down. "0" indicates no interaction between ZFR2^{A771T} and that

specific protein. Table 3.3 also depicts whether the protein interaction with $ZFR2^{WT}$ versus $ZFR2^{A771T}$ is significantly different, i.e. there is a ≥4-fold change in the binding of a specific protein between $ZFR2^{WT}$ and $ZFR2^{A771T}$, and is indicated be a "yes" or "no". These have also been summarised in Figure 3.23, which depicts those proteins which there is a ≥4-fold change in the binding of a specific protein between $ZFR2^{WT}$ and $ZFR2^{A771T}$, and is summarised in Figure 3.24, where the protein networks that are dysregulated by the variant are depicted with a red circle. In summary, the ILF2, ILF3, NFAT, AP-1 and ETS1 network and the SOCS3 and CUL5 pathways were the main pathways affected by the A771T variant, however the variant also altered binding to other proteins and protein families including the ANP32 family, which is important for chromatin modification, cellular growth and apoptosis, and CALR which is important for calcium homeostasis in the endoplasmic reticulum.

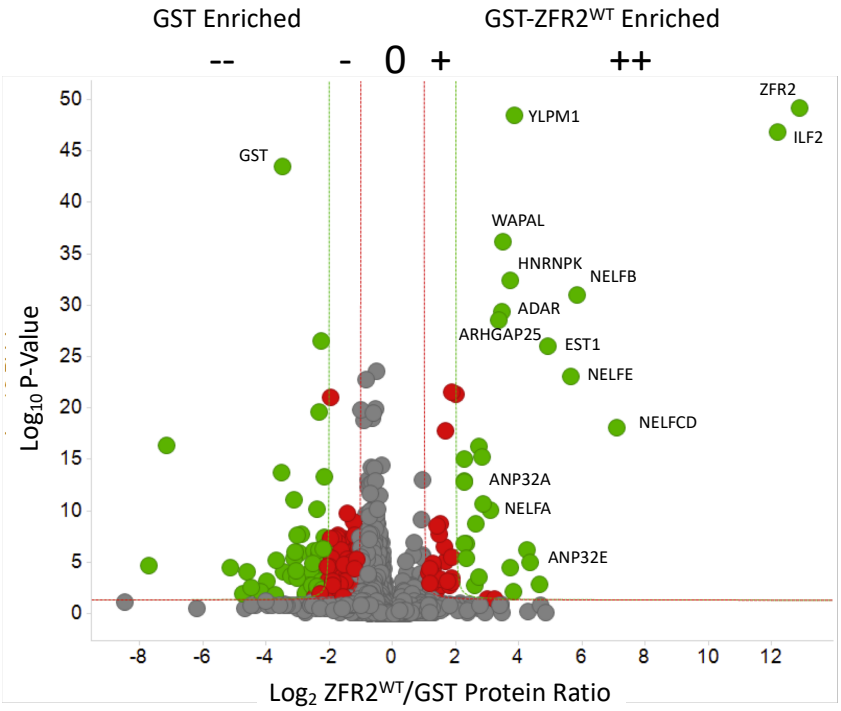


Figure 3.20. ZFR2^{WT} interacting proteins determined by mass spectrometry

Glutathione-S-transferase (GST) was used to determine the physical interaction between a GST tagged probe protein and the target protein (ZFR2^{WT}). Pull-downs were performed on Jurkat lysates in triplicates and the abundance of proteins was measured by mass spectrometry. X axis shows the $\log_2$ of the ZFR2^{WT}/GST protein ratio, and the y axis shows the $\log_{10}$ of the calculated probability (p-value), associated with Student's t-test. Proteins with a Log_{10} p-value ≥2.0 (the red line) indicates a statistically significant ($p<0.01$) difference in binding. Dots in red represent ≥2-fold change in protein ratio, and dots in green represent ≥4-fold change in protein ratio, and correspond to scoring -/+ and --/++ respectively, with negative indicating the protein is more enriched with the GST control pull-down, and positive indicating more enriched in ZFR2^{WT} pull down. Figure produced by E. Linossi.

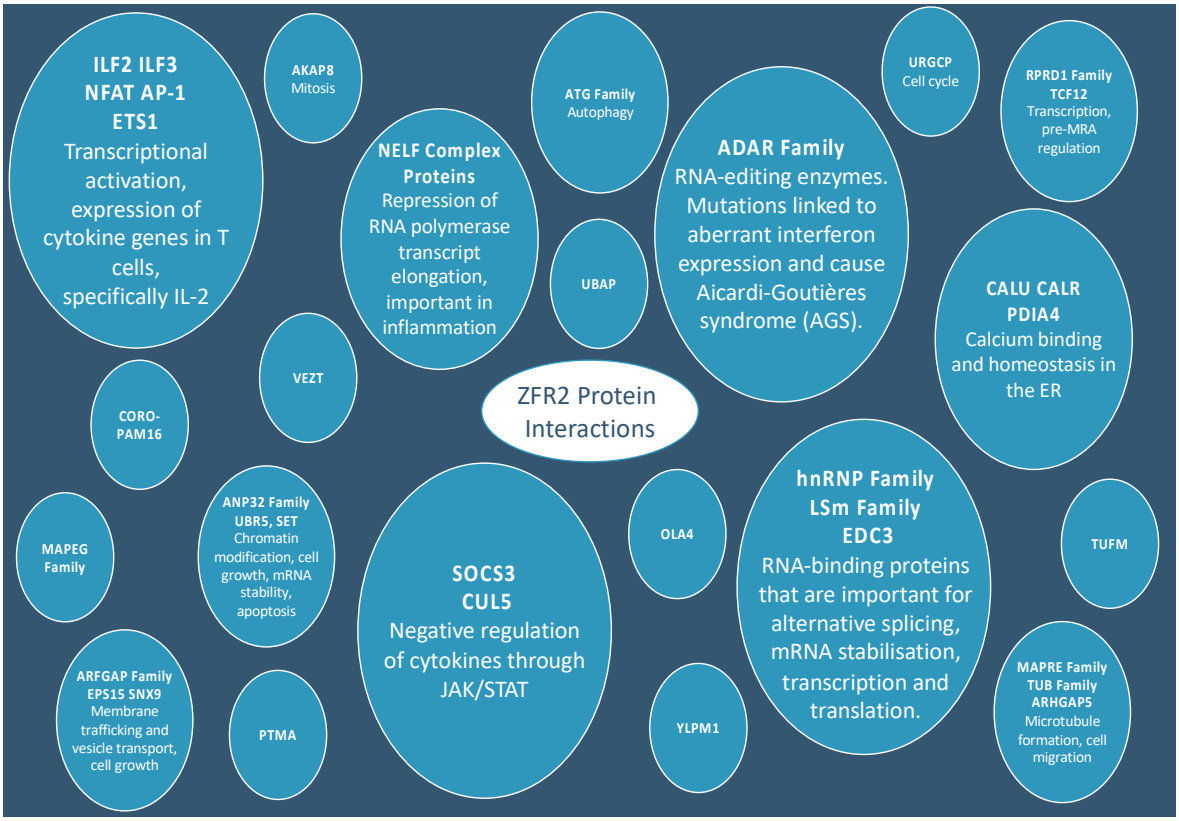


Figure 3.21: Summary of proteins and pathways that ZFR2^{WT} interacts with.

Protein interactions and pathways were determined by inputting all ZFR2^{WT} proteins into the STRING Consortium 2018. Information about each protein was obtained from GeneCards Human Gene Database (Weizmann Institute of Science). The size of the circle indicates the relevance of the protein/s to an immune-related pathway, with the larger circles being most relevant to the immune system and the smallest circles having no known role in the immune system.

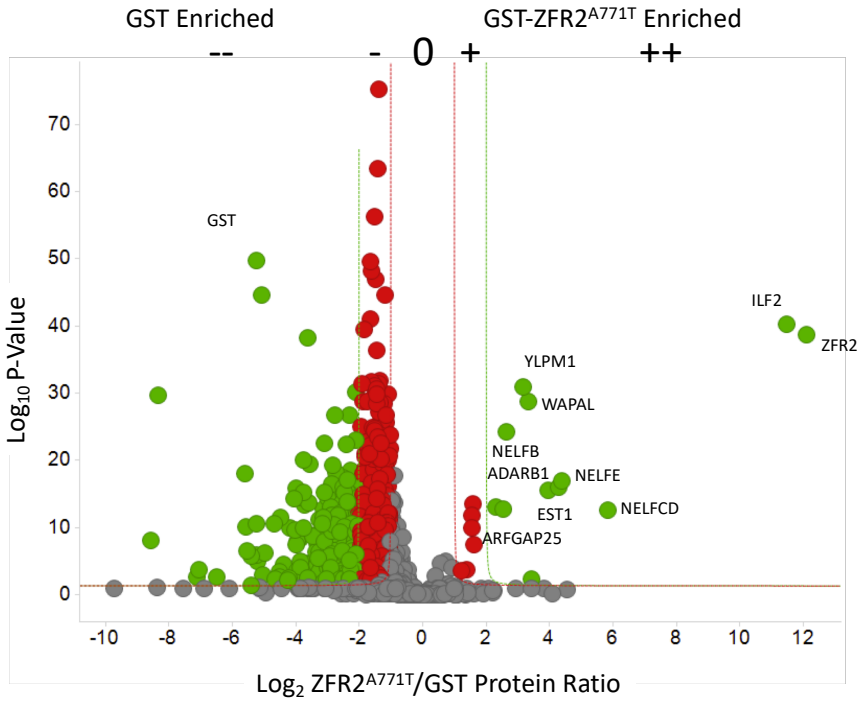


Figure 3.22. ZFR2^{A771T} interacting proteins determined by mass spectrometry.

Glutathione-S-transferase (GST) was used to determine the physical interaction between a GST tagged probe protein and the target protein (ZFR2^{A771T}). Pull-downs were performed on Jurkat lysates in triplicates and the abundance of proteins was measured by mass spectrometry. X axis shows the $\log_2$ of the ZFR2^{A771T}/GST protein ratio, whereas the y axis shows the $-\log_{10}$ of the calculated probability (p-value), associated with Student's t-test. Proteins with a Log_{10} p-value ≥2.0 (the red line) indicates a statistically significant ($p<0.01$) difference in binding. Dots in red represent ≥2-fold change, and dots in green represent ≥4-fold change in protein ratio, and correspond to scoring -/+ and --/++ respectively, with negative indicating the protein is more enriched with the GST control pull-down, and positive indicating more enriched in ZFR2^{A771T} pull down. Figure produced by E. Linossi.

Gene	Significant GST-ZFR2^{WT}	Significant GST-ZFR2^{A771T}	Significant ZFR2^{A771T}/ZFR2^{WT}
ADAR	++	+	No
ADARB1	++	++	No
AKAP2	++	0	Yes
AKAP8	++	0	Yes
ANP32A	+	--	Yes
ANP32B	+	--	Yes
ANP32E	++	--	Yes
ARFGAP1	++	++	No
ARFGAP3	+	0	No
ARHGAP25	++	+	No
ATG101	+	0	No
ATG13	++	+	No
CALR	++	--	Yes
CALU	+	--	No
CORO7-PAM16	+	0	No
CUL5	++	0	Yes
EDC3	+	0	No
EPS15	+	0	No
ETS1	++	++	No
HNRNPK	++	++	No
HNRNPR	+	0	No
ILF2	++	++	No
KHDRBS1	+	0	No
LSM7	++	++	No
MAPRE1	+	--	Yes
MAPRE2	+	0	No
MGST3	+	0	Yes
NELFA	++	+	No
NELFB	++	++	No
NELFCD	++	++	No
NELFE	++	++	No
NFATC3	+	0	Yes
NUDT5	+	0	No
OLA1	+	--	Yes
PDIA4	+	0	No
PTMA	++	--	Yes
RPRD1B	+	0	No
RPRD2	++	+	No
SEC16A	+	0	No

SET	++	--	Yes
SNX9	+	0	No
SOCS3	++	0	Yes
TCF12	++	+	No
TNKS1BP1	+	0	Yes
TTC4	+	0	No
TUBB8	+	0	Yes
TUFM	++	--	Yes
UBAP2	++	0	Yes
UBR5	+	0	No
URGCP	++	0	No
VEZT	+	0	No
WAPAL	++	++	No
YLPM1	++	++	No
ZFR2	++	++	No

Table 3.3. Table representing ZFR2 protein interactions determined by mass spectrometry

Glutathione-S-transferase (GST) was used to determine the physical interaction between a GST tagged probe protein and the target protein ($ZFR2^{WT}$ or $ZFR2^{A771T}$). Pull downs were performed on Jurkat lysates in triplicates and the abundance of proteins was measured by mass spectrometry. Analysis revealed a number of interacting proteins, which are listed in this table. The strength of the interaction is given as ≥2-fold change (-/+) and ≥4-fold change (--/++), with negative indicating the protein is more enriched with the GST control pull-down, and positive indicating the protein is more enriched in $ZFR2^{WT}$ or $ZFR2^{A771T}$ pull down. The yes or no indicate whether the $ZFR2^{WT}$/$ZFR2^{A771T}$ protein ratio is significant (i.e. there is a ≥4-fold change in the binding of a specific protein between $ZFR2^{WT}$ and $ZFR2^{A771T}$).

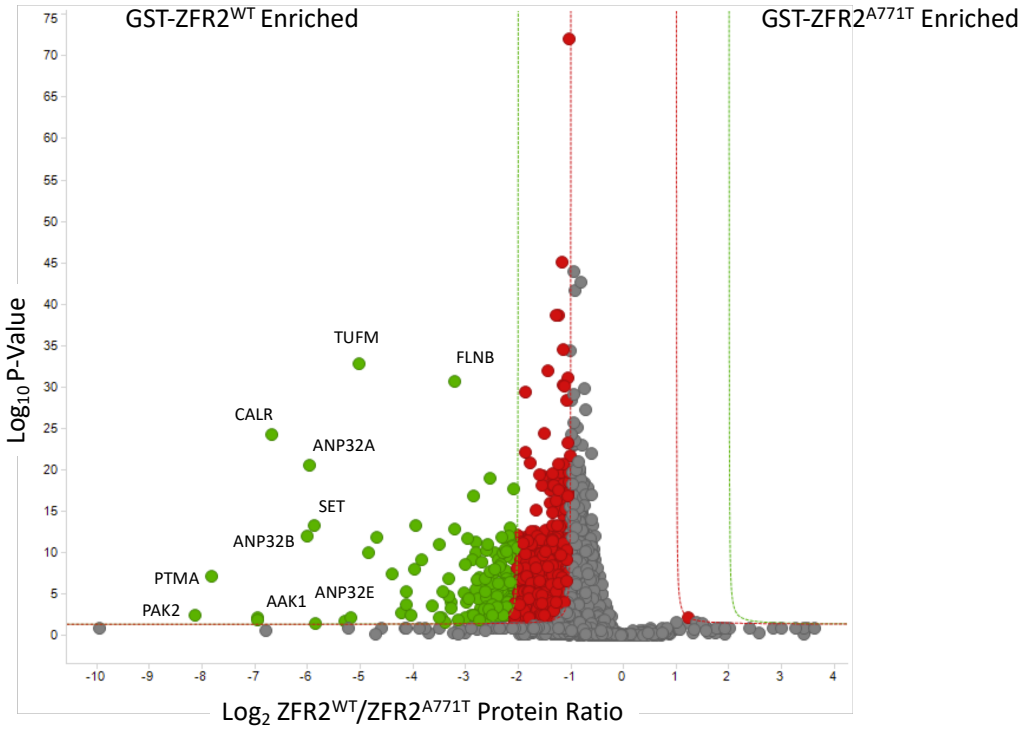


Figure 3.23. Protein interactions that are altered by the ZFR2^{A771T} variant

Glutathione-S-transferase (GST) was used to determine the physical interaction between a GST tagged probe protein and the target protein (ZFR2^{WT} and ZFR2^{A771T}). Pull-downs were performed on Jurkat lysates in triplicates and the abundance of proteins was measured by mass spectrometry. X axis shows the $\log_2$ of the ZFR2^{WT}/ZFR2^{A771T}protein ratio, whereas the y axis shows the -$\log_{10}$ of the calculated probability (p-value), associated with Student's t-test. Proteins with a Log_{10} p-value ≥2.0 (the red line) indicates a statistically significant ($p<0.01$) difference in binding. Dots in red represent ≥2-fold change, and dots in green represent ≥4-fold change in protein ratio. Figure produced by E. Linossi.

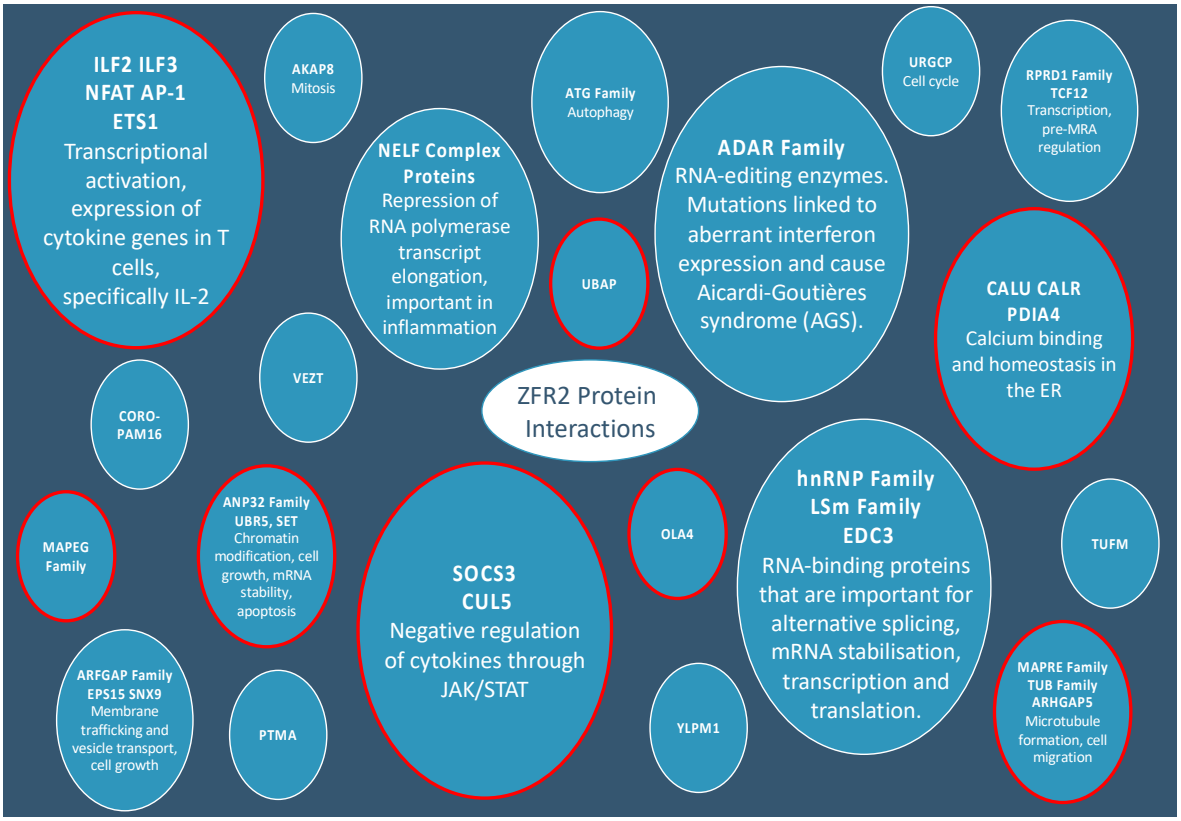


Figure 3.24. Summary of proteins and pathways that are dysregulated by the A771T variant.

Protein interactions and pathways were determined by inputting all ZFR2^{A771T} proteins into the STRING Consortium 2018. Information about each protein was obtained from GeneCards Human Gene Database (Weizmann Institute of Science). The size of the circle indicates the relevance of the protein/s to an immune-related pathway, with the larger circles being most relevant to the immune system and the smallest circles having no known role in the immune system. Circles with a red border are pathways and protein interactions that the A771T variant alters.

3.4.3 ZFR2 plays a role in mRNA splicing

To test whether the A771T variant alters protein localisation, we used site-directed mutagenesis to generate a pCMV6-Entry plasmid vector expressing the $ZFR2^{A771T}$ variant with a MycDDK tag at the C-terminus. Human embryonic kidney 293 (HEK293) cells were transfected with either $ZFR2^{WT}$ or $ZFR2^{A771T}$, and then cells were stained with a mouse monoclonal antibody against FLAG (DDK) and DAPI.

Figure 3.25 demonstrates that the A771T variant does not appear to affect protein localisation, however it does reveal that ZFR2 has a very unique localisation within the nucleus of the cell. Firstly, ZFR2 localises to nuclear speckles (Figure 3.25A), also known as interchromatin granule clusters or splicing speckles, which are nuclear domains that are enriched in pre-messenger RNA splicing factors located in the interchromatin regions of the nucleoplasm (Spector and Lamond, 2011). We confirmed the localisation of ZFR2 to nuclear speckles by co-staining with SC35, a splicing factor that is commonly used as a nuclear speckle marker, with ZFR2 and SC35 co-localising in the cell (Figure 3.25B). Figure 3.25 reveals that in addition to localising to nuclear speckles, ZFR2 also congregates and forms a rim around nucleoli, which was confirmed with co-transfection of NF-κBBS-TATA-GFP-NLS (the NF-κB binding site reporter driving expression of GFP with an NLS sequence at the C-terminus) which is known be expressed in the nucleoli (Figure 3.25C).

Proteins that have this interesting localisation pattern have been shown to associate with the granular component or peri- nucleolar heterochromatin of the nucleolus, and play a role in mRNA processing and export (Huang et al., 1997). Thus, taken together these protein

localisation studies demonstrate the mutation does not alter protein localisation, however they suggest that ZFR2 plays a role in mRNA splicing, processing and export, consistent with the findings from the mass spectrometry and the roles of the paralogs.

To further investigate the splicing functions of ZFR2, we used a biochromatic splicing reporter (RG6) system, designed by Orengo et al., 2006, that is able to express two different fluorescent proteins from a single alternative splicing event (Orengo et al., 2006). This reporter allows the ratio of two alternative splicing pathways to be determined, with enhanced green fluorescent protein (EGFP) expressed from one splicing pathway and dsRED expressed from the other. Inclusion or skipping of the exon by alternative splicing upstream of the dsRED/EGFP cassette allows for toggling between the reading frames of dsRED or EGFP. Simply, cells expressing RG6 alone will have an approximate 50:50 ratio between dsRED and EGFP. The more the exon is retained, the more EGFP will be expressed, and the more the exon is excluded, the more dsRED that will be expressed.

Figure 3.26 demonstrates that there is more exon retention in cells co-transfected with the RG6 reporter and $ZFR2^{WT}$ ($p=0.0009$) however there was no significant difference in splicing between cells expressing $ZFR2^{WT}$ and cells expressing $ZFR2^{A771T}$ ($p=0.3418$). ILF2 and ILF3 have been shown to play important roles in mRNA splicing (Rigo et al., 2012), and therefore ILF2 and ILF3 were co-transfected with the RG6 reporter as positive controls. As expected, transfection of ILF2 and ILF3 led to more exon retention ($p=0.0085$ and $p=0.0262$ respectively), however knocking out the DZF domain of ZFR2 did not significantly alter the proteins' splicing function ($p=0.1811$). We also used CRISPR interference (CRISPRi) to

selectively repress ZFR2 protein expression as a negative control ($ZFR2^{KD}$). The ratio of splicing between dsRED and EGFP in $ZFR2^{KD}$ cells was similar to the cells transfected with RG6 alone (baseline), demonstrating that knocking down ZFR2 in this overexpression system abolished splicing ($p=0.9999$). Therefore, these results confirmed that ZFR2 plays a role in splicing, however the variant is not affecting splicing. Furthermore, the DZF domain does not appear to be essential for the splicing function of ZFRF2.

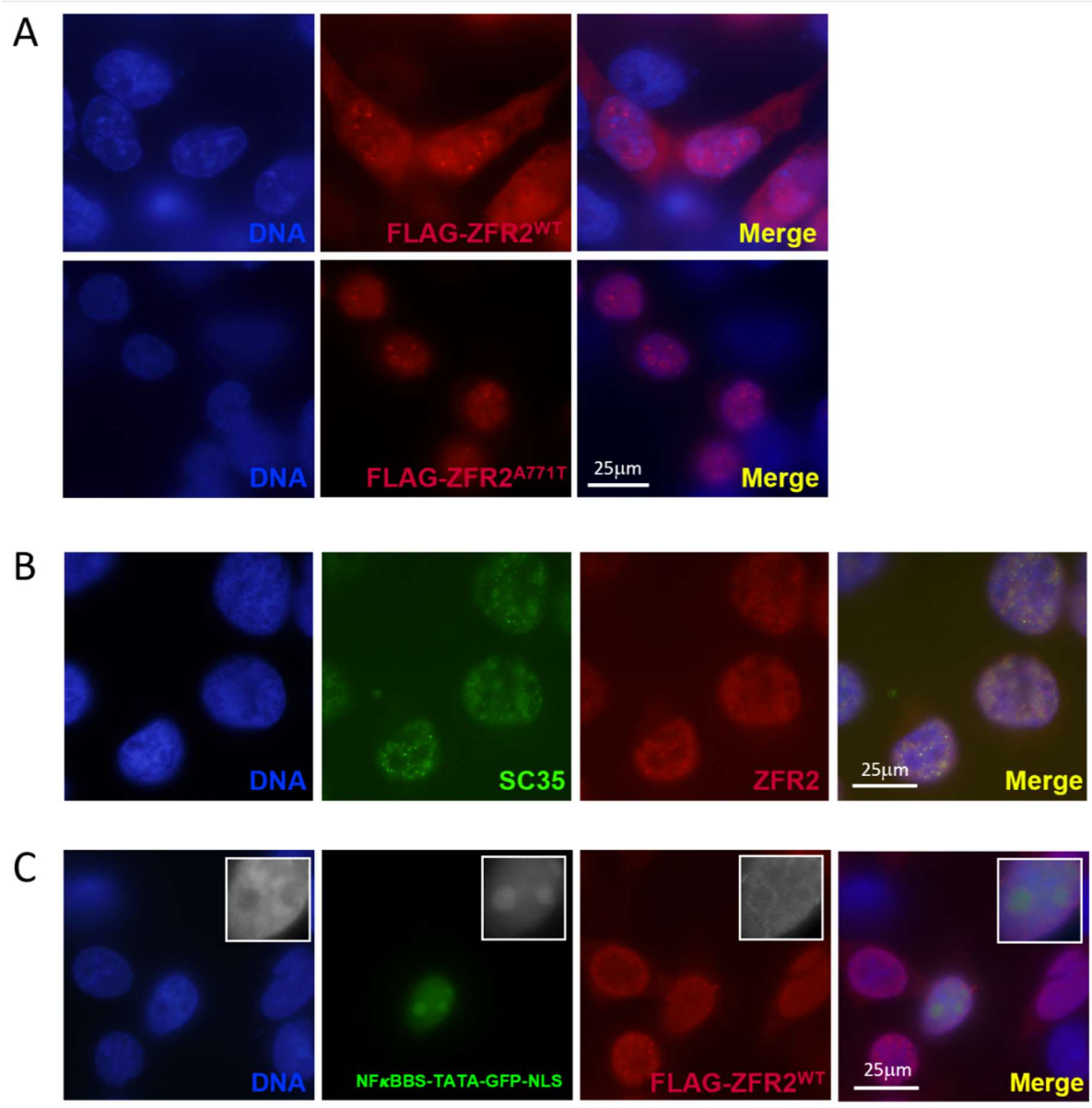


Figure 3.25. Localisation of ZFR2^{WT} and ZFR2^{A771T} in HEK 293 Cells

(A) Overexpression of FLAG-ZFR2^{WT} and FLAG-ZFR2^{A771T} in HEK 293 cells and subsequent staining with anti-FLAG (red) and nuclei staining with DAPI (blue). (B) Endogenous staining of nuclei with DAPI (blue), SC35 (green) and ZFR2 (red). (C) Overexpression of NFκBBS-TATA-GFP-NLS and FLAG-ZFR2^{WT} and subsequent staining with anti-FLAG (red) and nuclei staining with DAPI (blue) (performed and imaged by V. Athanasopoulos)

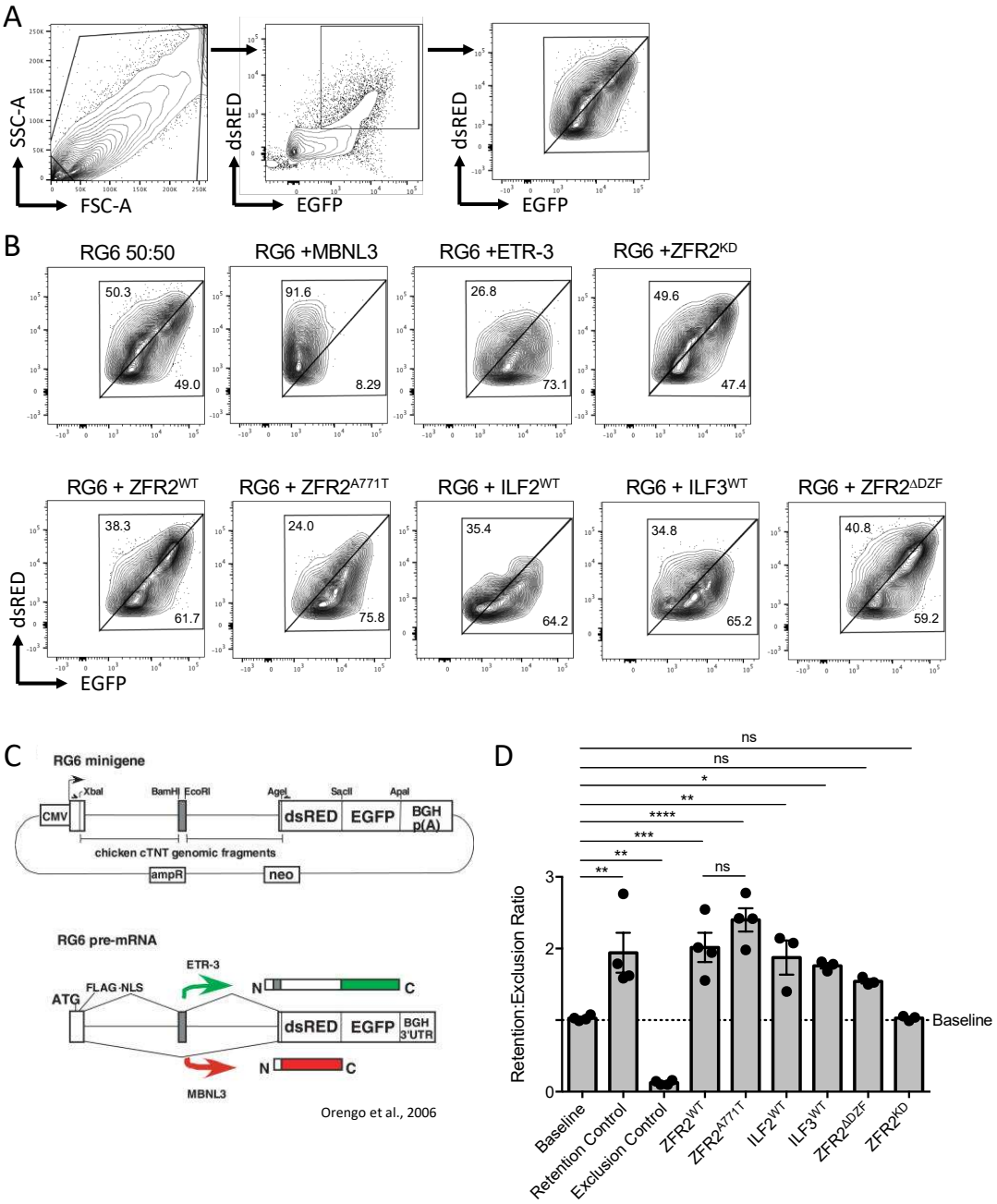


Figure 3.26. Bichromatic reporter to measure alternative splicing by flow cytometry.

(A) Gating strategy to analyse RG6 expressing HEK 293 cells by flow cytometry. (B) Representative plots for each of the cells expressing RG6 and co-expressing the indicated plasmid. Gates were set on the RG6 50:50 control so that ~50% of cells were in each gate i.e. 50% dsRED$^+$ and 50% expressing EGFP$^+$ (C) Diagram of RG6 minigene. Alternative splicing of a 28-nucleotide cassette exon shifts the reading frame between dsRED and EGFP. Restriction sites shown are unique to the plasmid. (D) Ratio of retention to exclusion for indicated plasmids. Each dot represents the mean of triplicate technical replicates from a separate experiment. Bars represent mean ± SEM. (ns= not significant) (*p≤0.05) (**p≤0.01) (***p≤0.001) (****p≤0.0001) One-way ANOVA with Dunnett's multiple comparison test.

3.4.4 The ZFR2 variant increases *IL-2* mRNA and protein independently of *IL-2* 3'UTR

ILF2 and ILF3 have been shown to regulate *IL-2* mRNA stability via the 3'UTR, by binding to the 3'UTR and stabilising the *IL-2* mRNA transcript (Shim et al., 2002b). We therefore asked whether the increase in plasma IL-2 observed in the patients carrying the ZFR2 mutations was due to the ZFR2 A771T variant affecting the stability of the *IL-2* mRNA transcript via the 3'UTR. To investigate this, human *IL-2* cDNA (Origene) was amplified by PCR from the full-length template (primers listed in the methods section). This resulted in two products; the full-length *IL-2* cDNA (IL-2 full length), and the *IL-2* cDNA lacking the 3'UTR (*IL-2* Δ3'UTR), each with restriction enzyme sites so the cDNA could be cloned upstream of the IRES in the MSCV-IRES-GFP vector. Human $ZFR2^{WT}$ and $ZFR2^{A771T}$ cDNA was also amplified by PCR (primers listed in the methods section) with restriction enzyme sites so the cDNA could be cloned upstream of the IRES in the MSCB-IRES-mCherry vector. mCherry labelled $ZFR2^{WT}$ and $ZFR2^{A771T}$ were co-transfected with *IL-2* full length or IL-2 Δ3'UTR (Figure 3.27). MFI of IL-2 (with or without the 3'UTR) was measured by flow cytometry for each population in cells transfected with mCherry empty vector (Figure 3.27A), $ZFR2^{WT}$ mCherry (Figure 3.27B) or $ZFR2^{A771T}$ mCherry (Figure 3.27C). MFI of IL-2 (with or without the 3'UTR) in each population was normalised to the counterpart population from cells expressing empty vector mCherry. We found that $ZFR2^{A771T}$ altered the expression of GFP fluorescence (IL-2), with an increase in GFP fluorescence in cells transfected with $ZFR2^{A771T}$ compared to cells transfected with $ZFR2^{WT}$ (Figure 3.27E). However, that increased GFP fluorescence induced by mutant ZFR2 was independent of the *IL-2* 3'UTR (Figure 3.27F) as the increase was comparable between full-length constructs and those lacking the 3'UTR.

Finally, we sought to test whether ZFR2 and the mutant form found in patients caused an increase in IL-2 protein secretion in human T cells. For this, we transfected a human T cell line (Jurkat cells) using the Neon^{TM} Transfection System (ThermoFisher) with ZFR2^{WT}, ZFR2^{A771T} or ZFR2^{KD} plasmid. An eGFP labelled empty vector was also co-transfected with each of these plasmids. After 24 hours the cells were sorted on GFP expression (to enrich for those which had been effectively transfected) and were stimulated with/without PMA + Ionomycin for 12 hours. The supernatant was collected, and IL-2 was measured with a human Th1/Th2/Th17 cytokine bead array (CBA, BD Biosciences). Figure 3.28 demonstrates that cells expressing ZFR2^{WT} secreted IL-2, however there was a significant increase in IL-2 production by cells expressing ZFR2^{A771T} ($p=0.0002$). Cells transfected with ZFR2^{KD} had less IL-2 production, similar to cells expressing ZFR2^{WT}. Unfortunately, this experiment was technically challenging and could only be repeated twice, mainly because overexpression of the ZFR2 plasmids in Jurkat cells caused significant cell death and whilst we controlled for transfection by sorting on eGFP, we were unable to control for cell death as no markers for cell death could be used on these transfected cells. This could explain why cells expressing pcDNA secreted more IL-2 compared to cells expressing ZFR2^{WT} or ZFR2^{A771T}, and why the ZFR2^{KD} didn't completely abolish IL-2 production.

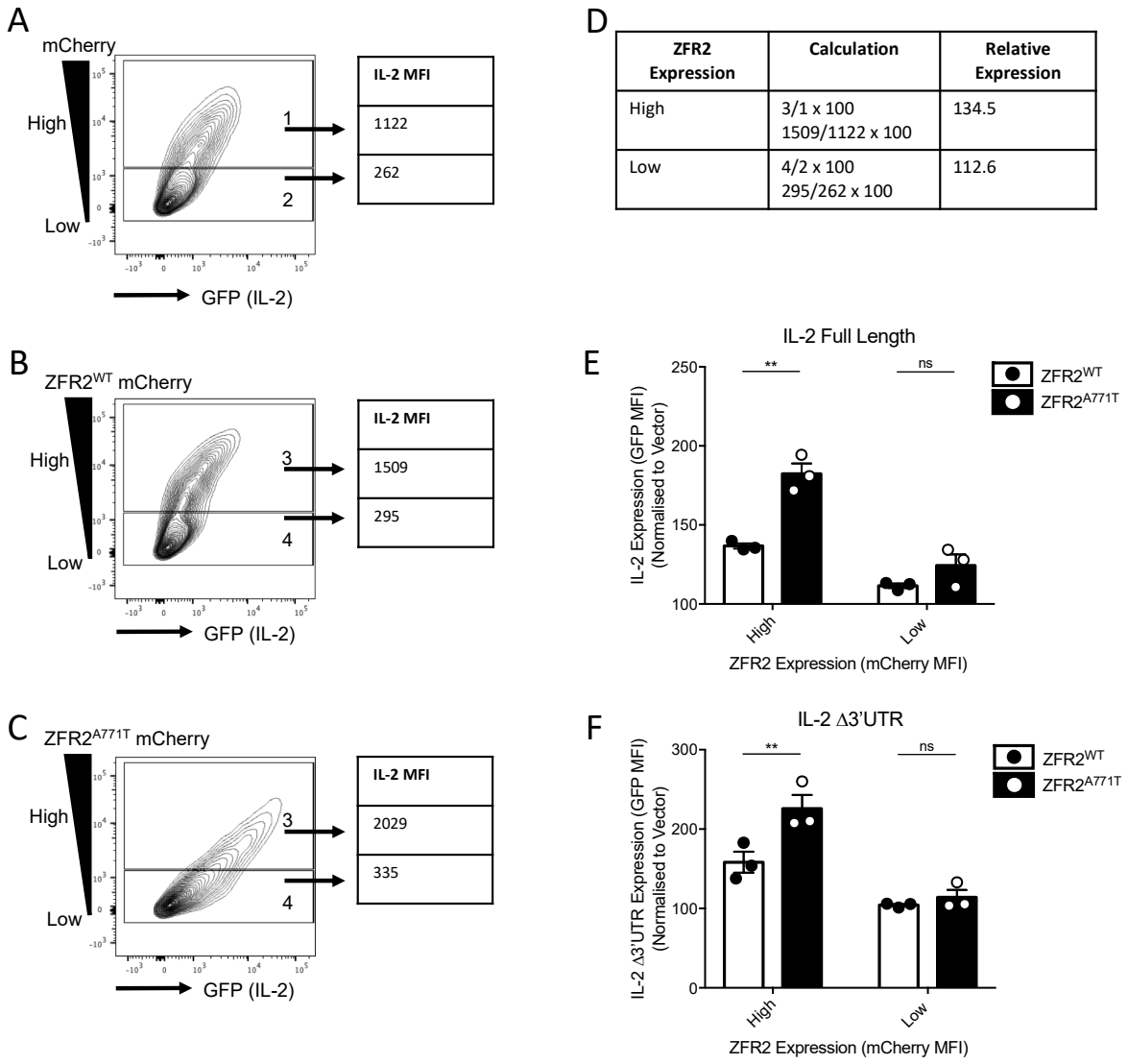


Figure 3.27. ZFR2^{A771T} alters *IL-2* mRNA expression, independently of the IL-2 3'UTR

Quantification of ZFR2-mediated activation. In cells transfected with mCherry vectors, the population of cells with the highest mCherry expression was defined as the mCherry high population, while the population of cells with lower mCherry expression was defined as the low population. MFI of IL-2 (with or without the 3'UTR) was measured for each population in cells transfected with (A) mCherry empty vector, (B) ZFR2^{WT} mCherry or (C) ZFR2^{A771T} mCherry. (D) MFI of IL-2 (with or without the 3'UTR) in each population was normalised to the counterpart population from cells transfected with empty vector. (E-F) Column graphs showing normalised (E) IL-2 and (F) IL-2 Δ3'UTR expression for high and low ZFR2^{WT} and ZFR2^{A771T} expression. Each dot represents a technical replicate. This is representative of three repeat experiments. (ns=not significant) (**p≤0.01) Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with Q=1%.

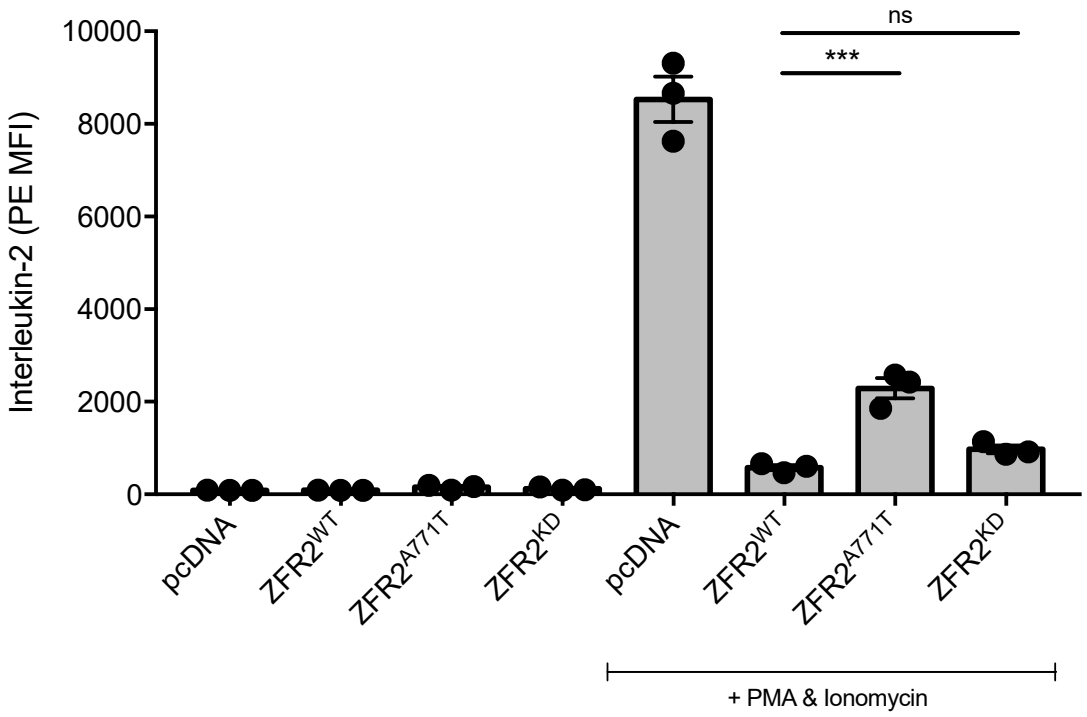


Figure 3.28. IL-2 production in Jurkat cells following overexpression of ZFR2 constructs

Jurkat cells were transfected using the Neon^{TM} Transfection System (ThermoFisher). Jurkat cells were transfected with ZFR2^{WT}, ZFR2^{A771T} or CRISPRi ZFR2^{KD} plasmids. Cells were also transfected with an eGFP labelled empty vector, and 24 hours after transfection were sorted on GFP expression. Cells were then cultured with or without PMA + Ionomycin for 12 hours. A Human Th1/Th2/Th17 CBA (BD Biosciences) was performed on the supernatant collected, with the MFI of PE representing the amount of IL-2 in the supernatant. Each dot represents a technical replicate, with the bars indicating the mean ± SEM. Representative of two repeat experiments. (***p≤0.001) (ns=not significant) one-way ANOVA with a Dunnett's multiple comparison test to stimulated ZFR2^{WT}.

3.4.5 The A771T variant augments NFAT and AP-1 activity

Given the role that ILF2 and ILF3 play in regulating *IL-2* transcription at the antigen receptor response element (ARRE)/NFAT DNA target sequence (Kao et al., 1994), we hypothesised that ZFR2 would also regulate NFAT activity, and that the A771T variant in ZFR2 would increase NFAT activity to increase transcription of NFAT targets such as *IL-2*. Hyperactivation of NFAT has been associated with inflammatory and autoimmune conditions such as ulcerative colitis (Liu et al., 2011) (Shih et al., 2008), rheumatoid arthritis (Yarilina et al., 2011) and lupus (Mehta et al., 2010) (Kyttaris et al., 2007), and would explain the increased proinflammatory cytokines (in particular the increased IL-2) in the individuals from Family A and Family B. To investigate this theory, we performed a NFAT luciferase assay. Luciferases are oxidative enzymes, which catalyse the conversion of a luciferin substrate to oxyluciferin; a reaction which releases light as a measurable by-product. The amount of light produced from this reaction is proportional to the level of luciferase activity. By harnessing the bioluminescent properties of the luciferase reaction, luciferase reporter assays can provide a quantitative readout of gene expression at the transcriptional level. Cells are also transfected with a Renilla luciferase control plasmid as an internal control for transfection efficiency in this assay, as it is constitutively expressed within the cells it enters. The ratio between the two luciferases is an effective control for the amount of transfection between the different transfections.

We used the same plasmids described above ($ZFR2^{WT}$, $ZFR2^{A771T}$, $ZFR2^{\Delta DZF}$, CRISPRi $ZFR2^{KD}$ or pcDNA as the control) and co-expressed them with an NFAT luciferase reporter to test whether ZFR2 regulates NFAT activity and whether the A771T variant alters NFAT activity. We confirmed that ZFR2 activates NDAT and found that cells transfected with $ZFR2^{A771T}$ had

increased NFAT activity compared to cells transfected with ZFR2^{WT} (p=0.0290) (Figure 3.29), suggesting that the mutation is a gain-of-function in terms of NFAT activation. NFAT activity was abolished in cells transfected with CRISPRi ZFR2^{KD} (p=0.0358) and ZFR2 lacking the DZF domain (ZFR2$^{\Delta DZF}$) (p=0.0355) (Figure 3.29) demonstrating that this domain is required for ZFR2 NFAT activity.

AP-1 is the main transcriptional partner of NFAT during T cell activation, forming stable complexes that regulate the expression of many inducible genes, including IL-2 and other proinflammatory cytokines (Jain et al., 1992, Paliogianni et al., 1993, Gonsky et al., 1998). As cells transfected with ZFR2^{A771T} had augmented NFAT activity compared to cells transfected with ZFR2^{WT}, we investigated the effect of the A771T variant on AP-1 luciferase activity using the same plasmids described above (Figure 3.30). The cells transfected with ZFR2^{WT} had augmented AP-1 activity compared to pcDNA (p=0.0016), and interestingly cells transfected with ZFR2^{A771T} had more AP-1 activity that cells transfected with ZFR2^{WT} (p=0.0001), suggesting that the mutation confers a gain-of-function. AP-1 activity was abolished in cells transfected with CRISPRi ZFR2^{KD} (p=0.0008) and ZFR2$^{\triangle DZF}$ (p=0.0017) (Figure 3.30), again suggestion that the DZF domain is required for ZFR2 AP-1 activity.

In early 2018, ZFR was shown to prevent excessive type I interferon activation by regulating alternative pre-mRNA splicing, with interferon stimulated genes becoming constitutively activated in the absence of ZFR (Haque et al., 2018). We therefore investigated the effect of ZFR2^{WT} and ZFR2^{A771T} on interferon $\boldsymbol{\beta}$ (IFN$\boldsymbol{\beta}$) luciferase activity using the same plasmids described above (Figure 3.31). Interestingly, cells expressing ZFR2^{WT} had a reduction in IFN$\boldsymbol{\beta}$

luciferase activity compared to pcDNA (p=0.0003), and cells transfected with ZFR2^{A771T}displayed a further reduction in IFN$\boldsymbol{\beta}$ luciferase activity compared to cells transfected with ZFR2^{WT} (p=0.0014), suggesting that ZFR2 acts with ZFR to prevent excessive type I interferon activation, and that the mutation also confers a gain of this IFN-regulatory function.

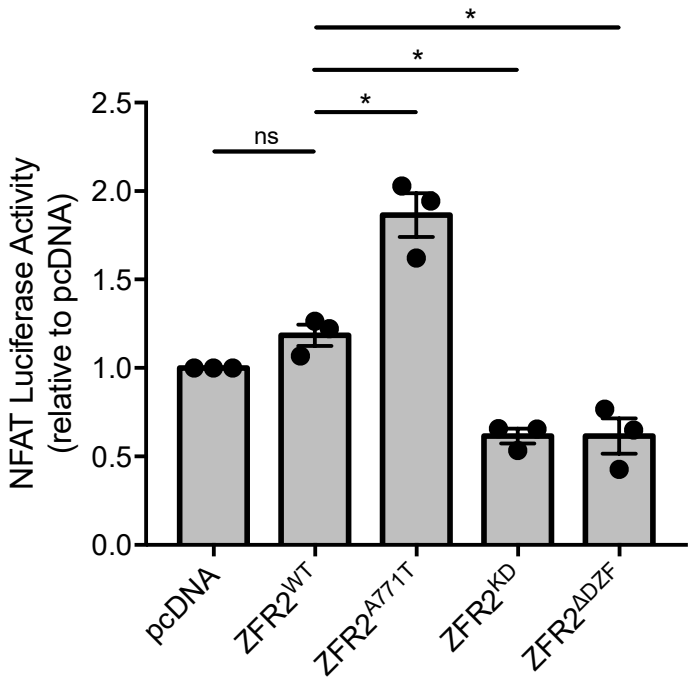


Figure 3.29. Dual luciferase reporter assay to quantify NFAT activation.

Dual luciferase reporter assay to quantify NFAT activation in response to ZFR2^{WT}, ZFR2^{A771T}, ZFR2^{KD} (a knockdown of ZFR2 using CRISPRi) and ZFR2$^{\Delta DZF}$ (ZFR2 with a deletion of the DZF domain) in HEK 293 cells. Each dot represents the mean of three technical replicates, with the bars indicating the mean ± SEM of NFAT luciferase activity, normalised to pcDNA. Representative of five repeat experiments. (*p≤0.05) (ns=not significant) one-way ANOVA with a Dunnett's multiple comparison test to ZFR2^{WT}.

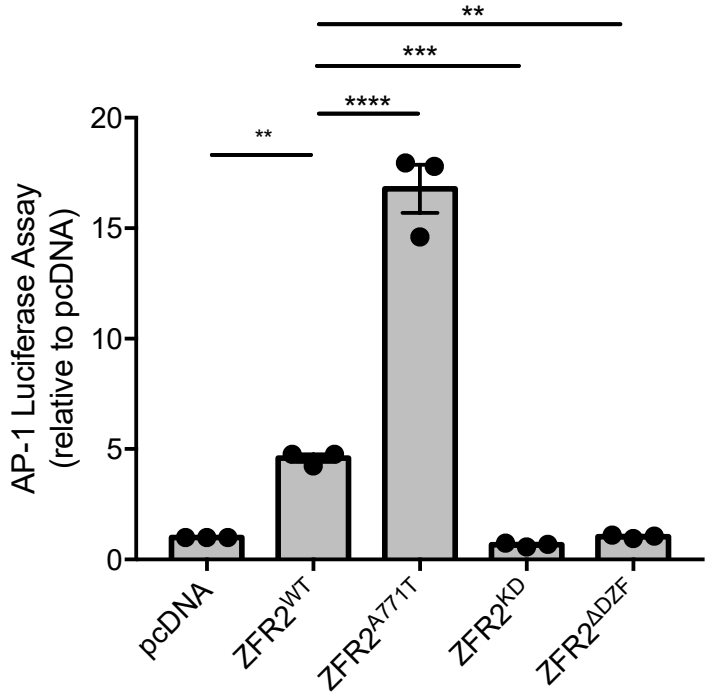


Figure 3.30. Dual luciferase reporter assay to quantify AP-1 activation.

Dual luciferase reporter assay to quantify AP-1 activation in response to ZFR2^{WT}, ZFR2^{A771T}, ZFR2^{KD} (a knockdown of ZFR2 using CRISPRi) and ZFR2$^{\Delta DZF}$ (ZFR2 with a deletion of the DZF domain) in HEK 293 cells. Each dot represents the mean of three technical replicates, with the bars indicating the mean ± SEM of AP-1 luciferase activity, normalised to pcDNA. Representative of five repeat experiments. (**p≤0.01) (***p≤0.001) (****p≤0.0001) one-way ANOVA with a Dunnett's multiple comparison test to ZFR2^{WT}.

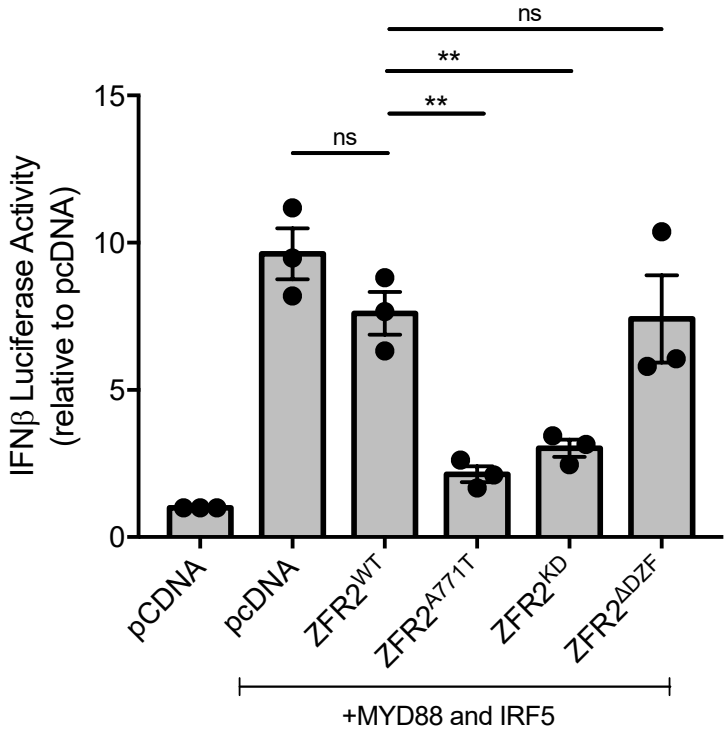


Figure 3.31. Dual luciferase reporter assay to quantify IFNβ activation.

Dual luciferase reporter assay to quantify IFNβ activation in response to ZFR2^{WT}, ZFR2^{A771T}, ZFR2^{KD} (a knockdown of ZFR2 using CRISPRi) and ZFR2$^{\Delta DZF}$ (ZFR2 with a deletion of the DZF domain) in HEK 293 cells. Each dot represents the mean of three technical replicates, with the bars indicating the mean ± SEM of IFNβ luciferase activity, normalised to pcDNA. Representative of five repeat experiments. (**p≤0.01) (ns=not significant) one-way ANOVA with a Dunnett's multiple comparison test to ZFR2^{WT}.

3.5. Investigating hallmarks of autoimmunity in ZFR2^{A714T} mice

3.5.1 Investigation of unimmunized ZFR2^{A714T} mice.

Whilst these luciferase and other *in vitro* assays demonstrate that the ZFR2 mutation appears to alter the function of ZFR2, they may not accurately reflect the consequences of the ZFR2 mutation *in vivo*. Therefore, a CRISPR/Cas9 mouse model carrying the orthologous ZFR2 variant (ZFR2A714T) was created by Gaétan Burgio and his team at JCSMR, ANU. ZFR2mut/wt mice were crossed to generate ZFR2mut/mut mice, homozygous for the A714T variant. Investigation of the major splenic immune cell subsets, including T cell subsets, B cell subsets, NK cells and monocytes was performed, as well as serological investigation of cytokines and antinuclear antibodies (ANAs). Data was pooled from both male and female mice due to small litters. No significant phenotypes were observed in the heterozygous or homozygous mice aged 12-28 weeks (data not shown) compared to wild-type age-matched littermates, except for a subtle (p=0.0290) increase in serum IL-2 in mice 12-14 weeks of age, however this phenotype was not observed in a separate cohort of mice 23-24 weeks of age (Figure 3.32). No differences in serum IL-4, IL-6, IL-10, TNF or IFNγ was observed (data not shown).

In an attempt to exacerbate disease in these mice, ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice were aged for 6 months, and the above phenotyping was performed. No significant difference in any B cell populations (Figure 3.33-Figure 3.36) or total CD3$^+$, CD4$^+$ or CD8$^+$ T cell populations (Figure 3.37) was observed. The ZFR2$^{mut/mut}$ mice did however exhibit a modest increase (p=0.0317) in CD4$^+$ CD44$^+$ activated cells (Figure 3.38) and CD44 MFI (p=0.0241) on CD4$^+$ cells (Figure 3.38) compared to the ZFR2$^{wt/wt}$ mice, and consequently a moderate decrease in naïve T cells in the ZFR2$^{mut/mut}$ mice (p=0.0259), suggesting that there are more antigen-experienced T cells in the ZFR2$^{mut/mut}$ mice. There was also a moderate increase (p=0.0317) in T_{regs} in the ZFR2$^{mut/mut}$ mice compared to the ZFR2$^{wt/wt}$ mice (Figure 3.39), however no other cellular or serological phenotypes were observed, including no difference in anti-nuclear antibodies or serum IL-2 (data not shown). Unfortunately, cell numbers for splenic cell subsets were unable to be determined due to significant variability in the processing of each spleen, which led to variability in cell numbers. This will be addressed in further studies.

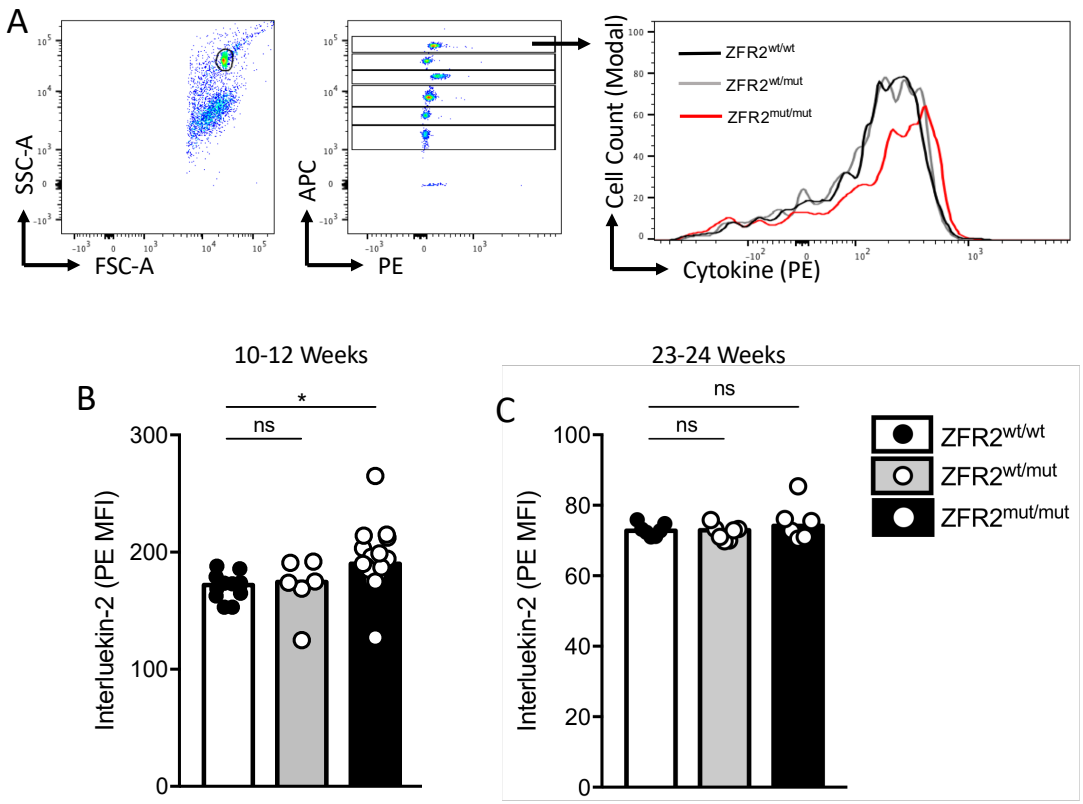


Figure 3.33. Young ZFR2$^{mut/mut}$ mice have a subtle increase in serum IL-2.

(A) APC fluorescence intensity indicates the cytokine measured, and the amount of PE corresponds to the amount of analyte bound, and thus the amount of cytokine present. IL-2 in mouse serum was measured, depicted as a representative histogram of the indicated mouse genotypes, as well as a column graph demonstrating the median PE MFI for IL-2 in mice aged (B) 10-12 weeks and mice aged (C) 23-24 weeks. Each dot represents a mouse. (*p≤0.05) (ns=not significant) Mann-Whitney U test.

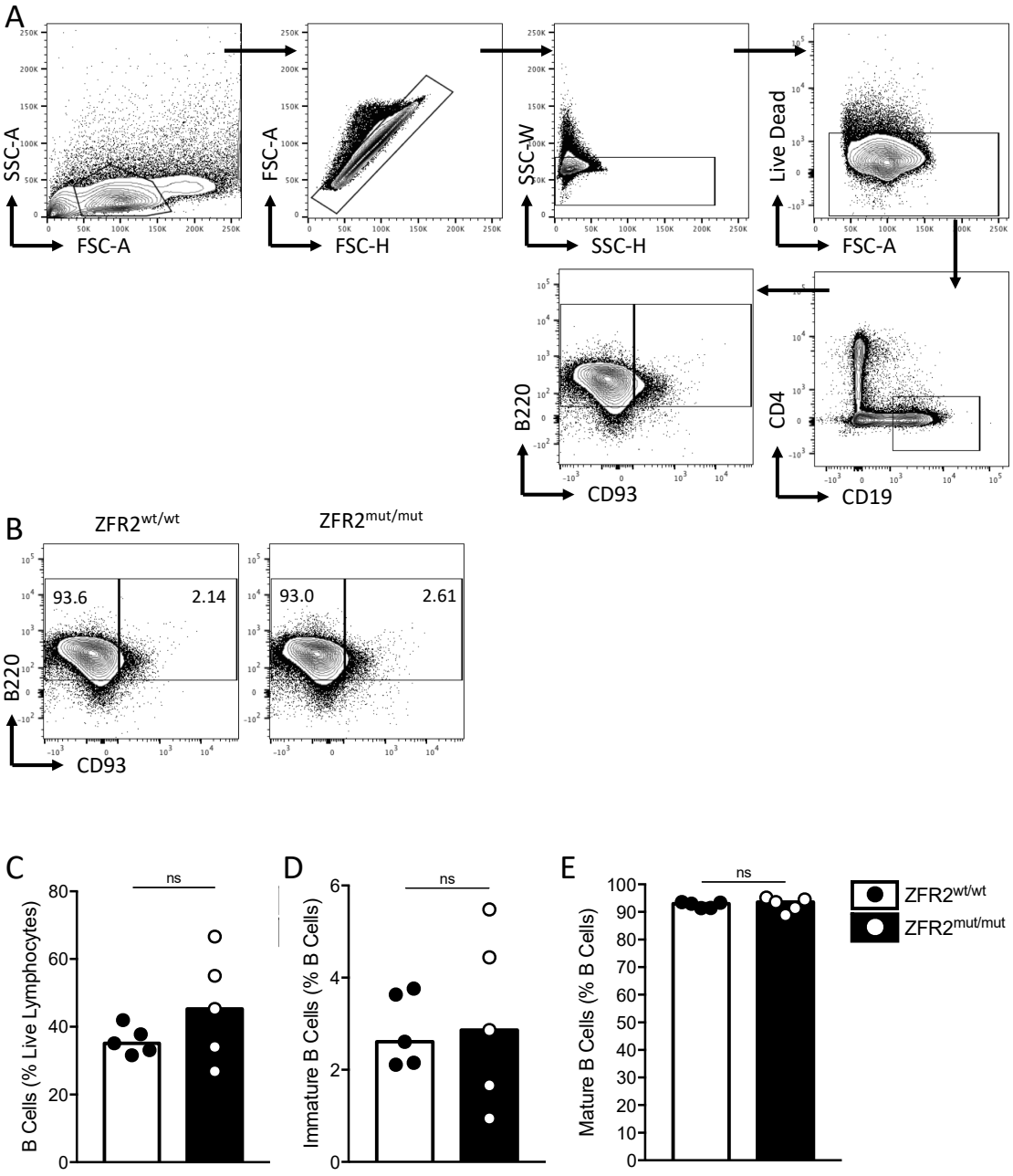


Figure 3.33. Flow cytometric analysis ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ B cells.

(A) Gating strategy used to quantify B cell subsets in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice.
(B) Representative plot of immature and mature B cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (C-E) Column graphs representing the median percentage of (C) total B cells, (D) immature B cells and (E) mature B cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse. (ns=not significant) Mann-Whitney U test.

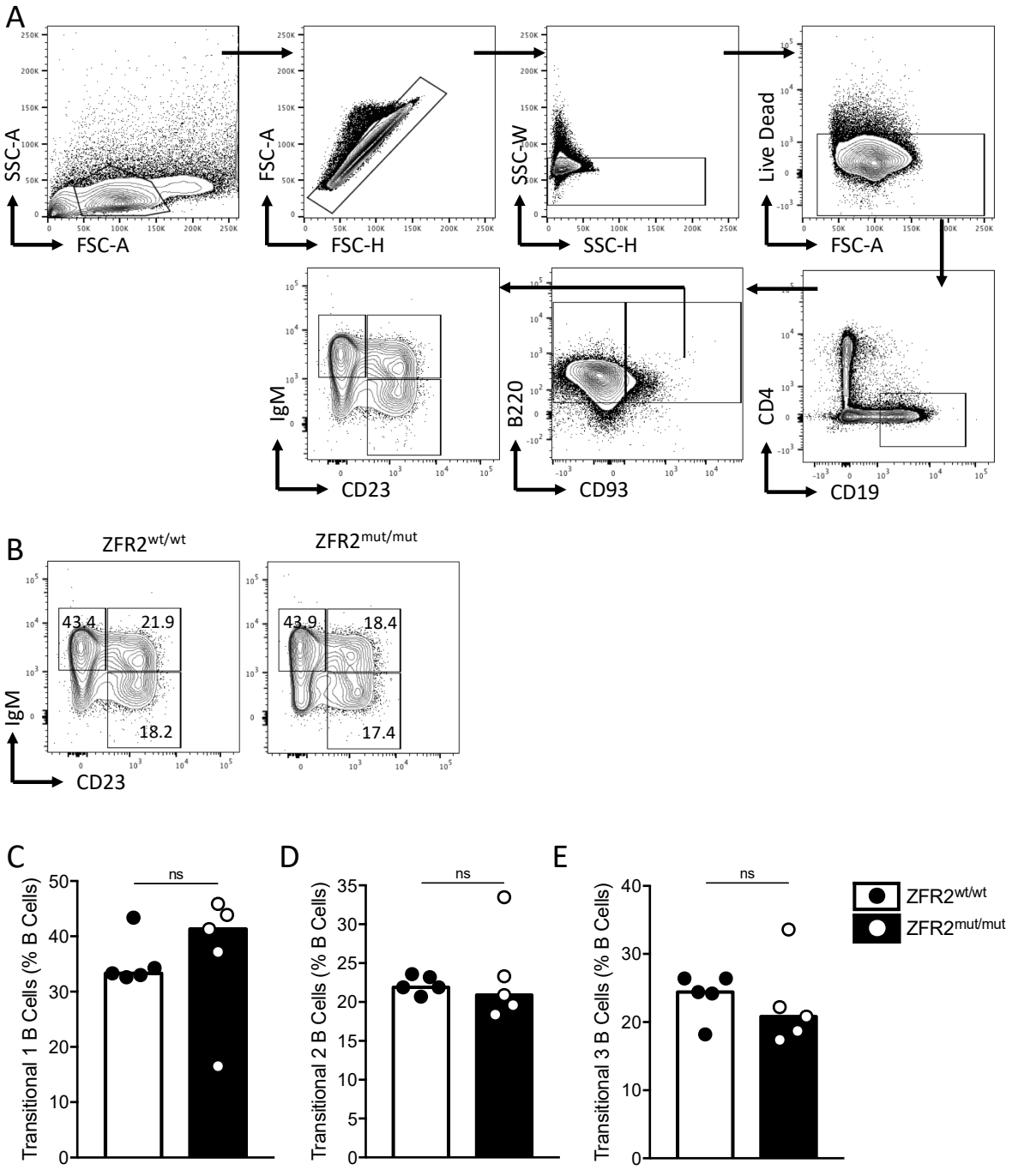


Figure 3.34. Flow cytometric analysis ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ transitional B cells.

(A) Gating strategy used to quantify transitional B cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (B) Representative plot of transitional B cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (C-E) Column graphs representing the median percentage of (C) transitional 1 B cells, (D) transitional 2 B cells and (E) transitional 3 B cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse. (ns=not significant) Mann-Whitney U test.

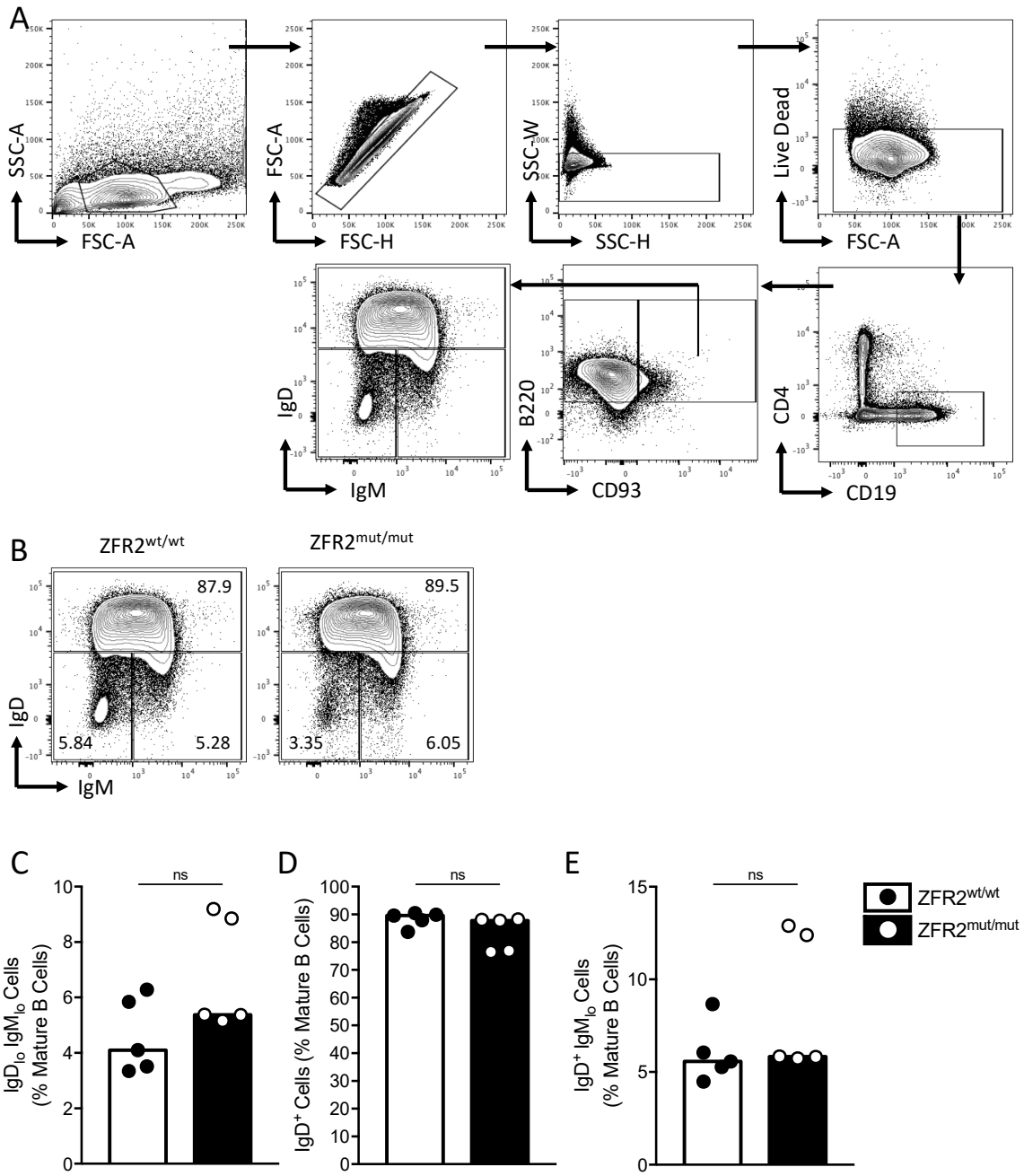


Figure 3.35. Flow cytometric analysis ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mature B cells.

(A) Gating strategy used to quantify mature B cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (B) Representative plot of mature B cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (C-E) Column graphs representing the median percentage of (C) IgD_{lo} IgM_{lo} cells, (D) IgD^+ cells and (E) IgD^+ IgM_{lo} B cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse. (ns=not significant) Mann-Whitney U test.

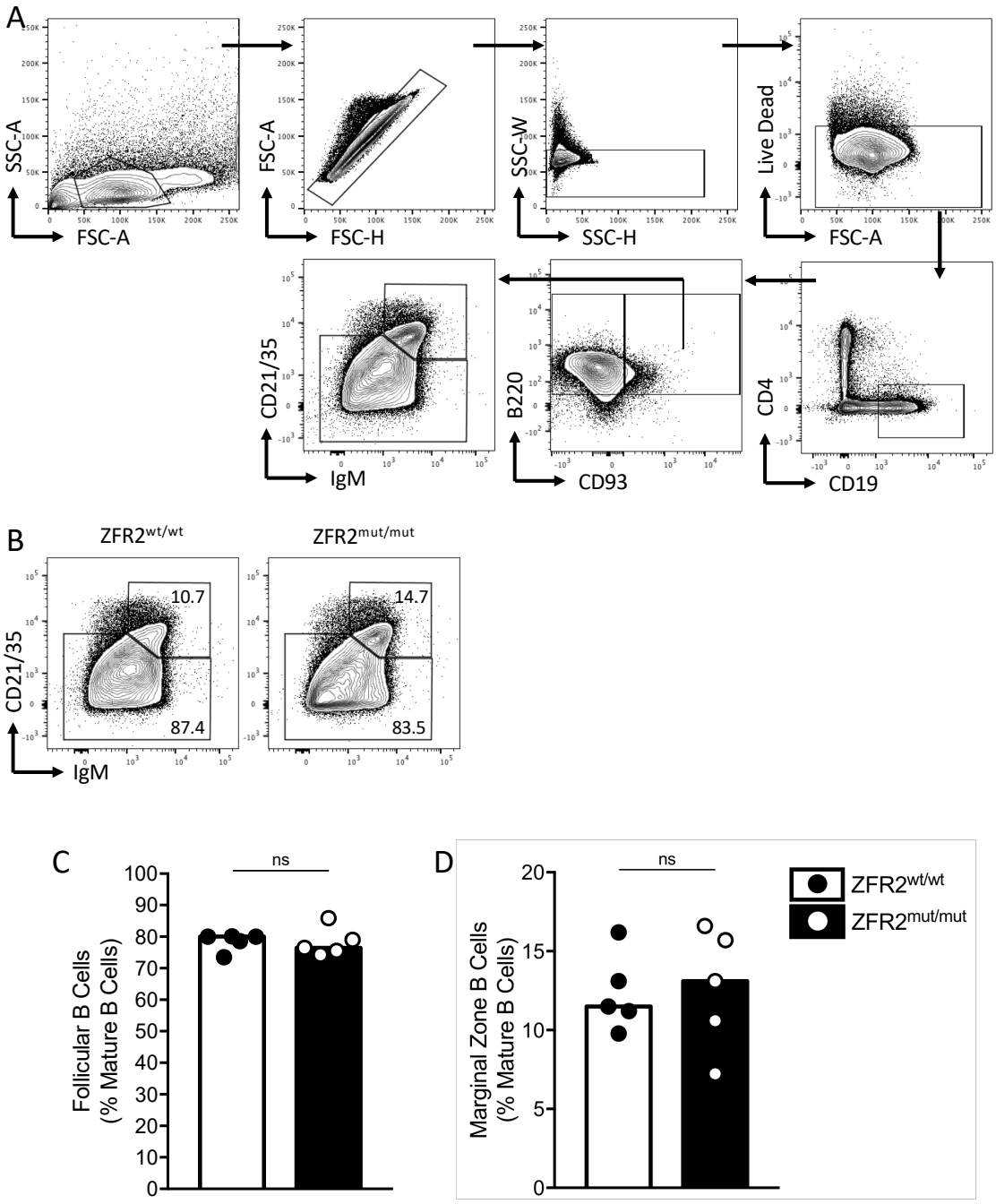


Figure 3.36. Flow cytometric analysis ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mature B cells.

(A) Gating strategy used to quantify follicular and marginal zone B cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (B) Representative plot of follicular B cells and marginal zone B cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (C-D) Column graphs representing the median percentage of (C) follicular B cells and (D) marginal zone B cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse. (ns=not significant) Mann-Whitney U test.

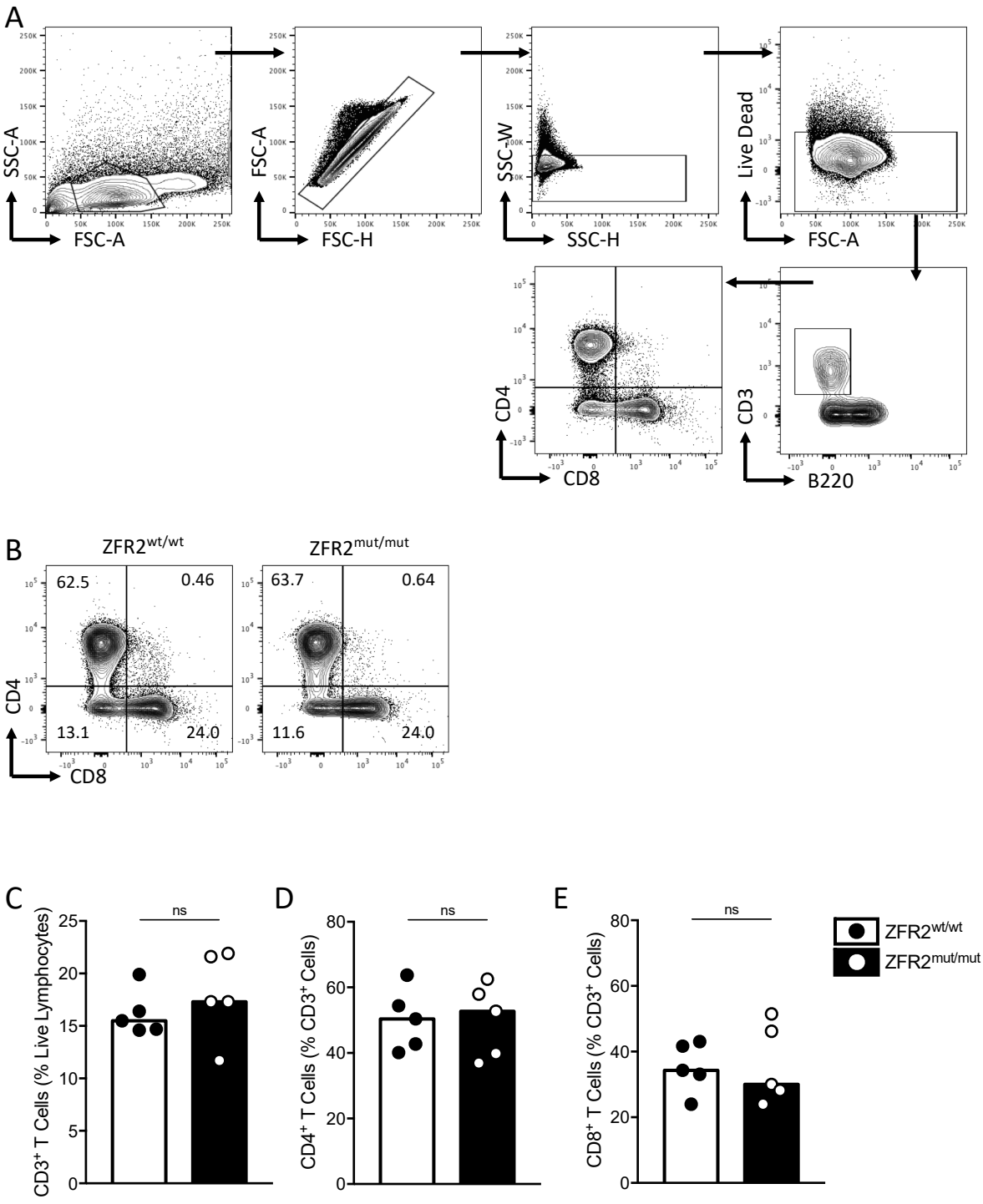


Figure 3.37. Flow cytometric analysis ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ T cells.

(A) Gating strategy used to quantify T cell subsets in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (B) Representative plot of CD4 and CD8 T cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (C-E) Column graphs representing the median percentage of (C) CD3$^+$ T cells, (D) CD4$^+$ T cells and (E) CD8$^+$ T cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse. (ns=not significant) Mann-Whitney U test.

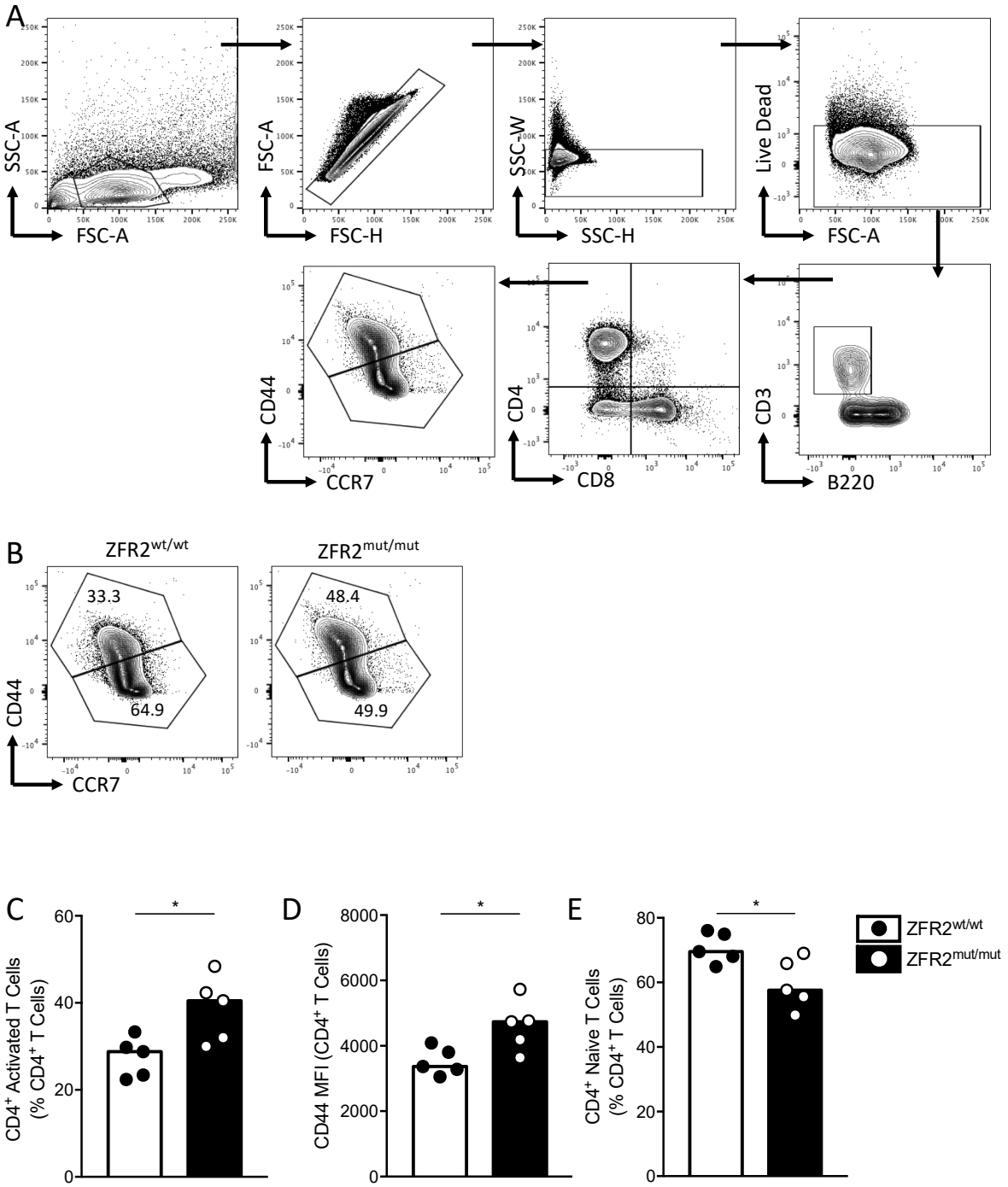


Figure 3.38. Flow cytometric analysis ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ CD4^{+} T cell subsets.

(A) Gating strategy used to quantify CD4^{+} activated and naïve T cell subsets in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (B) Representative plot of CD4 and CD8 T cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (C-E) Column graphs representing the median percentage of (C) CD3^{+} T cells, (D) CD4^{+} T cells and (E) CD8^{+} T cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse. (*p≤0.05) Mann-Whitney U test.

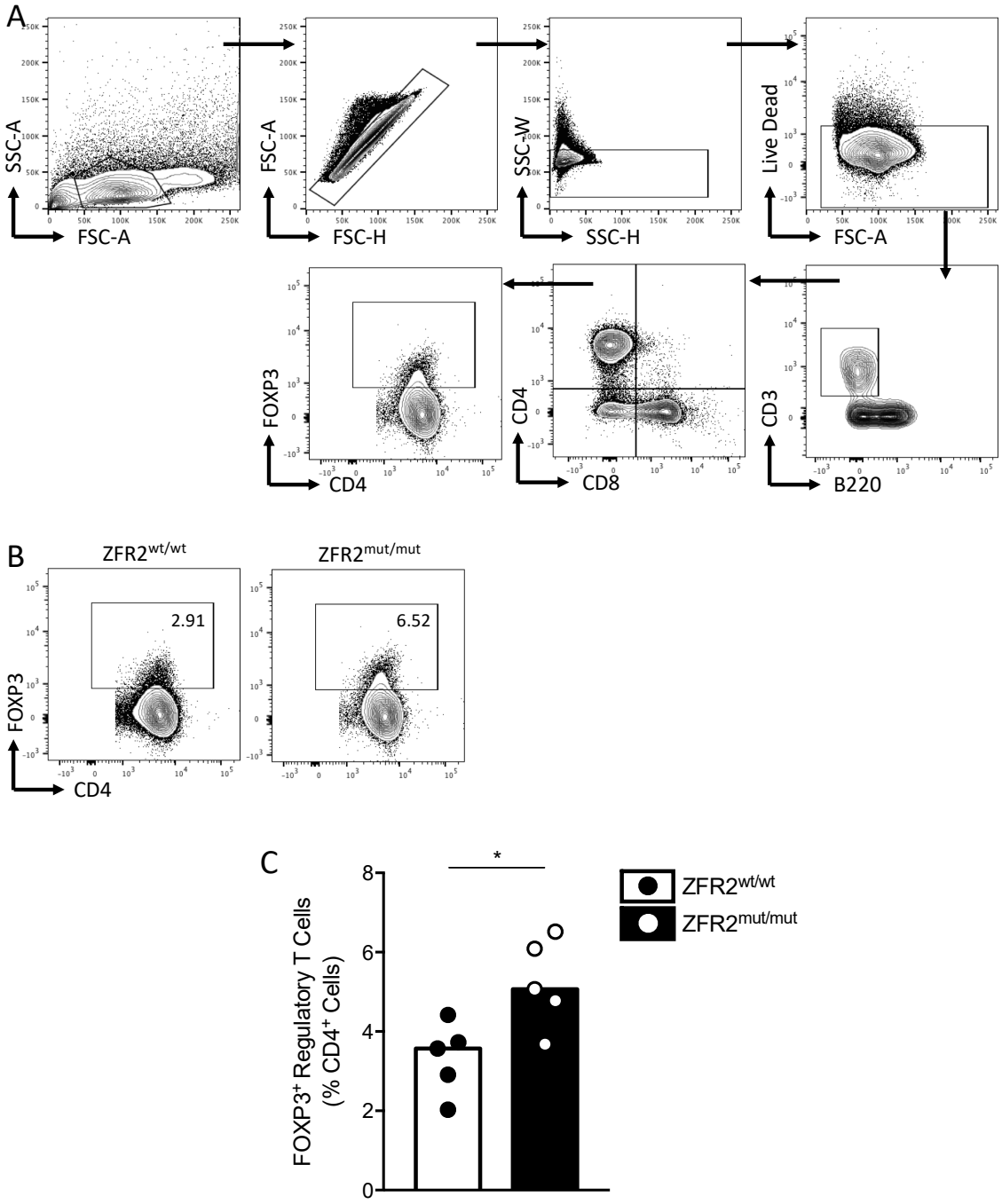


Figure 3.39. Flow cytometric analysis ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ FOXP3^{+} regulatory T cells.

(A) Gating strategy used to quantify FOXP3^{+} regulatory T cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (B) Representative plot of FOXP3^{+} regulatory T cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. (C) Column graphs representing the median percentage of FOXP3^{+} regulatory T cells in ZFR2$^{wt/wt}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse. (*p≤0.05) Mann-Whitney U test.

3.5.2 Investigation of immunized ZFR2^{A714T} mice

ZFR2$^{wt/mut}$ mice did not recapitulate the phenotypes observed in the SLE patients carrying the orthologous variant, and making the mouse homozygous for the ZFR2 variant and aging the mice in an attempt to exacerbate the phenotypes only revealed a very mild cellular phenotype with no signs of autoimmunity (anti-nuclear antibodies). In an attempt to possibly trigger or exacerbate the phenotypes in these mice, we collaborated with Dr Sarah Gabriel from the Walter and Eliza Hall Institute of Medical Research, Melbourne University, who infected ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice with chronic (Clone 13) lymphocytic choriomeningitis virus (LCMV) infection. Chronic LCMV infections are characterised by CD8$^+$ T cell dysfunction, with virus-specific CD8$^+$ T cells becoming exhausted, having poor effector function and losing their memory potential (Angelosanto et al., 2012). This exhaustion is an immunosuppressive mechanism by which chronic viruses escape the adaptive immune response. IL-2 has been shown to be crucial for the expansion of memory CD8$^+$ T cells, and for the induction and maintenance of virus-specific memory CD8$^+$ T cells (Kalia and Sarkar, 2018, D'Souza et al., 2002, D'Souza and Lefrancois, 2003). CD8$^+$ T cells in mice with chronic LCMV infections typically produce less IL-2, TNF and IFN-γ and express high levels of inhibitory PD-1 (Blackburn et al., 2009). Of note, low-dose IL-2 therapy in mice chronically infected with LCMV show enhanced CD8$^+$ T cell responses, increased regulatory T cells and decreased T_{FH} cells (West et al., 2013). Furthermore, it has been shown that RNA viruses can induce systemic lupus-like autoimmunity in mice (Schmitt et al., 2016). Thus, we hypothesised that LCMV infection might make subtle phenotypes conferred by the ZFR2 variant more apparent, particularly given the potential increased ability to produce IL-2 in mutant cells.

Five ZFR2$^{wt/wt}$, seven ZFR2$^{wt/mut}$ and five ZFR2$^{mut/mut}$ mice were infected with LCMV Clone 13, however unfortunately one of the ZFR2$^{wt/wt}$ and one of the ZFR2$^{mut/mut}$ mice had to be ethically culled and could not be included in the experiment, decreasing the power of the experiment and ability to perform statistics. After eight days of infection the mice were bled (although one of the remaining four wild-type mice could not be bled due to complications during the procedure). Although statistics could not be performed, the trends revealed by the analysis revealed a modest increase in total CD4$^+$ and CD8$^+$ cells in the ZFR2$^{mu/mut}$ mice compared to the ZFR2$^{wt/wt}$ mice (Figure 3.40), and a modest increase in virus-specific (gp33$^+$) CD8$^+$ T cells in the ZFR2$^{wt/mut}$ and ZFR2$^{mu/mut}$ mice compared to the ZFR2$^{wt/wt}$ mice (Figure 3.41) as well as a decrease in virus-specific exhausted memory T cells (T_{MEX}) in the ZFR2$^{wt/mut}$ and ZFR2$^{mu/mut}$ mice compared to the ZFR2$^{wt/wt}$ (Figure 3.42). All these findings are consistent with the mutant mice having increased IL-2 and resulting effector function.

Mice were sacrificed at day 28 and splenic analysis, although again lacking statistical power, revealed the following trends: a potential increase in FOXP3$^+$ regulatory T cells (Figure 3.43), a modest reduction in T_{FH} cells (Figure 3.44) and a reduction in T_{MEX} cells (Figure 3.50) in the ZFR2$^{wt/mut}$ and ZFR2$^{mu/mut}$ mice compared to the ZFR2$^{wt/wt}$. No changes were observed in total splenic CD4$^+$ T cells or CD8$^+$ T cells, T_{FR} cells, plasma cells or germinal centre B cells (Figure 3.45-Figure 3.50). Unfortunately, the serum from these immunised mice was unable to be obtained and therefore analysis of cytokines or anti-nuclear antibodies could not be performed.

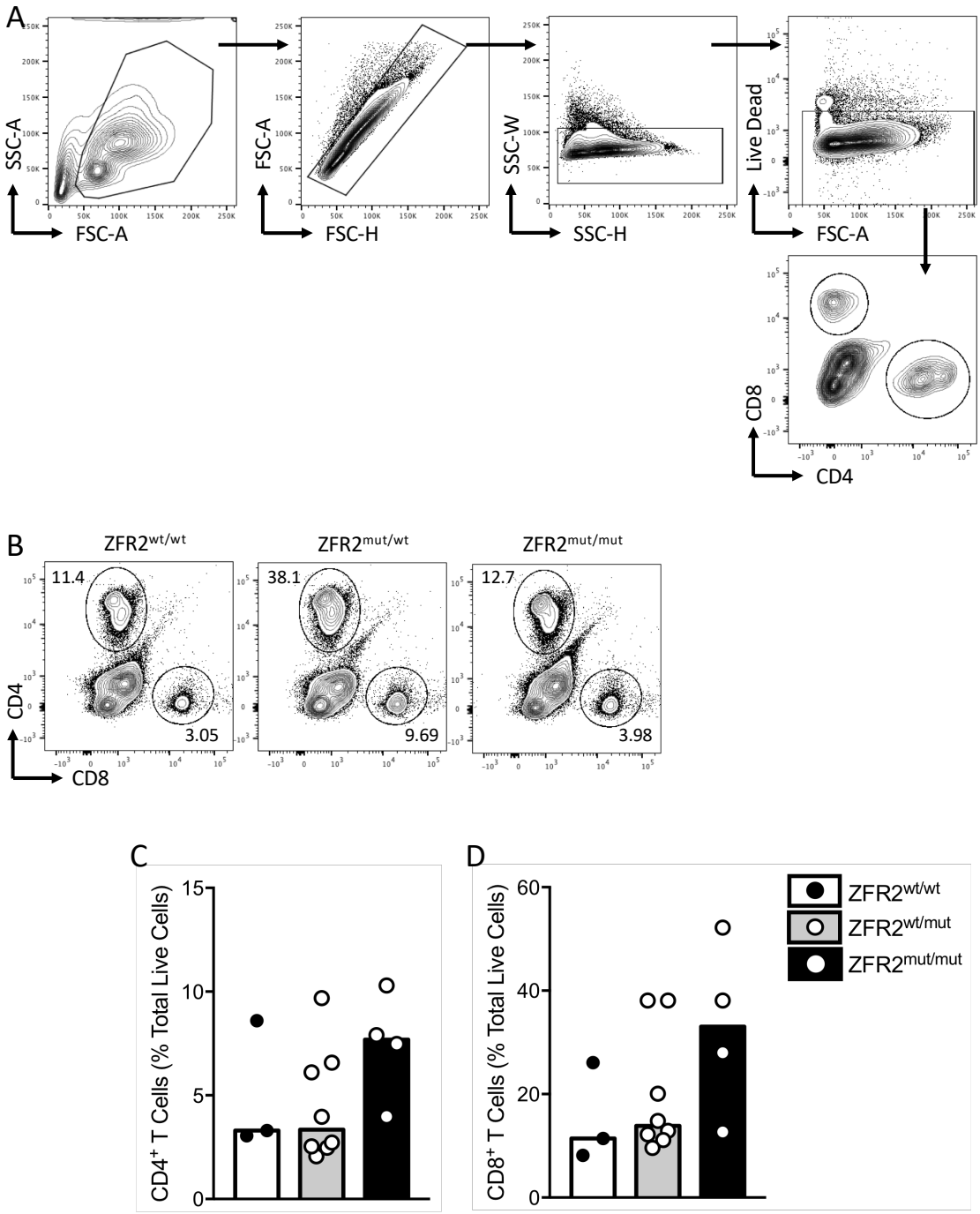


Figure 3.40. Flow cytometric analysis of ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ circulating CD4$^+$ and CD8$^+$ cells eight days after LCMV infection.

(A) Gating strategy used to quantify CD4$^+$ and CD8$^+$ cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice eight days after LCMV infection. (B) Representative plot of CD4$^+$ and CD8$^+$ cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. (C-D) Column graphs representing the median percentage of (C) CD4$^+$ and (D) CD8$^+$ cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse.

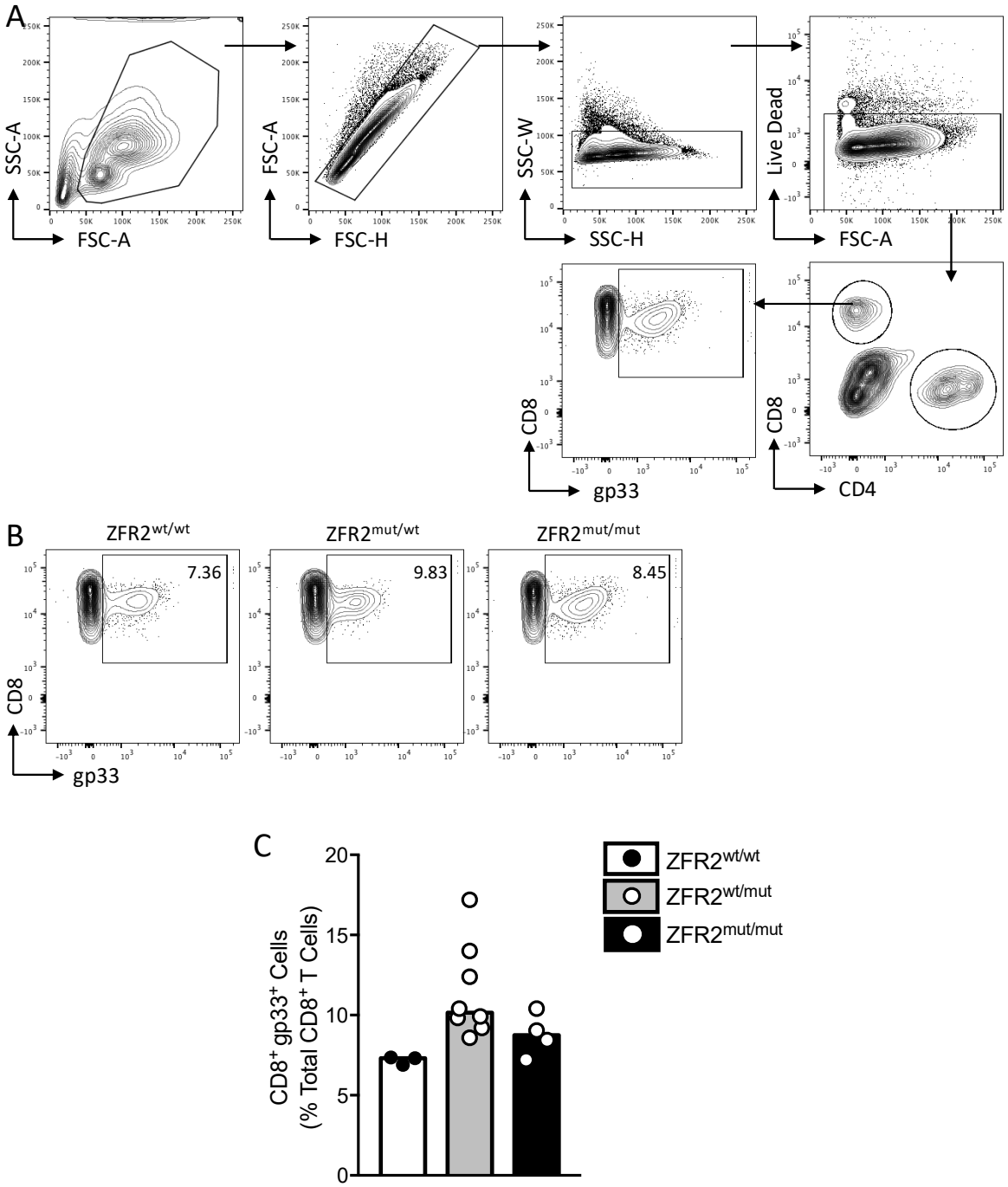


Figure 3.41. Flow cytometric analysis of ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ circulating virus-specific CD8$^+$ cells eight days after LCMV infection.

(A) Gating strategy used to quantify virus-specific (gp33$^+$) CD8$^+$ cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice eight days after LCMV infection. (B) Representative plot of virus-specific (gp33$^+$) CD8$^+$ cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. (C) Column graphs representing the median percentage of virus-specific (gp33$^+$) CD8$^+$ cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse.

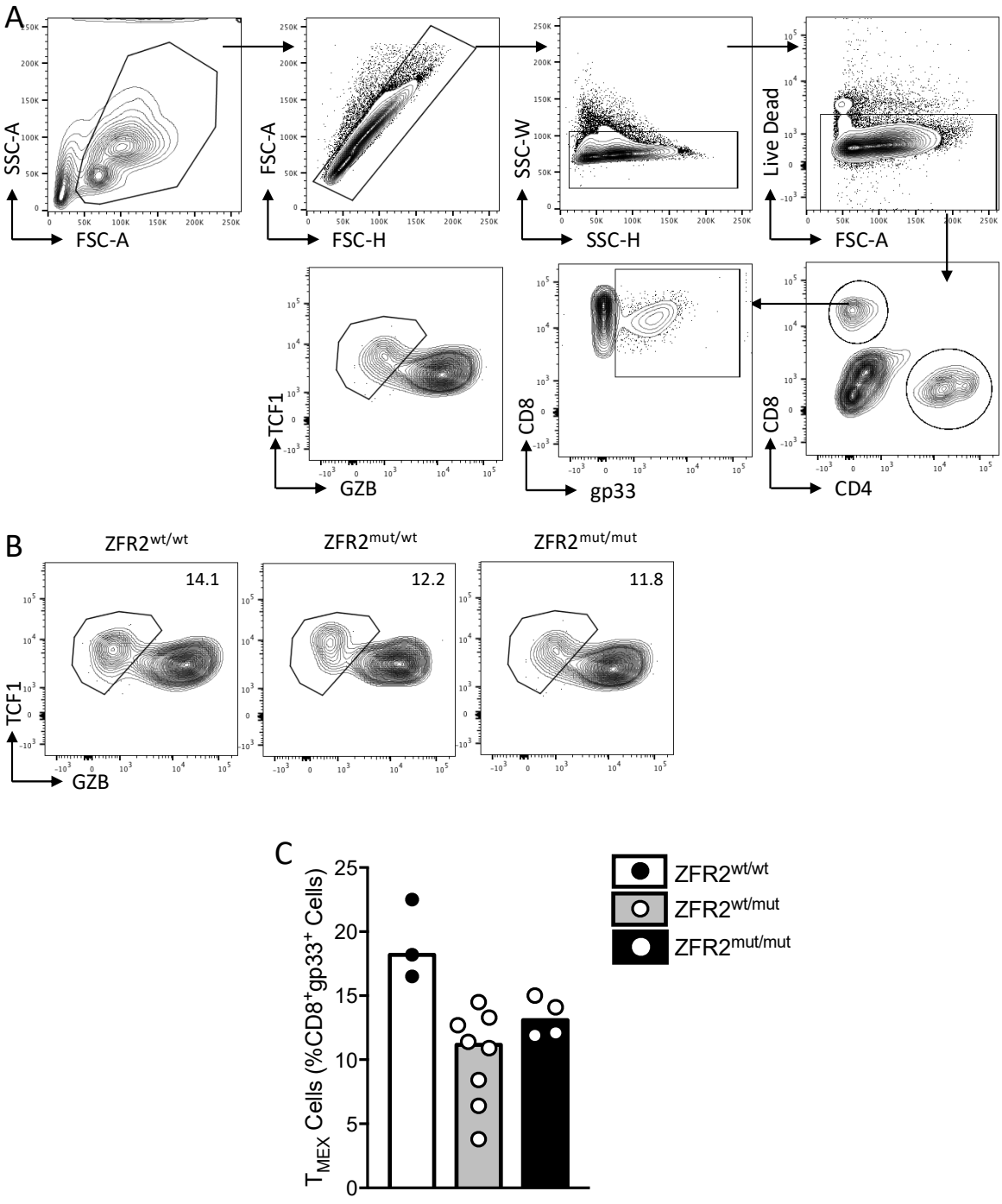


Figure 3.42. Flow cytometric analysis of ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ circulating virus-specific exhausted memory T cells (T_{MEX}) eight days after LCMV infection.

(A) Gating strategy used to quantify virus-specific exhausted memory T cells (T_{MEX}) in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice eight days after LCMV infection. (B) Representative plot of virus-specific exhausted memory T cells (T_{MEX}) in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. (C) Column graphs representing the median percentage of virus-specific exhausted memory T cells (T_{MEX}) in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse.

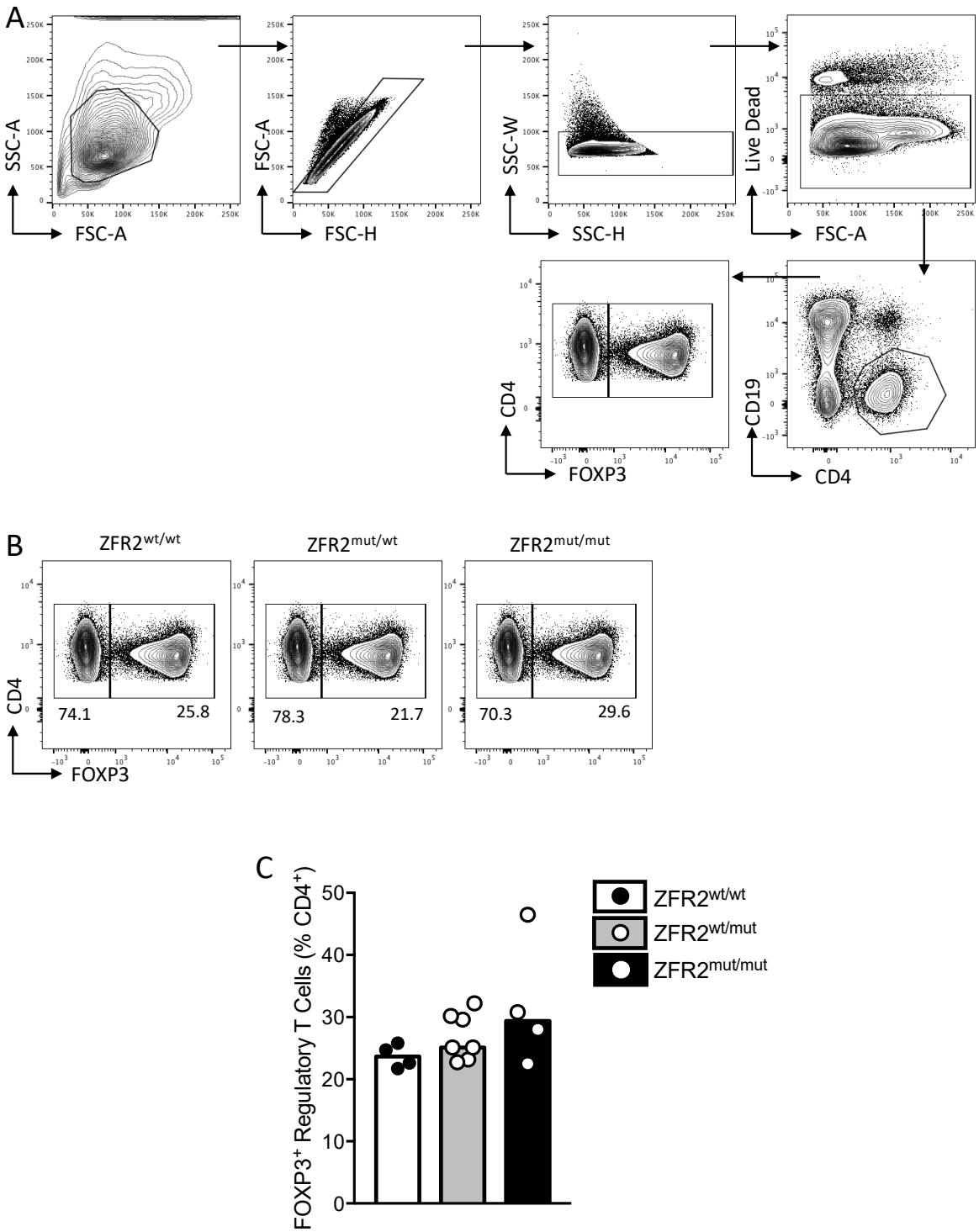


Figure 3.43. Flow cytometric analysis of ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ splenic regulatory T cells 28 days after LCMV infection.

(A) Gating strategy used to quantify splenic regulatory T cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice 28 days after LCMV infection. (B) Representative plot of splenic regulatory T cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. (C) Column graphs representing the median percentage of splenic regulatory T cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse.

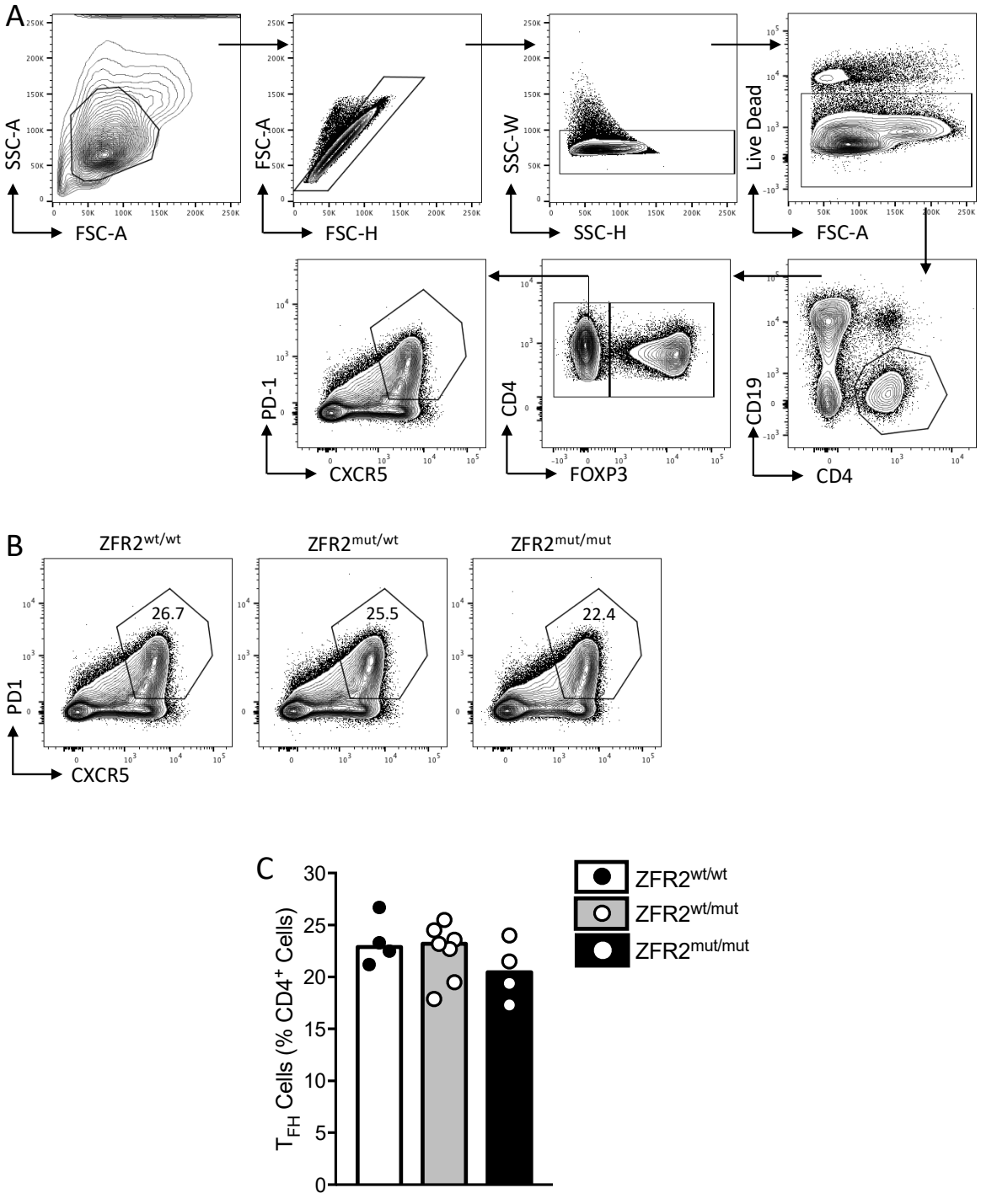


Figure 3.44. Flow cytometric analysis of ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ splenic T_{FH} cells 28 days after LCMV infection.

(A) Gating strategy used to quantify splenic T_{FH} cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice 28 days after LCMV infection. (B) Representative plot of splenic T_{FH} cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. (C) Column graphs representing the median percentage of splenic T_{FH} cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse.

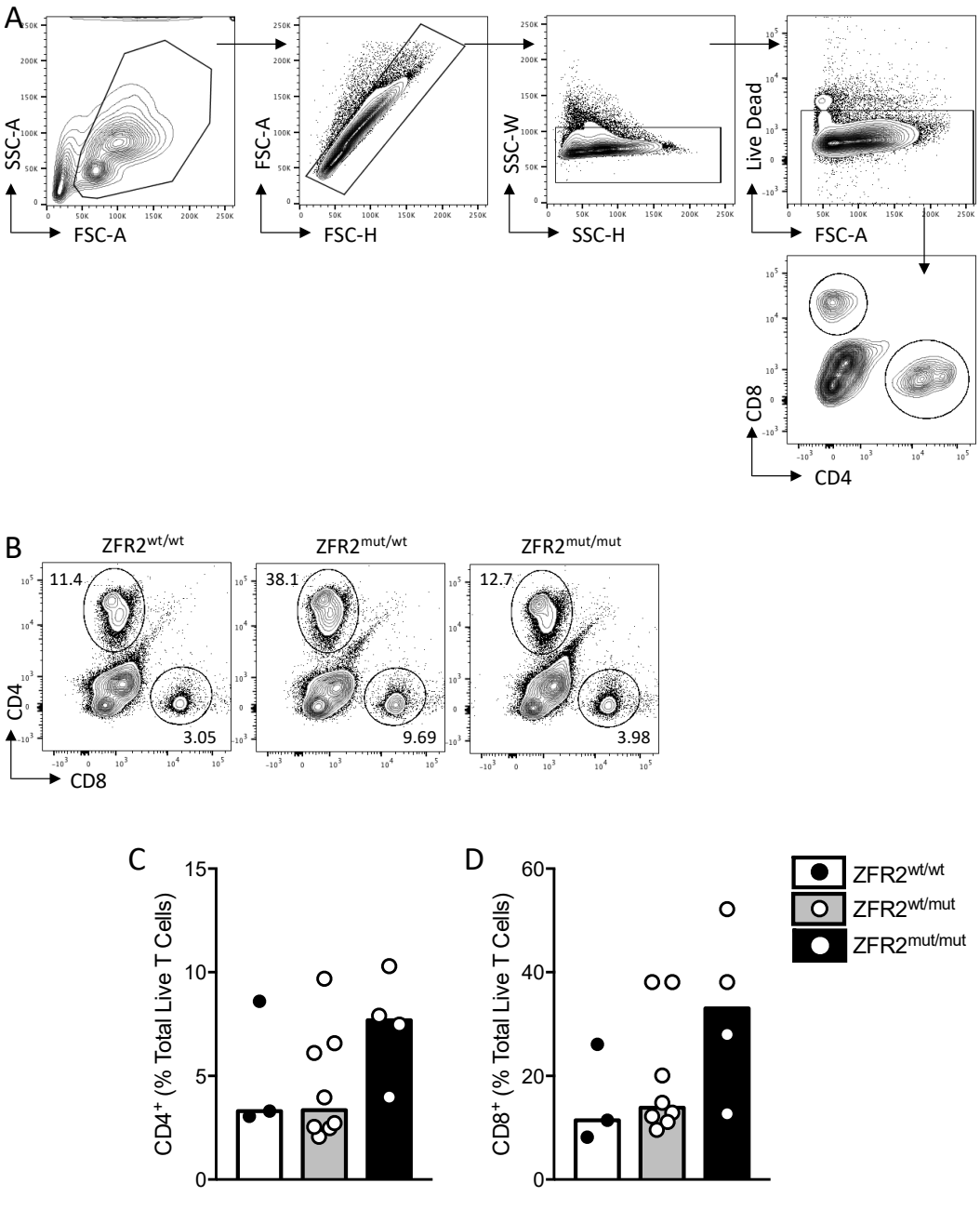


Figure 3.45. Flow cytometric analysis of ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ splenic CD4^{+} and CD8^{+} T cells 28 days after LCMV infection.

(A) Gating strategy used to quantify splenic CD4^{+} and CD8^{+} T cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice 28 days after LCMV infection. (B) Representative plot of splenic CD4^{+} and CD8^{+} T cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. (C) Column graphs representing the median percentage of splenic CD4^{+} and CD8^{+} T cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse.

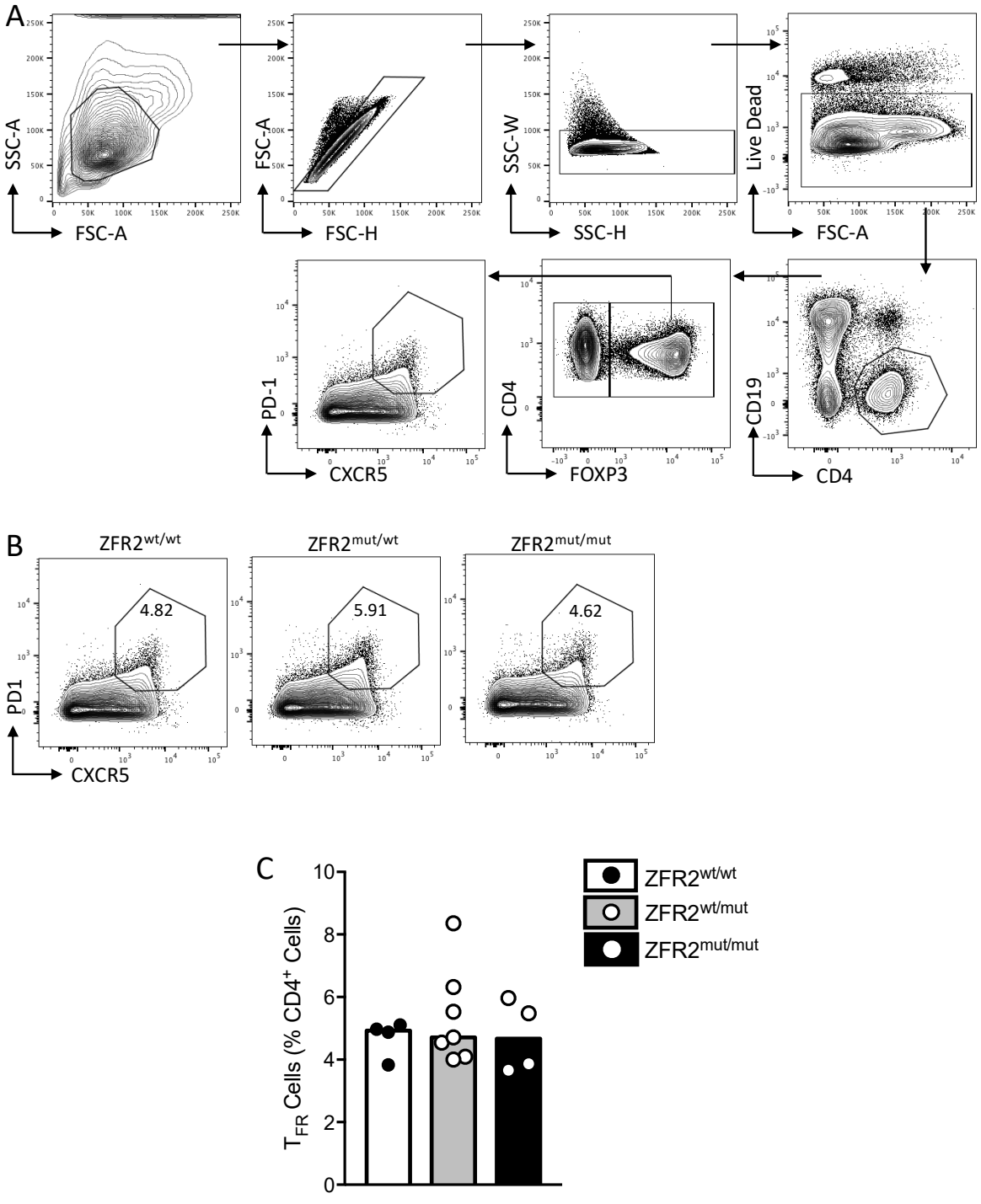


Figure 3.46. Flow cytometric analysis of ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ splenic T_{FR} cells 28 days after LCMV infection.

(A) Gating strategy used to quantify splenic T_{FR} cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice 28 days after LCMV infection. (B) Representative plot of splenic T_{FR} cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. (C) Column graphs representing the median percentage of splenic T_{FR} cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse.

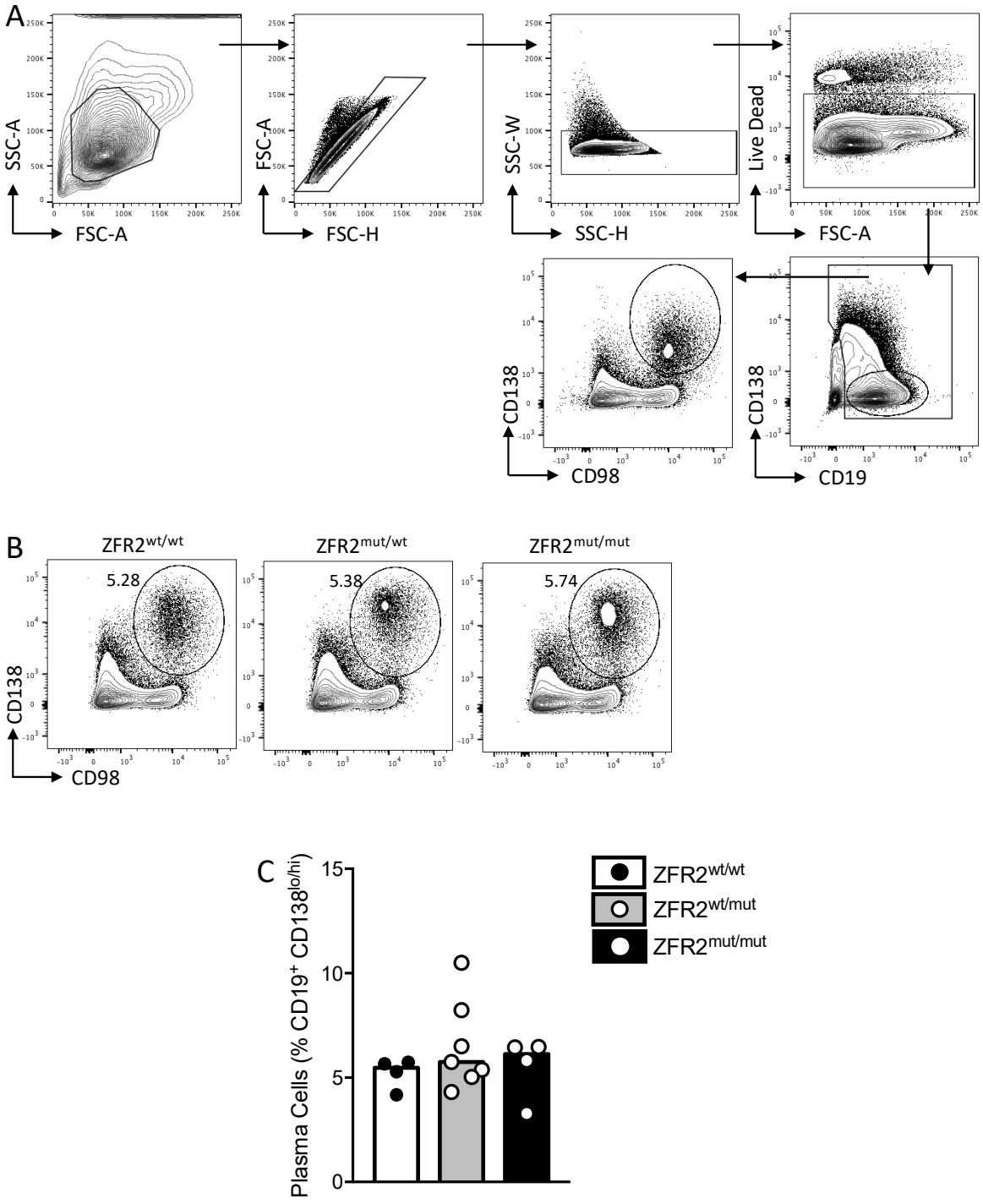


Figure 3.47. Flow cytometric analysis of ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ splenic plasma cells 28 days after LCMV infection.

(A) Gating strategy used to quantify splenic plasma cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice 28 days after LCMV infection. (B) Representative plot of splenic plasma cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. (C) Column graphs representing the median percentage of splenic plasma cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse.

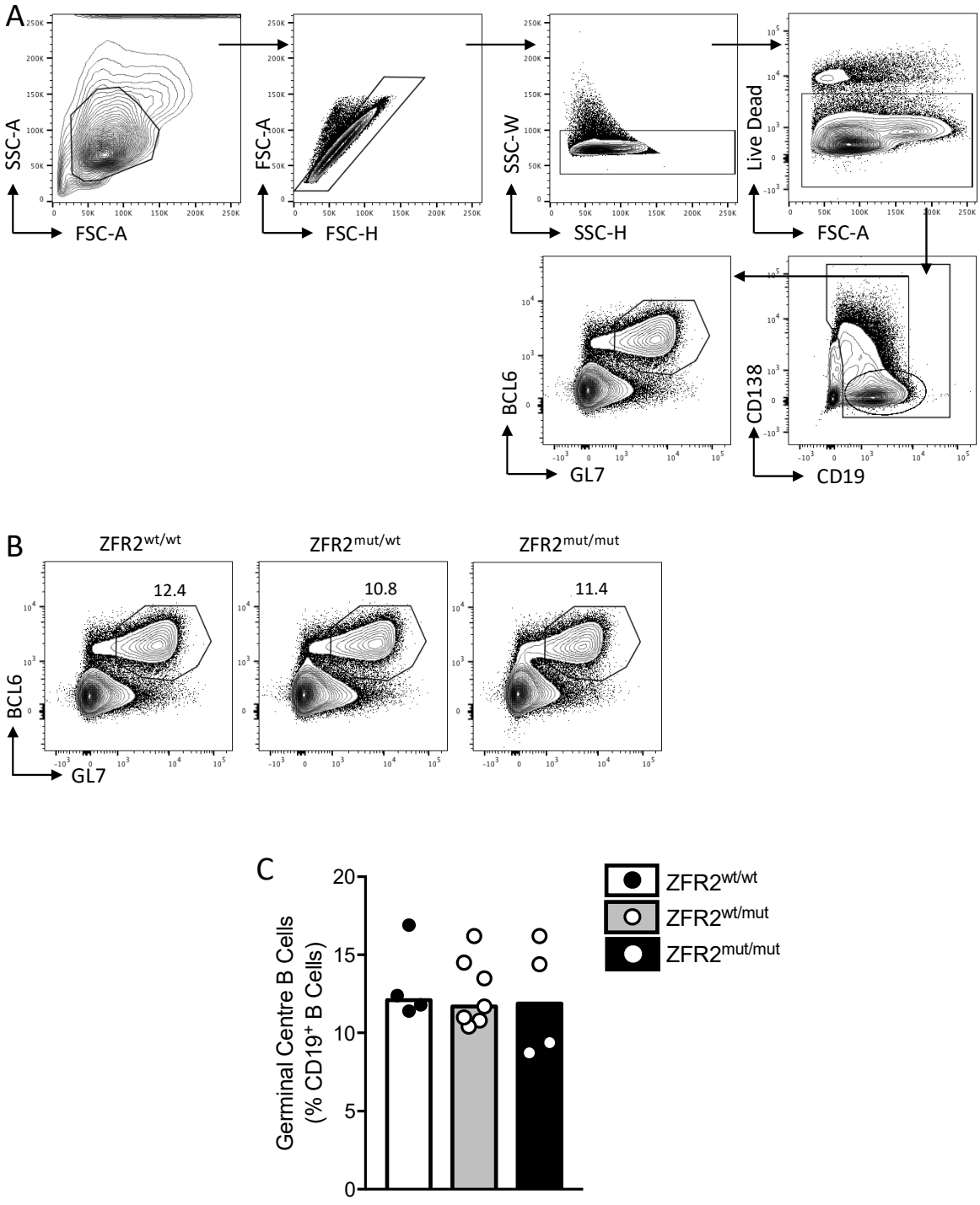


Figure 3.48. Flow cytometric analysis of ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ splenic germinal centre B cells 28 days after LCMV infection.

(A) Gating strategy used to quantify splenic germinal centre B cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice 28 days after LCMV infection. (B) Representative plot of splenic germinal centre B cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. (C) Column graphs representing the median percentage of splenic germinal centre B cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse.

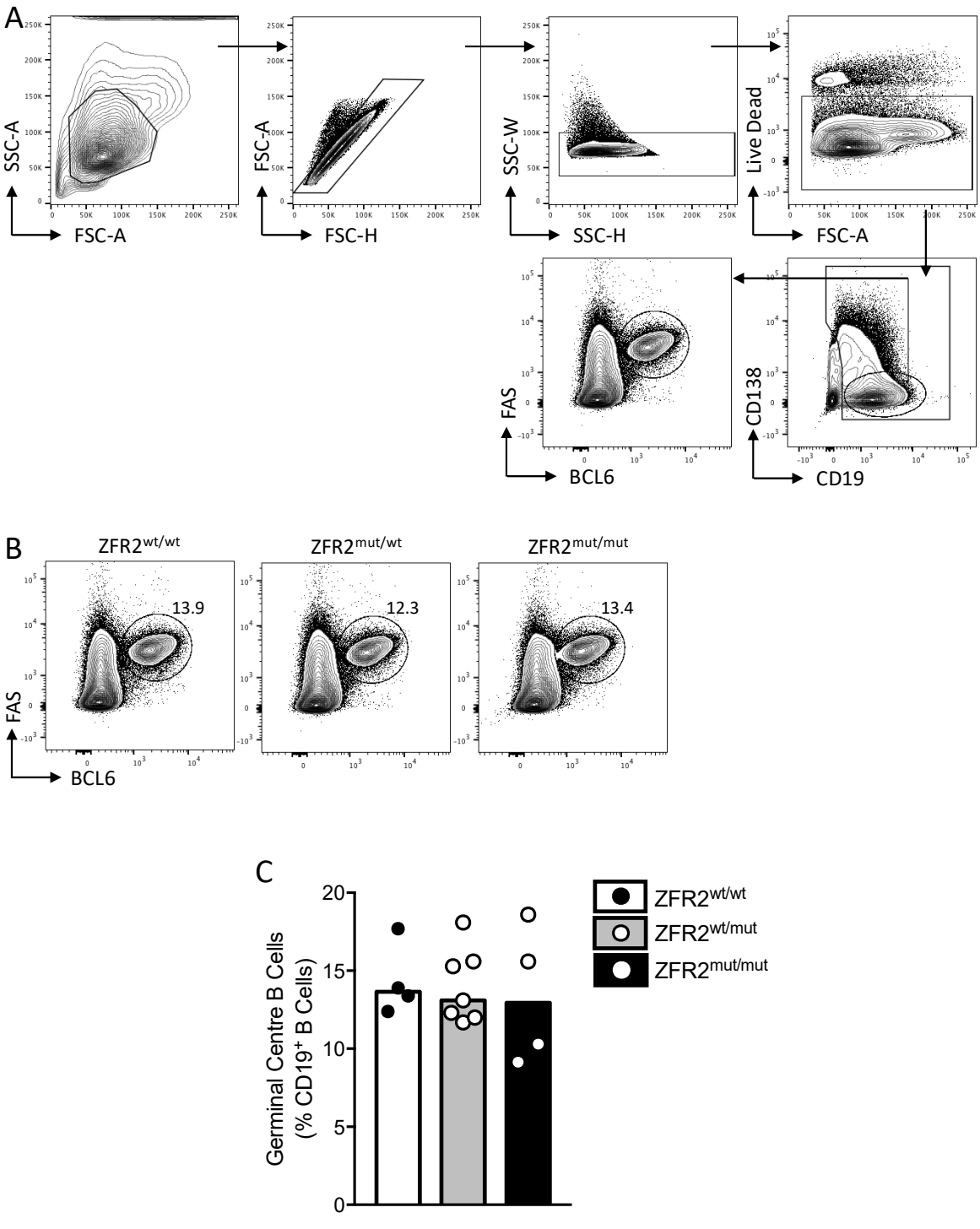


Figure 3.49. Flow cytometric analysis of ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ splenic germinal centre B cells 28 days after LCMV infection.

(A) Gating strategy used to quantify splenic germinal centre B cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice 28 days after LCMV infection. (B) Representative plot of splenic germinal centre B cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. (C) Column graphs representing the median percentage of splenic germinal centre B cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse.

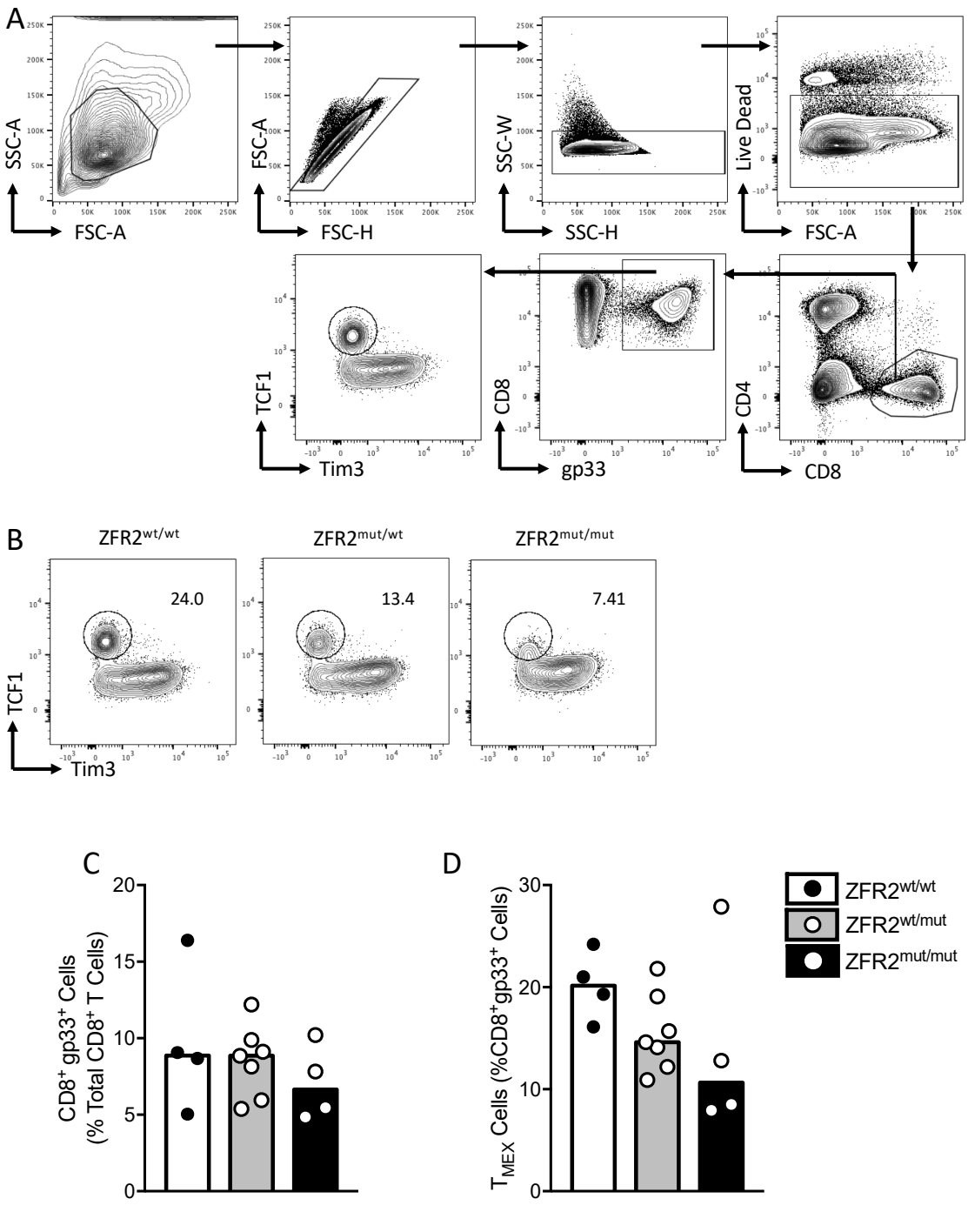


Figure 3.50. Flow cytometric analysis of ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ virus-specific splenic CD8$^+$ and exhausted memory T cells 28 days after LCMV infection.

(A) Gating strategy used to quantify virus-specific splenic CD8$^+$ T cells and exhausted memory (T_{MEX}) CD8$^+$ T cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice 28 days after LCMV infection. (B) Representative plot of virus-specific splenic CD8$^+$ T cells and exhausted memory (T_{MEX}) CD8$^+$ T cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. (C-D) Column graphs representing the median percentage of virus-specific splenic (C) CD8$^+$ T cells and (D) exhausted memory (T_{MEX}) CD8$^+$ T cells in ZFR2$^{wt/wt}$, ZFR2$^{wt/mut}$ and ZFR2$^{mut/mut}$ mice. Each dot represents a mouse.

These experiments were performed using the LCMV clone 13 strain for chronic LCMV infection. The Armstrong strain of LCMV causes an acute infection, and if often used in experiments aimed at assessing the effector response, which is quite sensitive to differences in the amounts of IL-2 present (Lieberman et al., 2001). Immunisation of ZFR2 mutant and control littermates with the Armstrong strain of LCMV are underway, but the results have not been included in this thesis due to time constrains.

3.6 Chapter Summary

This chapter concerns two families affected by SLE. The first family (Family A) is a multiplex family with four affected females (three with SLE and one with ANAs) and a deceased male who also had SLE. Whole exome/genome sequencing of these individuals identified a novel heterozygous SNV in a poorly understood protein called ZFR2 that segregated with disease in this family. This mutation lies within the DZF domain, a domain that is important for protein-protein interactions, especially with the paralogs ILF2 and ILF3. Analysis of the Centre for Personalised Immunology Variant Database (Centre for Personalised Immunology) revealed another male lupus patient from a different family (Family B) carrying a different mutation within the DZF domain of ZFR2, inherited from the patient's unaffected father. Whilst the *in vitro* and *in vivo* effects of this second mutation were not addressed, this individual was included in cellular and plasma phenotyping where possible to identify similar trends.

Interestingly, patients from both families possessed similar and unique cellular and plasma phenotypes, specifically an increase in regulatory T cells, an expanded $CD4^+$ $CD57^+$ cytotoxic T cell population, and an increase in plasma IL-2 (which may explain the increase T_{regs} and $CD4^+$ $CD57^+$ cytotoxic population as IL-2 has been shown to promote expansion of both these populations). Using mass spectrometry we identified a number of proteins ZFR2 interacts with, and with our knowledge of the roles of the paralogs in regulating IL-2 production transcriptionally and post-transcriptionally, we were able to predict the pathways that ZFR2 is involved in. Specifically, we demonstrated that ZFR2 plays a role in mRNA splicing, NFAT, AP-1 and IFNβ activity, and that the A771T variant from Family A is a gain-of-function

mutation, likely leading to increased IL-2, at least in human T cells, via increased NFAT and AP-1 activity as opposed to stabilising the *IL-2* mRNA transcript via the 3'UTR.

Finally, whilst we demonstrated that the CRISPR/Cas9 mice carrying the orthologous ZFR2 variant did not develop autoimmunity, they possessed some cellular phenotypes similar to the patient phenotypes, specifically an increase in T_{regs}. To exacerbate disease in these mice we infected them with LCMV, and preliminary findings revealed several interesting cellular trends to suggest that these mice may have more IL-2, specifically decreased T_{FH} and increased in exhausted $CD8^+$ memory cells (T_{MEX}). Further experiments with more mice per group, as well as additional readouts such as serum IL-2 and antinuclear antibodies are yet to be performed on newly-infected mice so that statistics can be performed, and definitive conclusions can be reached regarding these mice.

Overall, in this chapter we have identified a number of signalling pathways ZFR2 is involved in, including NFAT, AP-1 and IFNβ and mRNA splicing. We also show that the ZFR2 variants in these patients may be leading to a dysregulation in cytokine gene expression and production, specifically IL-2 through augmented NFAT and AP-1 activity. This increase in IL-2, and potentially other cytokines, may explain some of the abnormal cellular phenotypes we observed in these patients, and may contribute to some aspects of disease pathogenesis.

Chapter Four: Investigating the role of human T_{FR}-derived IL-32 in B cell responses

4.1 Preamble

This chapter addresses the second project of this thesis carried out in parallel and investigates the function of interleukin-32 (IL-32), a novel cytokine that we have found to be highly expressed in a subset of human germinal centre T cells that resemble T_{FR} cells. IL-32 is a unique cytokine whose function is regulated by alternative splicing, of which there are nine isoforms that have currently been described, the gamma isoform being the full-length isoform. Whilst IL-32 has been linked to cancers and has been described as a proinflammatory cytokine that induces cytokines such as TNFα, IL-1$\boldsymbol{\beta}$, IL-8 and IL-6, its role in the germinal centre reaction or its expression in T_{FR} cells has never been described.

In the search for the function of IL-32 in the germinal centre reaction, we stimulated germinal centre B cell subsets with recombinant IL-32γ and looked at a number of cell-surface markers, transcription factors, survival and proliferation. Of note, we identified a rapid upregulation of PD-L1 on multiple B cell subsets. PD-L1 is one of the two ligands for PD-1, which is highly expressed on T_{FH} and T_{FR} cells in the germinal centre. The interaction between PD-1 and PD-L1 has been shown to have several different (and occasionally conflicting) roles in the germinal centre reaction, with a number of reports concluding it leads to the attenuation of T cell activation and the suppression of T_{FH} cell differentiation and help for immunoglobulin class switching. This study proposes a novel pathway by which IL-32, derived from T_{FR} cells in the germinal centre, acts on B cells to induce them to become a previously described subset of regulatory B cells that expresses elevated amounts of surface PD-L1 and BAFFR, acting as a BAFF 'sink' to sequester BAFF from the germinal centre milieu.

4.2 Background

4.2.1 The discovery of interleukin-32 and its interacting proteins

IL-32 is a unique cytokine that was first described by Dahl et al. in 1992 as a protein highly expressed by mitogen-activated human T cells and IL-2 activated NK cells, and was termed NK4 (Dahl et al., 1992). It was later in 2005 when Kim et al. discovered that this cytokine was induced by interleukin-18 (IL-18) in the lung carcinoma A549 cell line and was subsequently termed IL-32 (Kim et al., 2005). This cytokine is expressed in horses, cows, sheep, pigs, primates and humans (Kim et al., 2005), and although this cytokine is not present in rodents, they appear have the ability to respond to human IL-32, as demonstrated using IL-32 transgenic mice (Bai et al., 2015a). In humans, IL-32 is found in most organs of the body, including the spleen, thymus, leukocytes, lung, small intestine, colon, prostate, heart, placenta, liver, muscle, kidney, pancreas and brain. IL-32 however is more prominently expressed in immune cells compared to non-immune cells (Kim et al., 2005) (Kobayashi and Lin, 2009) (Shoda et al., 2006), specifically T cells, NK cells and monocytes, and is only produced by some B cells in very low amounts (Keswani et al., 2012).

The receptor for IL-32 is unknown, but IL-32 has been shown to bind to Proteinase 3 (PR3), a neutrophil granule serine protease which exists in both a soluble and membrane form (Novick et al., 2006). PR3 is believed to cleave and enhance the activity of IL-32 (Novick et al., 2006), and it is thought that Alpha-1-antitrypsin (AAT), an inhibitor of PR3, might also modulate the activity of IL-32 (Marcondes et al., 2011). PR3 is not expressed by most immune cell types apart from granulocytes, thus making this an unlikely receptor for IL-32 in B cells, which is the IL-32-reponsive cell-type investigated in this thesis. IL-32 also possesses RGD motifs, which

can bind to membrane integrins (Heinhuis et al., 2012), and can also interact with intracellular proteins involved in integrin and focal adhesion signalling, such as focal adhesion kinase (FAK) and paxillin (Heinhuis et al., 2012).

4.2.2 The function of interleukin-32 is regulated by alternative splicing

The structure, regulation and sequence of IL-32 is dissimilar to other cytokines. Located on chromosome 16 p13.3, IL-32 has nine isoforms; IL-32$\boldsymbol{\gamma}$, IL-32 $\boldsymbol{\alpha}$, IL-32$\boldsymbol{\beta}$, IL-32$\boldsymbol{\delta}$, IL-32$\boldsymbol{\varepsilon}$, IL-32$\boldsymbol{\zeta}$, IL-32s, IL-32θ and IL-32$\boldsymbol{\eta}$, generated by alternative splicing of the full-length IL-32$\boldsymbol{\gamma}$ transcript (El-Far et al., 2016). A yeast two-hybrid assay conducted by Kang et al. in 2014 revealed that some isoforms can interact with each other and 10 interacting pairs were confirmed by immunoprecipitation (Kang et al., 2014b) (see Figure 4.1), suggesting that IL-32 is likely to have diverse modulatory effects through the interactions of the different isoforms. These cytokines are predominantly expressed intracellularly, however the full-length isoform, IL-32$\boldsymbol{\gamma}$, possesses a hydrophobic signal peptide in the N-terminus that allows its secretion. This signal peptide is spliced out in all other isoforms (Heinhuis et al., 2016a). Thus, it is likely that the IL-32 isoform derived from T_{FH} cells and in acting on B cells (the focus of this chapter) is IL-32$\boldsymbol{\gamma}$. The functions of these isoforms have not been well described and the question of which isoform is the most abundant is still up for debate, however IL-32$\boldsymbol{\gamma}$, IL-32$\boldsymbol{\beta}$ and IL-32$\boldsymbol{\alpha}$ are the most studied isoforms and their roles are discussed below.

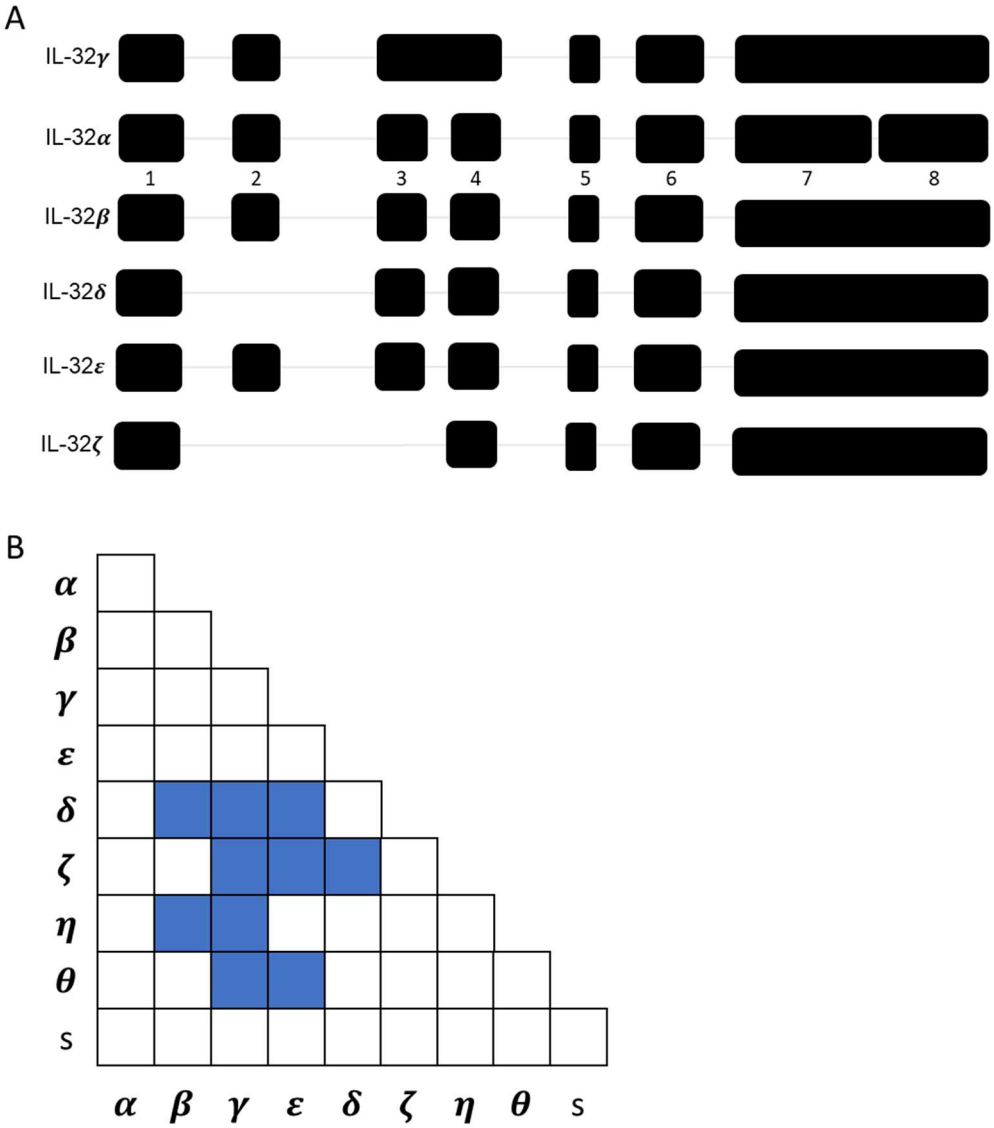


Figure 4.1. Isoforms of human IL-32.

(A) Two-dimensional schematic of IL-32 isoforms, based on findings from Goda et al., 2006 (Goda et al., 2006a). (B) The interacting pairs of IL-32 isoforms, identified by immunoprecipitation by Kang et al., 2014 (Kang et al., 2014a).

A biological function for IL-32 was first described by Netea et al. who showed that it induces proinflammatory cytokines such as TNF$\boldsymbol{\alpha}$ and IL-2 in RAW 264.7 macrophages (Netea et al., 2005). It has since been shown that IL-32 is a cytokine that exacerbates inflammation, inducing proinflammatory cytokines and playing a role in the immunopathogenesis and control of viral (Zepp et al., 2011, Li et al., 2008), bacterial (Heinhuis et al., 2011b, Bai et al., 2010, Joosten et al., 2006) and protozoan infections (Dos Santos et al., 2017). A number of factors have been shown to induce IL-32 including TNF$\boldsymbol{\alpha}$, IFN$\boldsymbol{\gamma}$, IL-1$\boldsymbol{\beta}$, IL-12 (Khawar et al., 2016), concanavalin A (Novick et al., 2006), CD3/CD28 (Moon et al., 2012), lipopolysaccharide (LPS) (Nakayama et al., 2013), PMA + ionomycin (Goda et al., 2006b), microbial molecules acting on TLR proteins and NOD2 receptors, and single and double stranded RNA (Ota et al., 2015). It has been demonstrated that IL-32 is induced by activating the NF$\boldsymbol{\kappa}$B, JAK/STAT, extracellular receptor kinases (ERK) and/or p38 MAPK pathways (Moschen et al., 2010).

The gamma isoform of IL-32 is considered to be the most potent and active with respect to inflammation, as it induces the production of proinflammatory cytokines such as TNFα, IL-1$\boldsymbol{\beta}$, IL-8 and IL-6 (Heinhuis et al., 2016a, Heinhuis et al., 2012, Heinhuis et al., 2011a, Choi et al., 2009a). A study by Joosten et al. demonstrated that injection of IL-32$\boldsymbol{\gamma}$ into the synovial joints of mice causes inflammation and swelling, however this swelling was reduced in TNF$\boldsymbol{\alpha}$ deficient mice (Joosten et al., 2006), demonstrating that this IL-32 induced inflammation is likely to be TNF$\boldsymbol{\alpha}$-dependent. This study by Joosten et al., in addition to others (Shoda et al., 2006), have elucidated a relationship between IL-32$\boldsymbol{\gamma}$ and TNF$\boldsymbol{\alpha}$, with IL-32$\boldsymbol{\gamma}$ inducing TNF$\boldsymbol{\alpha}$ (Shoda et al., 2006) and TNF$\boldsymbol{\alpha}$ driving the production of IL-32$\boldsymbol{\gamma}$ (Khawar et al., 2016), such that it becomes a self-perpetuating amplification of proinflammatory mediators.

The beta isoform of IL-32 is considered to have anti-inflammatory properties as it associates with PKC$\boldsymbol{\delta}$ and C/EBP$\boldsymbol{\alpha}$, resulting in the upregulation of IL-10 (Kang et al., 2013a) and downregulation of proinflammatory cytokine secretion (Kang et al., 2009). It is therefore thought that the splicing of the gamma isoform into the beta isoform acts as a safety switch or negative feedback loop. Interestingly, the beta isoform interacts with the delta isoform, inhibiting the beta function of IL-10 production, probably by inhibiting the association of IL-32$\boldsymbol{\beta}$ with PKC$\boldsymbol{\delta}$ (Kang et al., 2013b). The epsilon isoform is also believed to create a negative feedback for controlling proinflammatory activities, decreasing TNF$\boldsymbol{\alpha}$ mediated IL-8 transcriptions (Kim, 2014).

Studies have demonstrated that overexpression of the gamma and beta isoforms of IL-32 induces cell death, in a caspase-8 dependent way (Heinhuis et al., 2016b), inducing apoptosis and ROS/NOS (reactive oxygen species/ nitric oxide synthase) production (Ribeiro-Dias et al., 2017), as well as production of IL-8 (Heinhuis et al., 2016b). Interestingly, co-expression with CXCR1 (the IL-8 receptor) rescued cells overexpressing IL-32 from cell death (Heinhuis et al., 2016b). Furthermore, silencing IL-32$\boldsymbol{\beta}$, but not IL-32$\boldsymbol{\gamma}$, with siRNA reduced cell death and IL-8 secretion (Heinhuis et al., 2016b),

The alpha isoform of IL-32 associates with PKC$\boldsymbol{\varepsilon}$ and STAT3, as well as integrins and focal adhesion kinase 1 (FAK1), inducing IL-6 production (Kang et al., 2012a). IL-32$\boldsymbol{\alpha}$ also regulates the activity of the transcriptional repressor B Cell CLL/Lymphoma 6 (BCL6), which amongst many things negatively regulates IL-6 production by binding to consensus sites on IL-6, however in the presence of IL-32$\boldsymbol{\alpha}$ this is recovered in a PKC$\boldsymbol{\varepsilon}$ manner (Park et al., 2014a).

After PMA stimulation, IL-32$\boldsymbol{\alpha}$ also interacts with BCL6 in a PKC$\boldsymbol{\varepsilon}$ dependent manner, and when PKC$\boldsymbol{\varepsilon}$ is knocked down this interaction is not observed. It has been shown that PKC$\boldsymbol{\varepsilon}$, IL-32$\boldsymbol{\alpha}$ and BCL6 form a trimeric complex, inducing the SUMOylation (covalent modification with a small ubiquitin-like modifier (SUMO) protein) of BCL6. The interaction of PKC$\boldsymbol{\varepsilon}$, IL-32$\boldsymbol{\alpha}$ and BCL6 is thought to regulate IL-6 production, as IL-6 reporter activity was supressed in cells co-transfected with IL-32$\boldsymbol{\varepsilon}$ but was restored in cells co-transfected with IL-32$\boldsymbol{\alpha}$ (Park et al., 2012). IL-32$\boldsymbol{\alpha}$ also interacts with promyelocytic leukaemia zinc finger protein (PLZF), a transcriptional regulator that regulates the expression of interferon simulated genes and has a similar structure to BCL6, an interaction also regulated by PKC$\boldsymbol{\varepsilon}$ (Park et al., 2014b).

4.2.3 Known roles of IL-32 in human disease

IL-32 has been shown to play a role in infectious diseases, chronic inflammatory and autoimmune diseases such as irritable bowel syndrome (IBS) (Shioya et al., 2007), Crohn's disease (CD) (Shioya et al., 2007), Sjogren's syndrome (SS) and SLE, with SLE patients having lower plasma IL-32 levels compared to healthy controls (Wang et al., 2017, Inoue et al., 2014). Patients with rheumatoid arthritis have increased IL-32 levels in synovial biopsies (Joosten et al., 2006), and gene polymorphisms in IL-32 have been associated with systemic lupus erythematosus (Wang et al., 2016). Variants in NOD2 are implicated in Crohn's disease, and IL-32 has been shown to synergize with a NOD2 ligand MDP, suggesting a role for IL-32 in Crohn's disease (Netea et al., 2005). Furthermore, IL-32 is upregulated in inflamed mucosa of patients with irritable bowel disease, and is associated with the production of proinflammatory cytokines (Shioya et al., 2007).

IL-32 levels are also increased in the serum of patients with HIV, causing a T_H1 response and the induction of proinflammatory cytokines, triggering an anti-viral response, but has also been shown to induce immunosuppressive molecules to prevent extreme pathology, acting like a double-edged sword. In HEK293T cells and Jurkat T cells, silencing of IL-32 results in increased HIV replication (Palstra et al., 2018), and treatment of PBMCs with recombinant IL-32$\boldsymbol{\gamma}$ reduces viral load (Smith et al., 2011). Viral infections caused by Hepatitis C, B and influenza A have also been linked to IL-32 production. In Hepatitis C, IL-32 mRNA levels are associated with increased liver inflammation, fibrosis and damage. Increased IL-32 levels are also found in the serum of patients affected with Influenza A, and is thought to play a critical role in triggering proinflammatory cytokine storm (Wang et al., 2015). The gamma isoform is also considered to have the best antiviral effect, and is dependent on NF-□B and CREB (Li et al., 2010).

IL-32 is also elevated in patients with sepsis, along with other proinflammatory cytokines such as TNF$\boldsymbol{\alpha}$ and IL-6. Interestingly, in transgenic mice, the IL-32$\boldsymbol{\alpha}$ and IL-32β transgenic mice were susceptible to septic shock, but the IL-32$\boldsymbol{\gamma}$ mice were protected by an enhanced immune response that prevented the spread of the infection (Kobayashi et al., 2010). IL-32 also plays a role in Mycobacterium tuberculosis, specifically with latent infections, with silencing of IL-32 in infected macrophages resulting in decreased proinflammatory cytokines and proliferation of the Mycobacterium. Treating the macrophages with IL-32$\boldsymbol{\gamma}$ resulted in decreased Mycobacterium and apoptosis of cells, and IL-32$\boldsymbol{\gamma}$ transgenic mice had better survival rates and less alveolar macrophage damage when infected with Mycobacterium (Bai et al., 2010).

In chronic obstructive pulmonary disease (COPD), IL-32 is increased in the lung tissue, serum, sputum supernatants and bronchoalveolar lavage fluids, and has been shown to be produced by epithelial cells (Calabrese et al., 2008). IL-32 is also higher in the lung tissue of smokers and is associated with increased TNF$\boldsymbol{\alpha}$ and IFN$\boldsymbol{\gamma}$ in both COPD and lung disease in smokers (Calabrese et al., 2008). A number of studies have also implicated IL-32 in atherosclerosis and has been shown to enhance lipid accumulation (Xu et al., 2017, Damen et al., 2018, Damen et al., 2017).

Finally, since the first report of IL-32 in 2005, there has been increasing evidence that IL-32 plays an important role in the pathophysiology of cancers, including the initiation, proliferation and maintenance of cancer. IL-32 is highly expressed in various cancer tissues and has been correlated with both good and poor prognosis in cancers, depending on the tumour microenvironment and the isoform. For example, the gamma and beta isoforms have been associated with increased apoptosis of cancer cells in both colon cancer (Heinhuis et al., 2016b) and melanoma (Lee et al., 2016), whereas in breast cancer these same isoforms have been associated with increased invasion and migration of cancer cells. Whilst further investigations are required to elucidate the opposing roles of IL-32 in cancer, there is accumulating evidence that supports the importance of IL-32 in the development of cancers (Hong et al., 2017, Joosten et al., 2013, Marcondes et al., 2008, Sorrentino and Di Carlo, 2009b, Sloot et al., 2018).

4.3 Exploring a role for interleukin-32 in the germinal centre reaction

4.3.1 Interleukin-32 is highly expressed in germinal centre T cells

In a recent RNA sequencing (RNA-seq) experiment conducted by Pablo Fernández de Cañete Nieto from our laboratory, IL-32 was found to be present in large amounts in human germinal centre T cells, specifically in T_{FR}-like cells (Figure 4.2). This was confirmed with RT-PCR. IL-32 was also found to not be expressed in B cells, however it was detected in most T cell subsets (T_{reg}, T_{FR}, T_{Naive}, T_{FH} and $T_{Effector}$ cells), with the highest expression observed in the human tonsillar counterpart that is closest to mouse T_{FR} cells (Figure 4.2), as defined by a $CD4^+$ $CXCR5^{hi}$ $PD\text{-}1^{hi}$ $CD127^{lo}$ $CD25^+$ $BCL6^+$ phenotype. Despite these cells bearing most markers found in mouse T_{FR} and T_{reg} cells, including the highest expression of CTLA4 and lowest IL-2 production, human tonsil T_{FR}0like cells do not express FOXP3, unlike mouse T_{FR} cells. Thus, we are designating them T_{FR}-like cells for the purpose of this thesis.

As mentioned previously, mouse T_{FR} cells are a recently defined subset of $CD4^+$ T cells that are derived from regulatory T cells (Sage and Sharpe, 2015a) and are thought to limit T_{FH} and germinal centre B cell responses and limit the magnitude of germinal centre reactions (Linterman et al., 2011b, Wollenberg et al., 2011b, Chung et al., 2011a, Aloulou et al., 2016, Sage et al., 2014, Wing et al., 2014), and play important roles in the control of autoimmune responses (Fu et al., 2018, Sage and Sharpe, 2015b, Fonseca et al., 2017). Much of this understanding of T_{FR} cells has been obtained from murine studies, and whilst $FOXP3^+$ follicular T_{FR} cells have been reported in humans (Chung et al., 2011a, Carreras et al., 2006, Lim et al., 2004, Sayin et al., 2017), their function remains poorly understood. Even less is known about this subset in tonsils, which largely lacks FOXP3 expression.

Having detected IL-32 mRNA in cells with the characteristic germinal centre phenotype according to high PD-1 and CXCR5 expression, we asked whether IL-32-producing cells could indeed be found within germinal centres. Indeed, immunohistochemical analysis of human tonsils revealed expression of IL-32-producing cells within human germinal centres, specifically within the light-zone of the germinal centre (Figure 4.3). Interestingly, the majority of the IL-32 positive cells localised in the middle of the light zone rather than on the border of the light zone closer to the follicular mantle, also known as the outer zone, which is where the majority of the $CD4^+$ T cells localise in the germinal centre (MacLennan, 1994, Kerfoot et al., 2011). Co-staining of IL-32 (brown) with CD4 (red) revealed that only a small fraction of $CD4^+$ T cells in the germinal centre are double positive for IL-32 (Figure 4.4), suggesting that this IL-32 positive population of cells is a distinct population of $CD4^+$ follicular T cells within the germinal centre. Unfortunately, co-staining with CD25, a marker for T_{FR} cells, did not produce good quality stains. Some IL-32 producing $CD4^+$ T cells were also found outside the germinal centres, in areas likely representing the T cell zones.

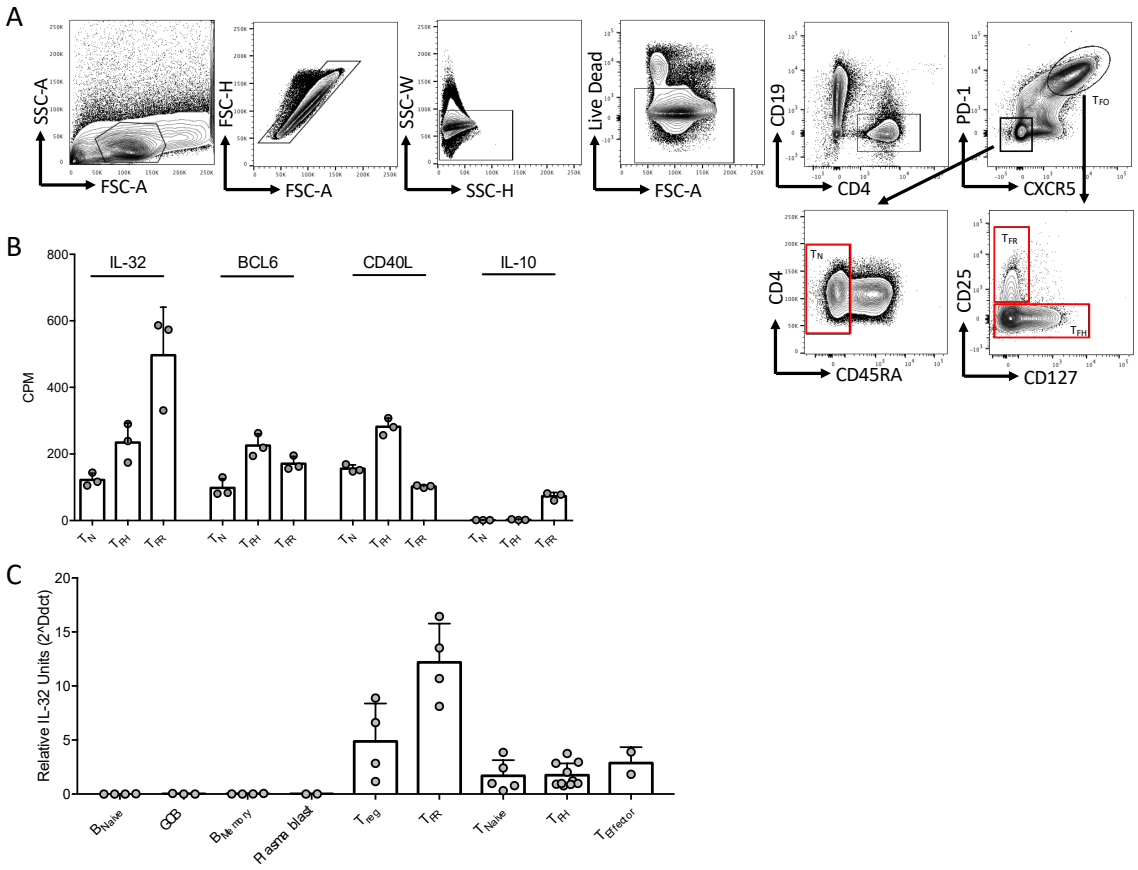


Figure 4.2. IL-32 is highly expressed in germinal centre T cells.

(A) Flow cytometric plots showing the gating strategy used to sort-purify naïve T cells (T_N), T_{FH} and T_{FR} cells for RNA-seq. (B) Selected transcripts (IL-32, BCL6, CD40L and IL-10) from the RNA-seq experiment in the indicated subsets from three tonsil donors (represented as RNA counts per million). (C) Confirmation of RNA-seq experiment with IL-32 RT-PCR on naïve B cells (B_{Naive}-$CD3^-$ $CD19^+$ $CD27^-$ $CD38^-$ IgD^+), germinal centre B cells (GCB-$CD3^-$ $CD19^+$ $CD38^+$ $CD27^{int}$) memory B cells (B_{Memory}- $CD3^-$ $CD19^+$ $CD38^-$ $CD27^+$), plasmablasts ($CD3^-$ $CD19^+$ $CD38^{hi}$ $CD27^{hi}$), regulatory T cells (T_{regs}-$CD4^+$ $CD19^-$ $CD25^+$ $CD127^-$), T_{FR}, T_{FH} and T_{Naive} (as gated in A), and effector T cells ($T_{Effector}$-$CD4^+$ $CD19^-$ $CD25^+$ $CCR7^-$ $CD45RO^-$). The housekeeping gene used was β1-Microglobulin. Each dot represents a single tonsil donor.

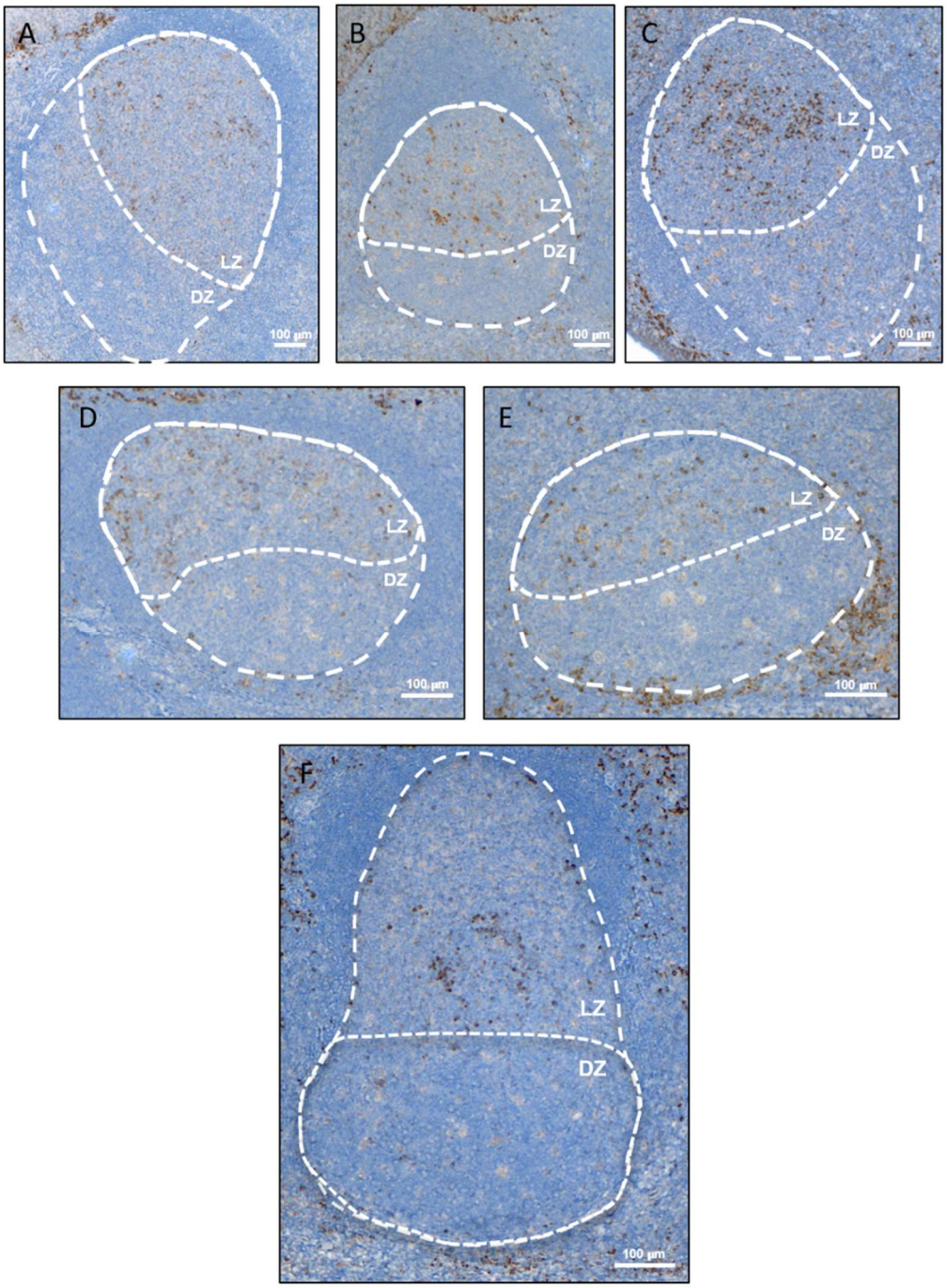


Figure 4.3. Identification of IL-32^{+} cells in human tonsils

Immunohistochemistry staining of human tonsil tissue showing localisation of IL-32 positive cells (brown). Tissue sections were counterstained with haematoxylin (blue). Each image represents a different tonsil donor. Original magnification was x100 for all tonsil sections, except for D which was x200. Immunohistochemistry was performed by A.Bullman, and imaging was performed by J. Dahlstrom.

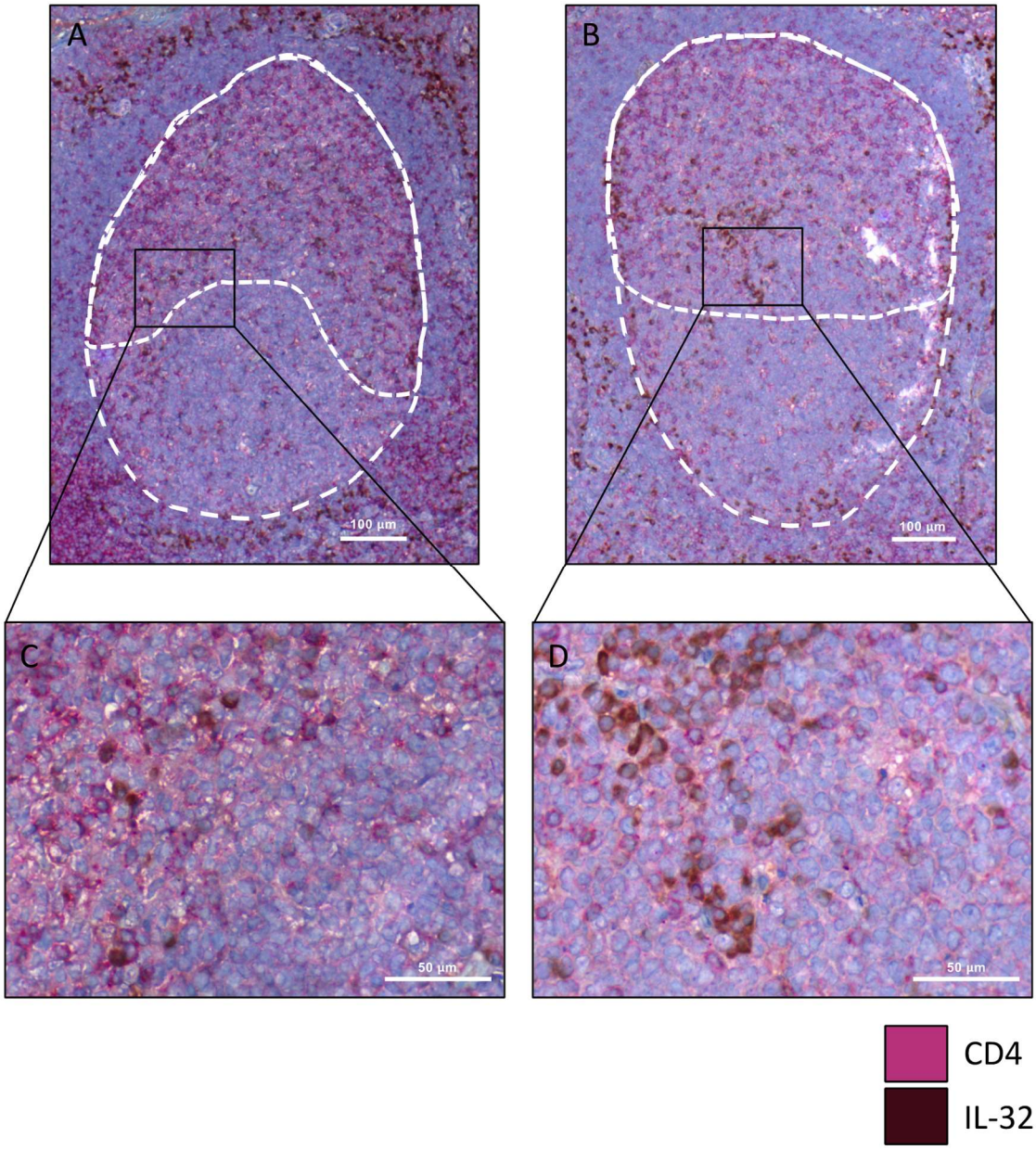


Figure 4.4. Co-localisation of IL-32$^+$ and CD4$^+$ cells in human tonsils.

Double immunohistochemistry staining of human tonsil tissue showing localisation of IL-32 positive cells (brown) and CD4 positive cells (red). Tissue sections were counterstained with haematoxylin (blue). Each image represents a different tonsil donor. Original magnification was x100 A and B, and x630 for C and D, representing a magnified portion of the x100 image. Immunohistochemistry was performed by A.Bullman, and imaging was performed by J. Dahlstrom.

4.3.2 IL-32 selectively binds to germinal centre B cell subsets

To investigate which cells IL-32 binds to and acts on we incubated fluorochrome-conjugated IL-32γ (1.5µg/mL) with unfractionated total human tonsil cells for two hours. Flow cytometric analysis of $CD4^+$ T cells and B cell subsets revealed that IL-32 does not bind strongly to $CD4^+$ T cells but binds to $CD19^+$ B cells. (Figure 4.5). Interestingly, the amount of bound IL-32 was slightly different between each B cell subset, with more IL-32 binding memory B cells over the other B cell subsets, as per the following order: memory B >> naïve B >> germinal centre B >> plasmablasts, with minimal albeit detectable expression in plasmablasts.

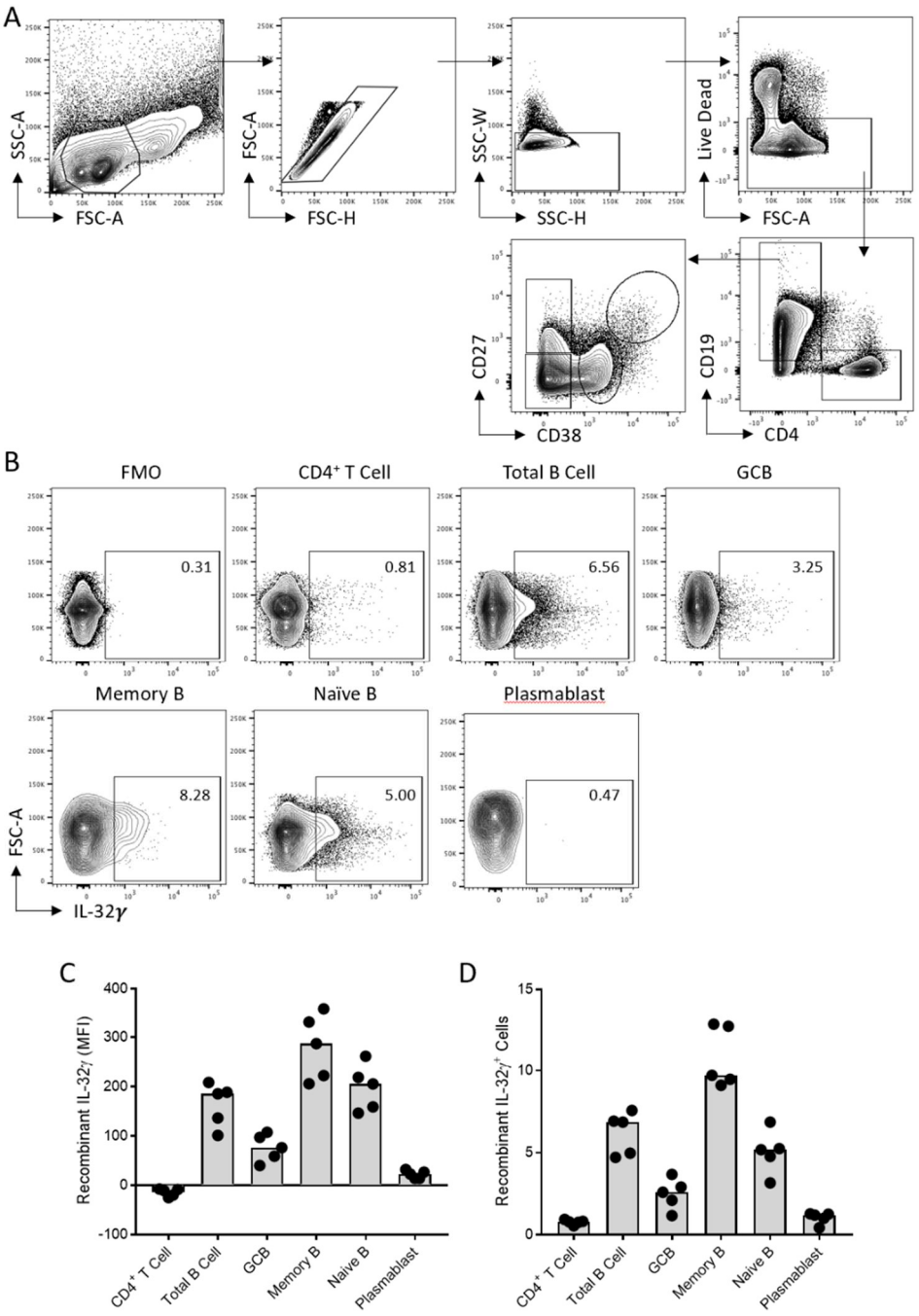


Figure 4.5. rIL-32γ binds to B cells.

(A) Flow cytometric gating strategy used to assess rIL-32γ expression on CD4$^+$ T cells, total CD19$^+$ B cells, germinal centre B cells (GCB), memory B cells, naïve B cells and plasmablasts following stimulation with 1500 ng/mL of conjugated (AF647) rIL-32γ for 2 hours (B) representative plots demonstrating the amount of rIL-32γ bound to the indicated cell subsets, as well as a fluorescence minus one (FMO) control. (C-D) Column graphs demonstrating the median (C) MFI and (D) percentage of rIL-32γ bound to each cell subset indicated. Each dot represents a single tonsil donor. Representative of pooled data from two experiments.

4.3.2 IL-32 selectively upregulates PD-L1 on human tonsil B cells

Having determined that IL-32γ mainly binds to B cells, we investigated the effect of human IL-32 on B cells by isolating human tonsil B cells by magnetic separation (Human B Cell Isolation Kit II, Miltenyi Biotec) and culturing them for 24 hours with IL-32γ. We chose the gamma isoform as this is the full-length isoform of IL-32 and has been shown to be the most potent in literature (Choi et al., 2009b) and the form that can be secreted. We then looked for changes in expression of several proteins, receptors and transcription factors important for B cell survival and/or function, including BAFFR, IL-21R, FAS, CD86, ICOSL, CTLA4, PD-L1, PD-L2, BCL6, FOXP3, CXCR4, CD83, PD-1, IgD and IgG. No changes were observed in the percentage or MFI for any of these molecules, with the exception of PD-L1. Indeed, we found a selective and statistically significant three-fold (median) increase in PD-L1 on the surface of the B cells ($p=0.0002$ for percentage, $p<0.0001$ for MFI) (Figure 4.6).

We then investigated the earliest time point this upregulation of PD-L1 occurred on the cell surface of the B cells by sorting cells by magnetic separation (Human B Cell Isolation Kit II, Miltenyi Biotec) and stimulating cells with or without IL-32γ for 5 minutes, 10 minutes, 30 minutes, 2 hours and 24 hours. Upregulation of PD-L1 occurred within five minutes of stimulation with IL-32γ, and was maintained at comparable amounts throughout all time points tested. Of note, PD-L2, the other ligand for PD-1, was not upregulated on B cells at any time point, and interestingly there was decreased expression of PD-L2 on the surface of B cells at five, 10 and 30 minutes (Figure 4.7). We also investigated changes in expression of BAFFR, IL-21R, FAS, CD86, ICOSL, CTLA4, CXCR4, CD83, PD-1, IgD and IgG on the B cells at

these time points and found no significant differences in the percentage or MFI of any of these surface markers (Figure 4.8-4.13).

This rapid upregulation of PD-L1 on the surface of B cells following stimulation with IL-32$\boldsymbol{\gamma}$ for 5 minutes was intriguing: it suggested that there are intracellular stores of PD-L1 within the cell. To investigate the possibility that IL-32 was inducing rapid translocation of intracellular PD-L1 to the cell surface, we sorted B cells by magnetic separation (Human B Cell Isolation Kit II, Miltenyi Biotec), incubated cells with or without IL-32$\boldsymbol{\gamma}$, stained the cell surface with saturating amounts of anti-PD-L1 (BV605), and then stained intracellularly with anti-PD-L1 conjugated to a different fluorophore (APC). This revealed that the majority of PD-L1 is stored intracellularly in B cells, and upon stimulation with IL-32$\boldsymbol{\gamma}$ it is translocated to the surface of the B cells (Figure 4.14).

Our next question was whether the other two main isoforms of IL-32, specifically IL-32$\boldsymbol{\alpha}$ and IL-32$\boldsymbol{\beta}$, were also able to induce PD-L1 on the surface of B cells, and what dose of each IL-32 isoform is most effective at inducing PD-L1. We performed a dose titration of rIL-32$\boldsymbol{\gamma}$, rIL-32$\boldsymbol{\alpha}$ and rIL-32$\boldsymbol{\beta}$, starting at 0 $\boldsymbol{\mu}$g/mL and increasing two-fold from 0.04 $\boldsymbol{\mu}$g/mL until 40.96 $\boldsymbol{\mu}$g/mL for each cytokine. Expression of PD-L1 was determined on human tonsil B cells sorted by magnetic separation (Human B Cell Isolation Kit II, Miltenyi Biotec) from three healthy tonsil donors, treated with the different doses and isoforms of IL-32. As expected, rIL-32$\boldsymbol{\gamma}$ was the most potent isoform with respect to inducing PD-L1 on B cells. rIL-32$\boldsymbol{\beta}$, and to a much smaller extent rIL-32$\boldsymbol{\alpha}$, also induced PD-L1 expression (Figure 4.15-4.17). Interestingly, the upregulation of PD-L1 started to plateau at the highest IL-32$\boldsymbol{\gamma}$ concentrations, around 10-20

$\boldsymbol{\mu}$g/mL. We decided to use a concentration of 1.5 $\boldsymbol{\mu}$g/mL of rIL-32$\boldsymbol{\gamma}$ for all further experiments as this elicited a modest response.

As IL-32 associates with some, but not all B cell subsets, we asked whether a) the B cells upregulating PD-L1 were those that preferentially bound IL-32; and/or b) whether only specific B cell subset/s were able to respond to IL-32 by upregulating PD-L1. First we tested whether the IL-32 induced PD-L1^{hi} B cells preferentially bound IL-32, compared to PD-L1^{lo} B cells. For this, we incubated B cells with the fluorochrome-conjugated rIL-32γ for two hours. Interestingly, PD-L1^{hi} B cells were found to have greatest association with conjugated rIL-32γ compared to the PD-L1^{lo} B cells, suggesting that these PD-L1^{hi} B cells are the preferential binders of IL-32γ.

Next we tested whether the increase in PD-L1 on B cells was specific to a subset of B cells, especially as different B cell subsets bound rIL-32$\boldsymbol{\gamma}$ at different efficiencies (Figure 4.18). B cells were isolated by magnetic separation (Human B Cell Isolation Kit II, Miltenyi Biotec), and then further sorted into germinal centre B cells (CD19$^+$ CD38$^+$ CD27$^-$), memory B cells (CD19$^+$ CD38$^-$ CD27$^+$), naïve B cells (CD19$^+$ CD38$^-$ CD27$^-$ IgD$^+$) and plasmablasts (CD19$^+$ CD38^{hi} CD27^{hi}) using fluorescence activated cell sorting (FACS). Cells were cultured for 30 minutes with or without rIL-32$\boldsymbol{\gamma}$, and then PD-L1 expression was measured and quantified. Figure 4.19 demonstrates that PD-L1 was upregulated on total B cells, germinal centre B cells (GCBs), memory B cells and naïve B cells, but not plasmablasts. Furthermore, whilst PD-L1 can also be expressed on T cell subsets (Rosignoli et al., 2009), human T cells did not upregulate PD-L1 on their cell surface following rIL-32$\boldsymbol{\gamma}$ stimulation (Figure 4.20).

We also investigated whether rIL-32γ can induce PD-L1 on the surface of mouse B cells as some studies have shown that mice respond to rIL-32γ (Joosten et al., 2006). Total mouse B cells were isolated by magnetic separation (Mouse B Cell Isolation Kit, Miltenyi Biotec) and were stimulated with or without human rIL-32γ for two hours, however no change in surface PD-L1 was observed between stimulated and unstimulated B cells (Figure 4.21).

Having demonstrated that PD-L1 is rapidly and selectively upregulated on human B cells, we then asked whether IL-32 has an effect on B cell proliferation and differentiation following B cell activation with CD40, BCR and IL-21 in the presence or absence of rIL-32γ for five days. We found a modest increase in PD-L1 expression in non-activated cells stimulated with rIL-32γ for five days and a statistically significant increase in PD-L1 expression on activated B cells in the presence of rIL-32γ (Figure 4.22), although there was however some variability between donors and between the B cell subsets, likely due to differences in cell survival. Notably, we did not observe any differences in B cell proliferation or differentiation. Stimulation with rIL-32γ for 18 hours revealed a modest increase in B cell survival (Figure 4.23) (ratio of live/(dead+apoptotic); $p<0.0001$).

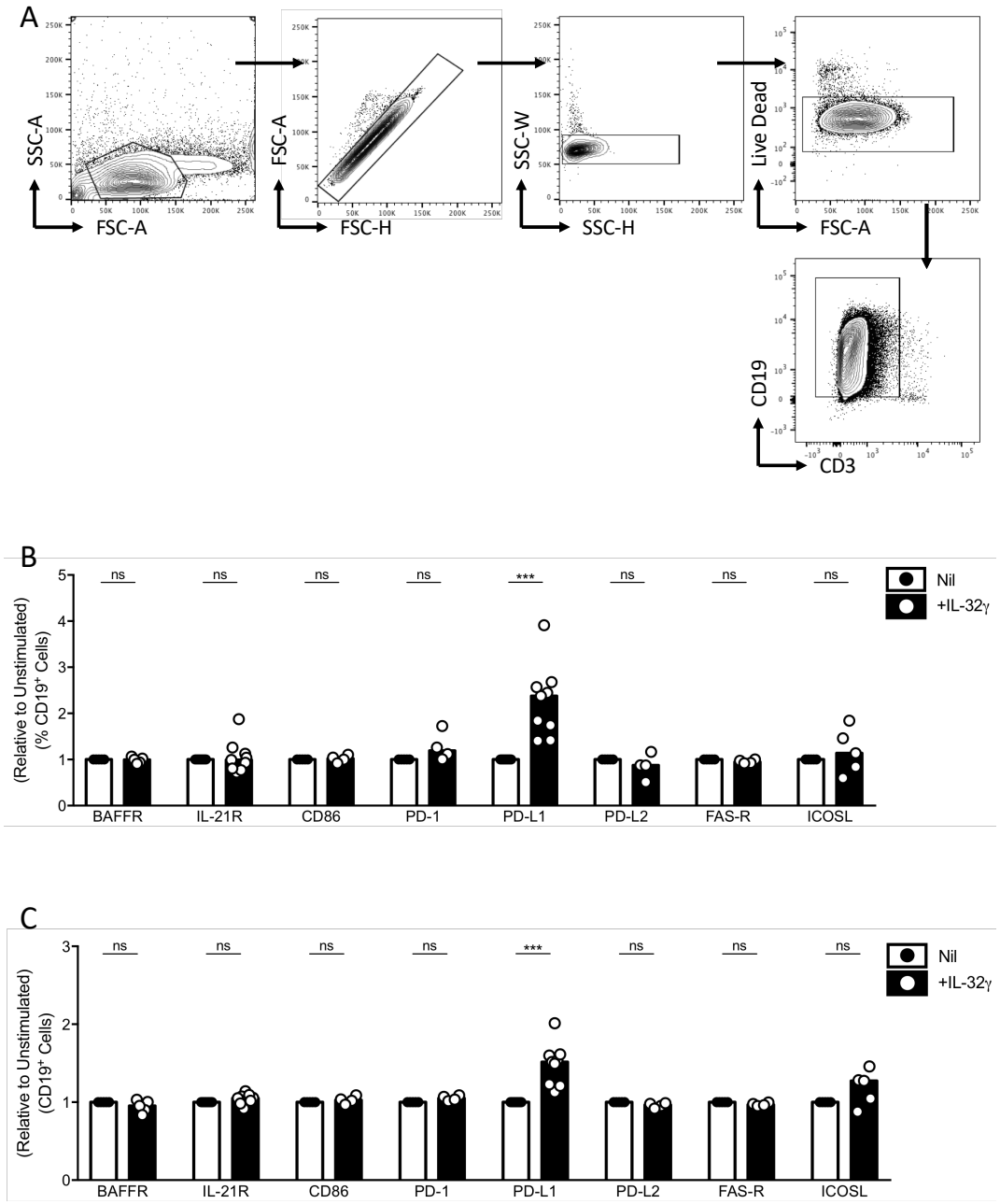


Figure 4.6. rIL-32γ selectively induces PD-L1 expression on the surface of human B cells after 24 hours.

(A) Flow cytometric gating strategy of $CD3^-$ $CD19^+$ B cells after MACS purification of B cells (Miltenyi Biotec) and stimulation with 500 ng/mL rIL-32γ for 24 hours to look at expression of BAFF-R, IL-21, CD86, PD-1, PD-L1, PD-L2, FAS-R and ICOSL as (B) percentage of total $CD19^+$ B cells, and (C) as MFI on total $CD19^+$ B cells. (***p≤0.001) (ns=not significant) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from three experiments.

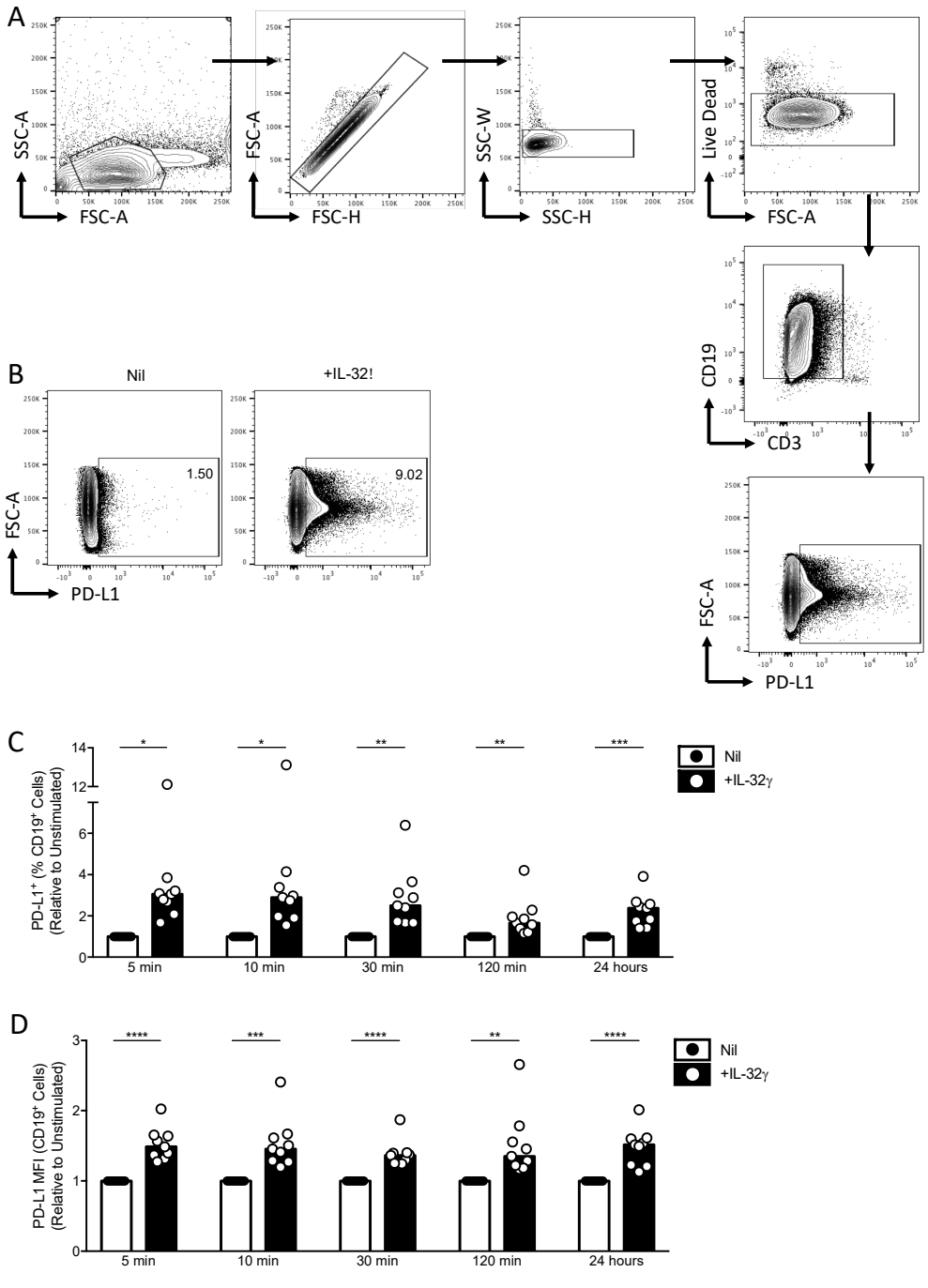


Figure 4.7. rIL-32γ induces PD-L1 expression on the surface of human B cells after 5 minutes.

(A) Flow cytometric gating strategy of $CD3^-$ $CD19^+$ B cells after MACS purification of B cells (Miltenyi Biotec) and stimulation with 500 ng/mL rIL-32γ to look at expression PD-L1 at 5 minutes, 10 minutes, 30 minutes, 120 minutes and 24 hours. (B) representative plot of unstimulated (Nil) and stimulated (rIL-32γ) (C-D) Column graphs representing PD-L1 expression as (C) percentage of total $CD19^+$ B cells, and (D) as MFI on total $CD19^+$ B cells. (*p≤0.05) (**p≤0.01) (***p≤0.001) (****p≤0.0001) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from three experiments.

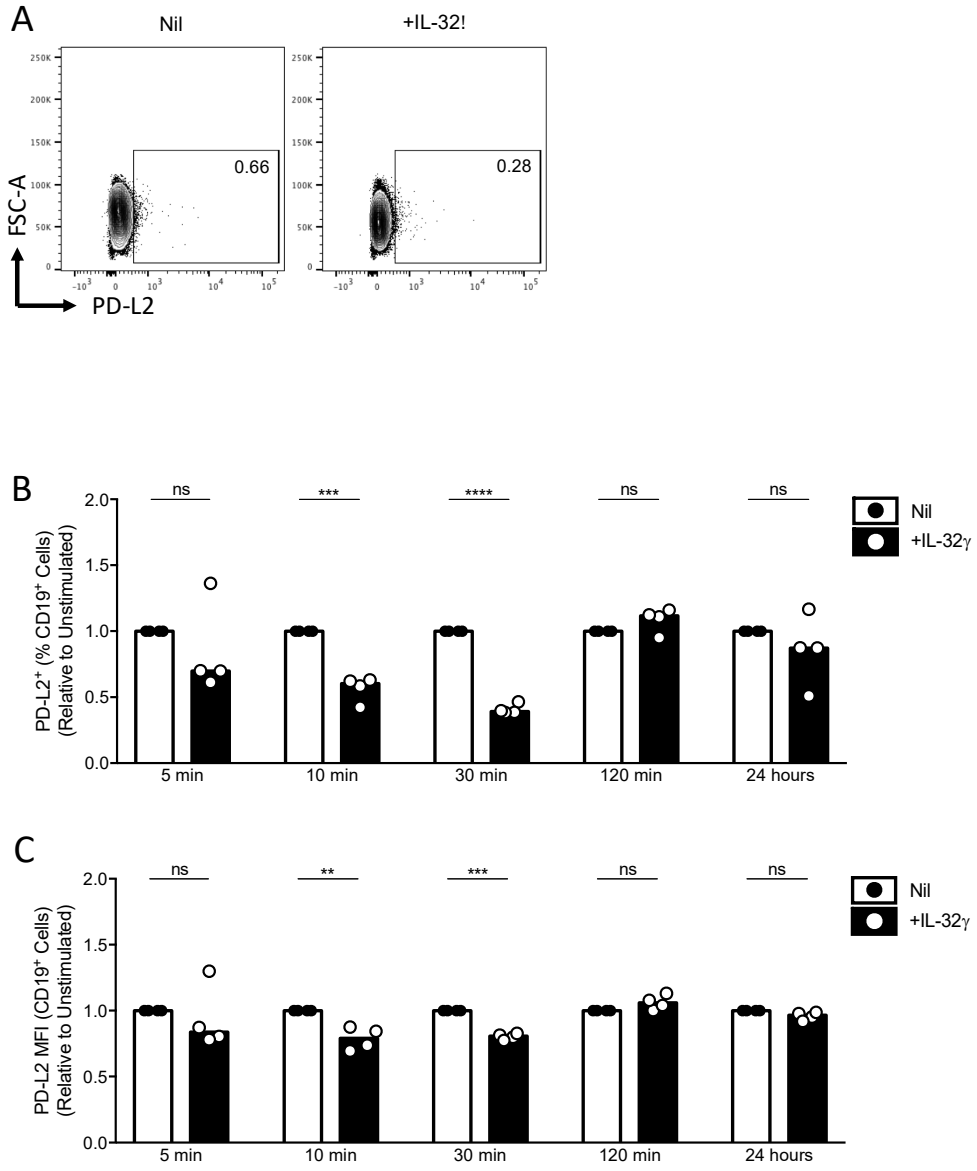


Figure 4.8. rIL-32γ does not alter expression of PD-L2 on human B cells.

$CD3^-$ $CD19^+$ B cells were MACS purified (Miltenyi Biotec) and B cells were gated as in Figure 4.5A to determine expression of PD-L2 on human B cells after stimulation with 500 ng/mL rIL-32γ for 5 minutes, 10 minutes, 30 minutes, 120 minutes and 24 hours. (A) Representative plot of unstimulated (Nil) and stimulated (rIL-32γ) (B-C) Column graphs representing PD-L2 expression as (C) percentage of total $CD19^+$ B cells, and (D) as MFI on total $CD19^+$ B cells. (**p≤0.01) (***p≤0.001) (****p≤0.0001) (ns=not significant) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from three experiments.

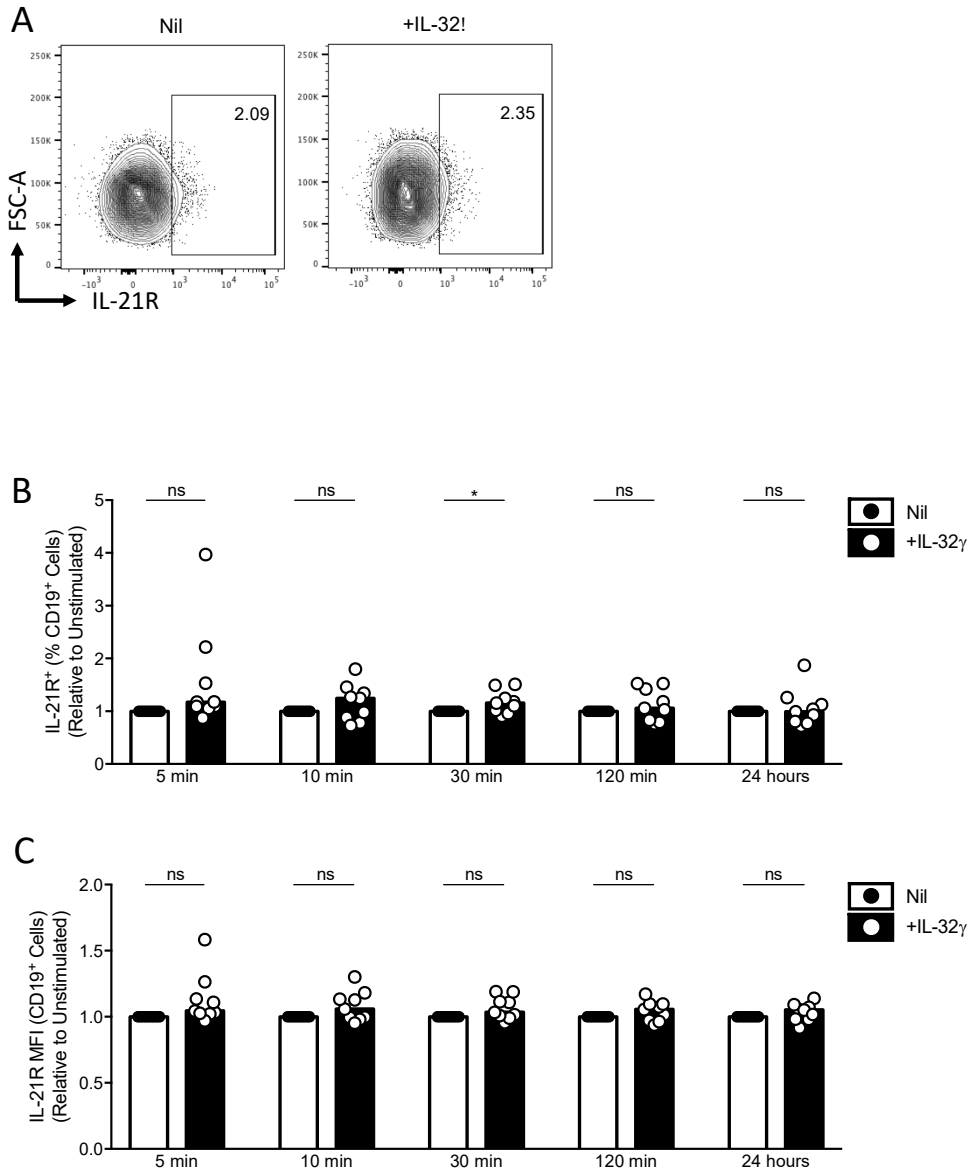


Figure 4.9. rIL-32γ does not alter expression of IL-21R on human B cells.

CD3$^-$ CD19$^+$ B cells were MACS purified (Miltenyi Biotec) and B cells were gated as in Figure 4.5A to determine expression of IL-21R on human B cells after stimulation with 500 ng/mL rIL-32γ for 5 minutes, 10 minutes, 30 minutes, 120 minutes and 24 hours. (A) Representative plot of unstimulated (Nil) and stimulated (rIL-32γ) (B-C) Column graphs representing IL-21R expression as (C) percentage of total CD19$^+$ B cells, and (D) as MFI on total CD19$^+$ B cells. (*p≤0.05) (ns=not significant) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from three experiments.

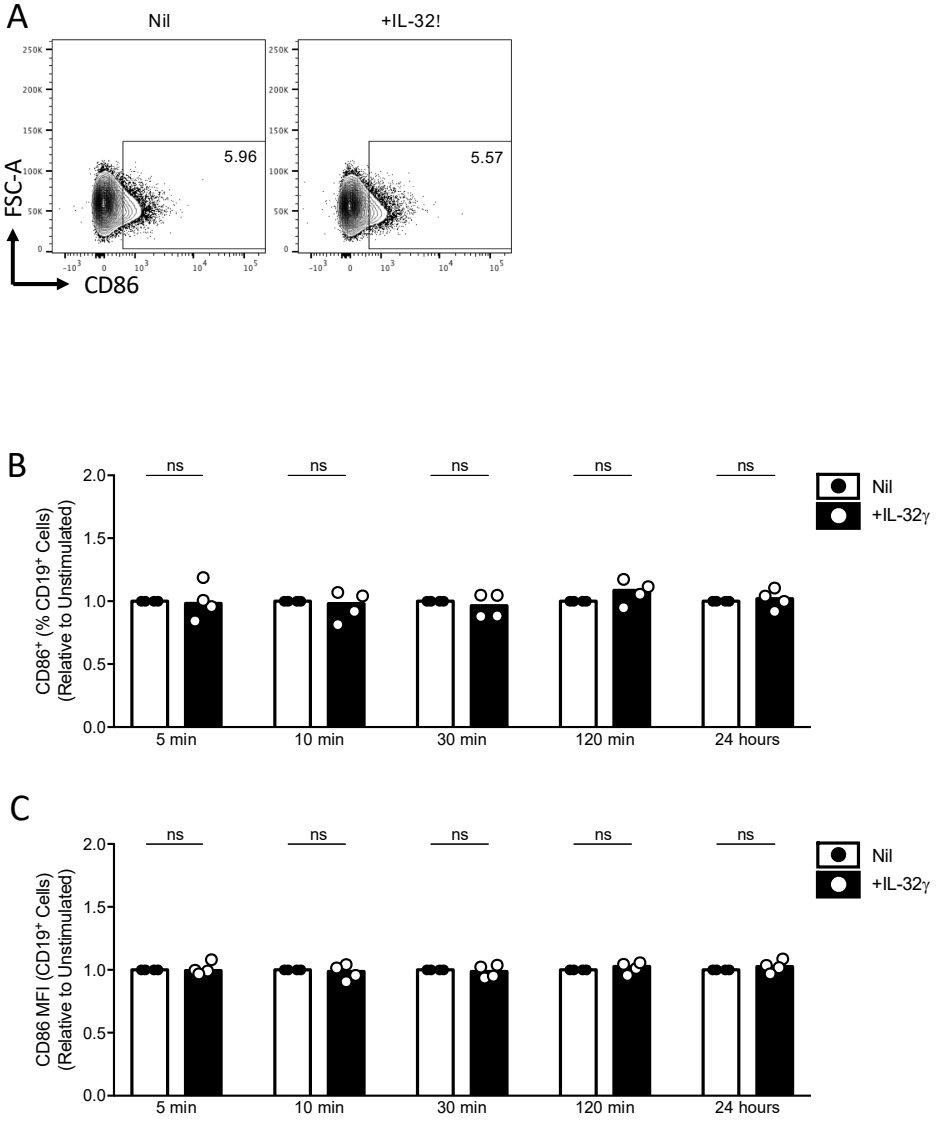


Figure 4.10. rIL-32γ does not alter expression of CD86 on human B cells.

CD3$^-$ CD19$^+$ B cells were MACS purified (Miltenyi Biotec) and B cells were gated as in Figure 4.5A to determine expression of CD86 on human B cells after stimulation with 500 ng/mL rIL-32γ for 5 minutes, 10 minutes, 30 minutes, 120 minutes and 24 hours. (A) Representative plot of unstimulated (Nil) and stimulated (rIL-32γ) (B-C) Column graphs representing CD86 expression as (C) percentage of total CD19$^+$ B cells, and (D) as MFI on total CD19$^+$ B cells. (ns=not significant) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from three experiments.

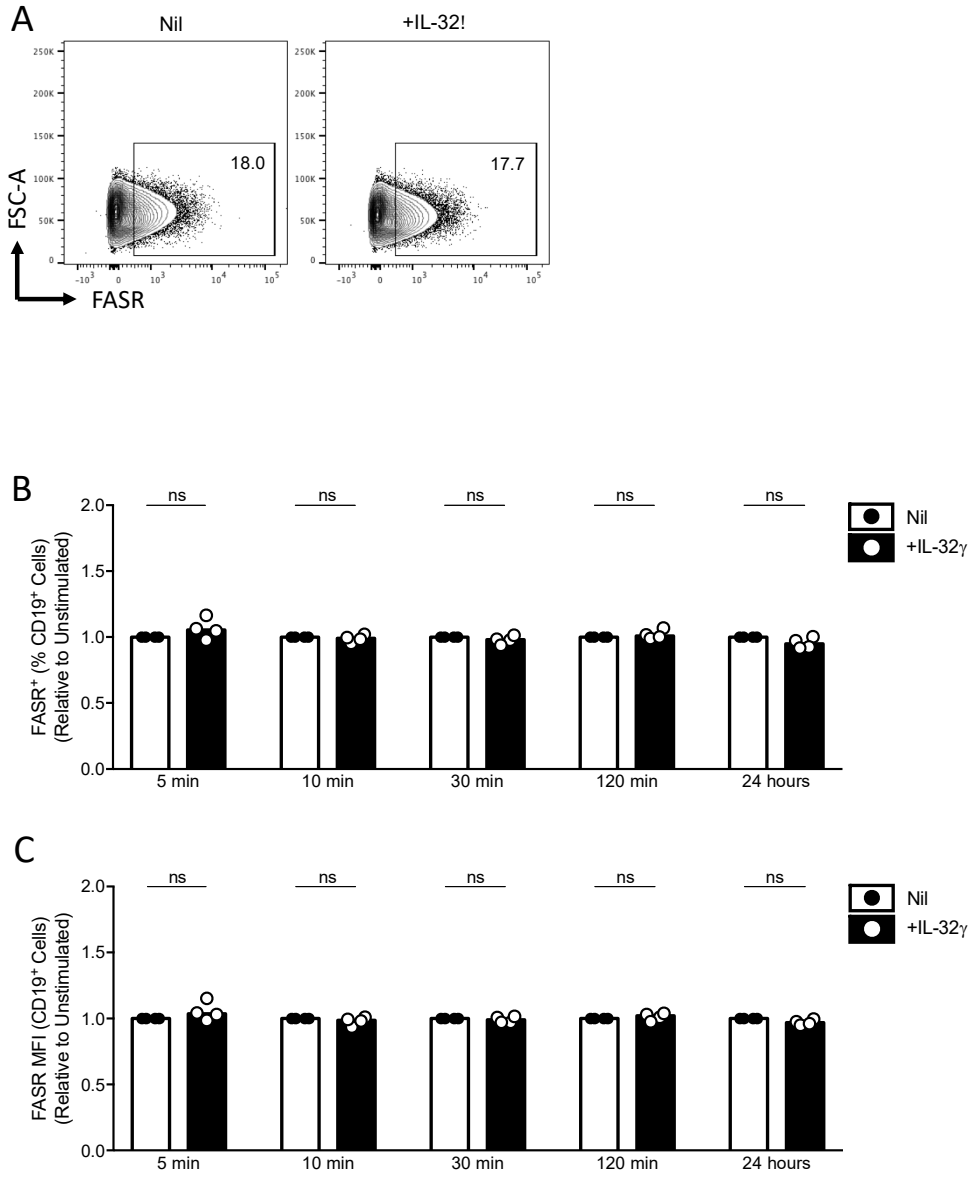


Figure 4.11. rIL-32γ does not alter expression of FAS on human B cells.

$CD3^-$ $CD19^+$ B cells were MACS purified (Miltenyi Biotec) and B cells were gated as in Figure 4.5A to determine expression of FAS on human B cells after stimulation with 500 ng/mL rIL-32γ for 5 minutes, 10 minutes, 30 minutes, 120 minutes and 24 hours. (A) Representative plot of unstimulated (Nil) and stimulated (rIL-32γ) (B-C) Column graphs representing FAS expression as (C) percentage of total $CD19^+$ B cells, and (D) as MFI on total $CD19^+$ B cells. (ns=not significant) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from three experiments.

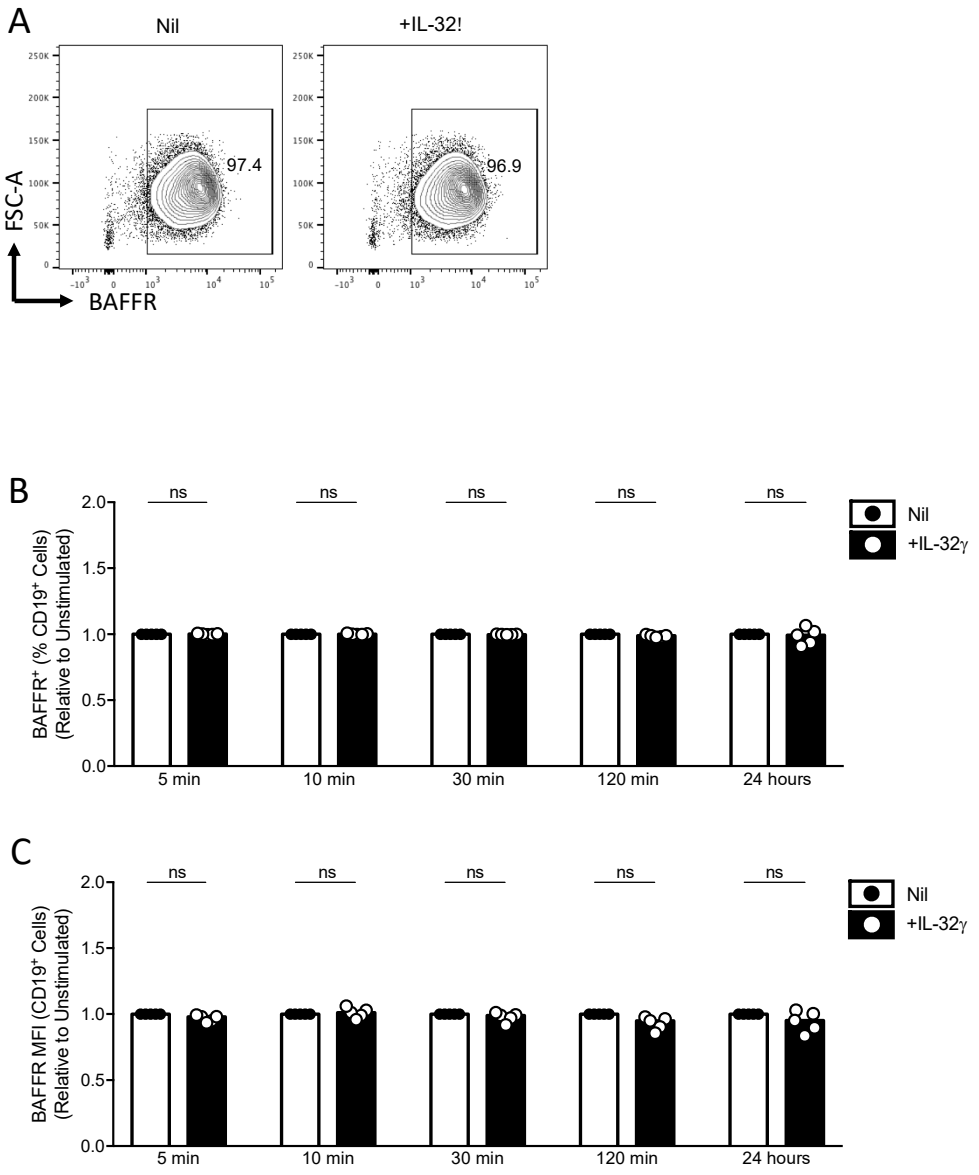


Figure 4.12. rIL-32γ does not alter expression of BAFFR on human B cells.

$CD3^-$ $CD19^+$ B cells were MACS purified (Miltenyi Biotec) and B cells were gated as in Figure 4.5A to determine expression of BAFFR on human B cells after stimulation with 500 ng/mL rIL-32γ for 5 minutes, 10 minutes, 30 minutes, 120 minutes and 24 hours. (A) Representative plot of unstimulated (Nil) and stimulated (rIL-32γ) (B-C) Column graphs representing BAFFR expression as (C) percentage of total $CD19^+$ B cells, and (D) as MFI on total $CD19^+$ B cells. (ns=not significant) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from three experiments.

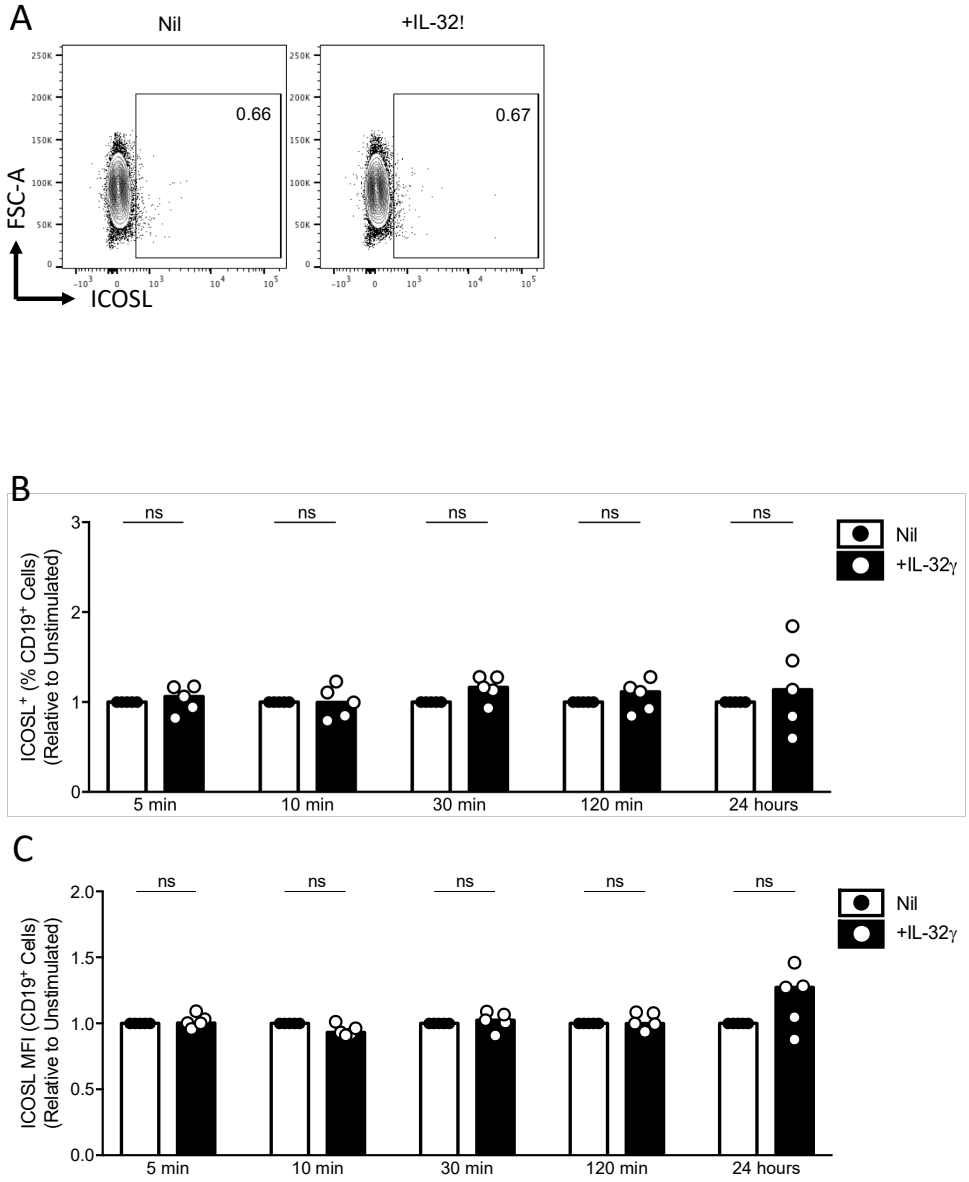


Figure 4.13. rIL-32γ does not alter expression of ICOSL on human B cells.

CD3$^-$ CD19$^+$ B cells were MACS purified (Miltenyi Biotec) and B cells were gated as in Figure 4.5A to determine expression of ICOSL on human B cells after stimulation with 500 ng/mL rIL-32γ for 5 minutes, 10 minutes, 30 minutes, 120 minutes and 24 hours. (A) Representative plot of unstimulated (Nil) and stimulated (rIL-32γ) (B-C) Column graphs representing ICOSL expression as (C) percentage of total CD19$^+$ B cells, and (D) as MFI on total CD19$^+$ B cells. (ns=not significant) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from three experiments.

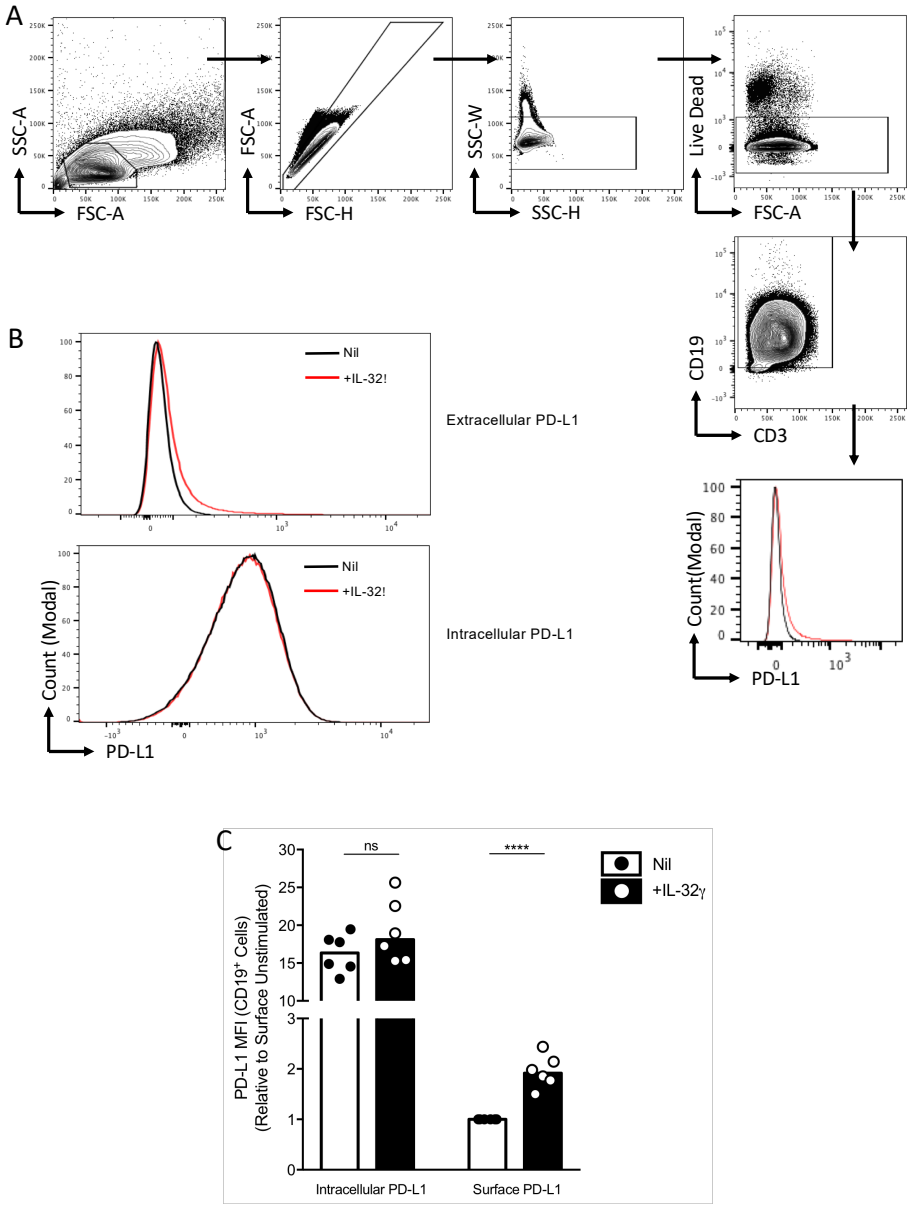


Figure 4.14. PD-L1 is stored intracellularly in B cells, and rIL-32γ induces PD-L1 expression on the cell surface

(A) Flow cytometric gating strategy of $CD3^-$ $CD19^+$ B cells after MACS purification of B cells (Miltenyi Biotec) and stimulation with 500 ng/mL rIL-32γ to look at expression PD-L1 both intracellularly and on the cell surface after 30 minutes. (B) Representative histograms of extracellular (top) and intracellular (bottom) PD-L1 from unstimulated (Nil) and stimulated (rIL-32γ) B cells (C) Column graph representing intracellular and surface PD-L1 expression as MFI on total $CD19^+$ B cells for both unstimulated and stimulated cells. (****p≤0.0001) (ns=not significant) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from three experiments.

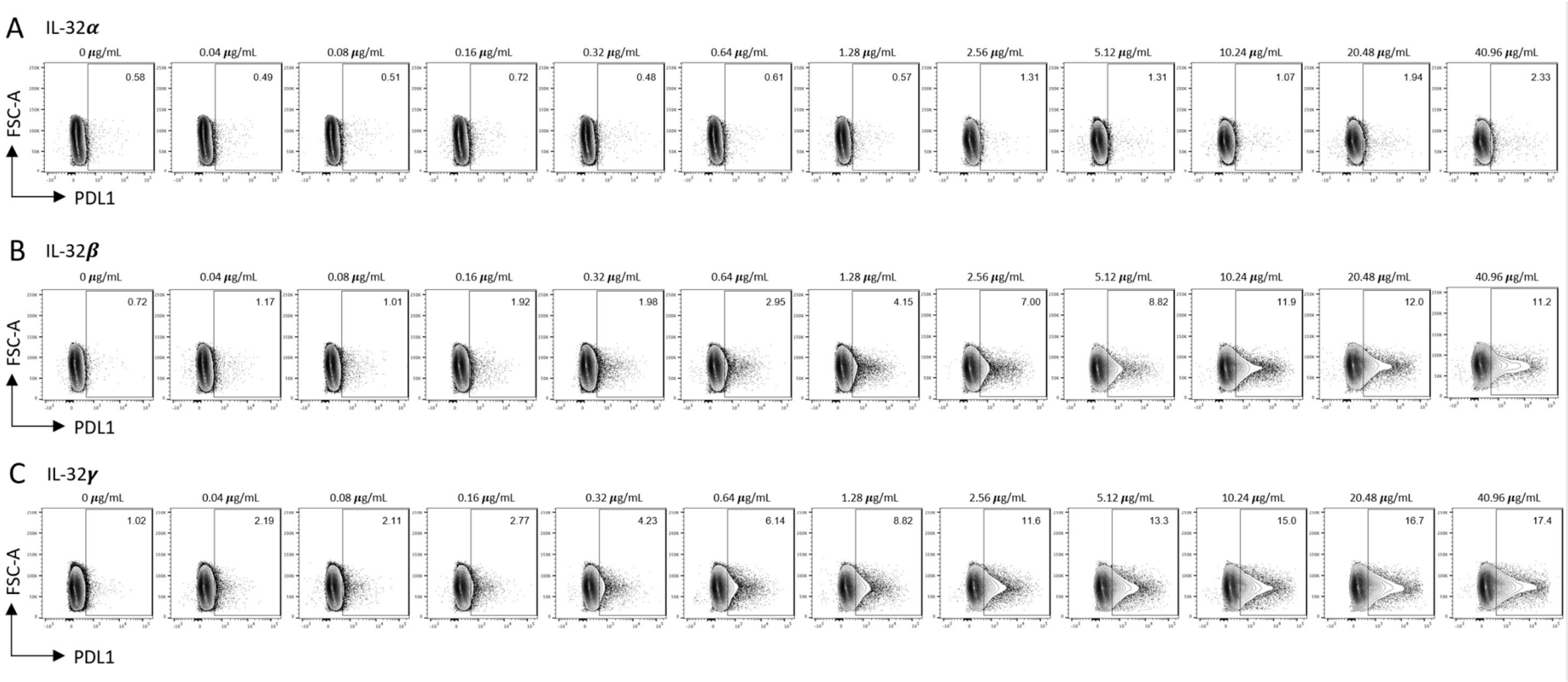


Figure 4.15. IL-32γ is the isoform that most potently induces PD-L1 expression on the surface of human B cells

$CD3^-$ $CD19^+$ B cells were MACS purified (Miltenyi Biotec) and B cells were gated as in Figure 4.5A to determine expression of PD-L1 on human B cells after stimulation with the indicated concentrations of (A) rIL-32α, (B) rIL-32β or (C) rIL-32γ for 30 minutes, and surface expression of PD-L1 was measured. Representative of two repeat experiments.

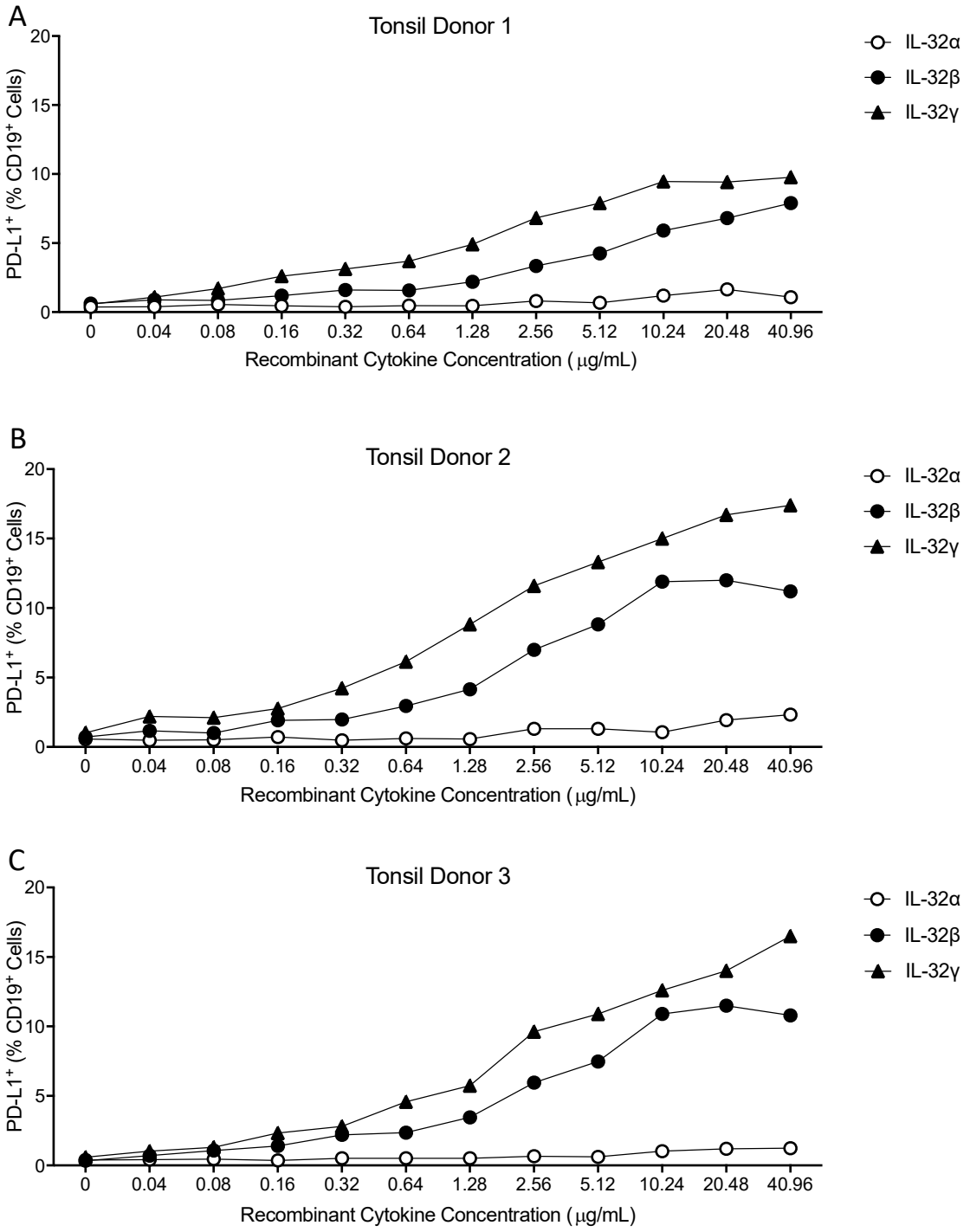


Figure 4.16. IL-32γ is the most potent isoform at inducing PD-L1 expression on the surface of human B cells.

$CD3^-$ $CD19^+$ B cells were MACS purified (Miltenyi Biotec) and B cells were gated as in Figure 4.5A to determine expression of PD-L1 on human B cells after stimulation with the indicated concentrations of rIL-32$\boldsymbol{\alpha}$, rIL-32$\boldsymbol{\beta}$ and rIL-32$\boldsymbol{\gamma}$ for 30 minutes, and surface expression of PD-L1 (as a percentage of total $CD3^-$ $CD19^+$ cells) was quantified for tonsil donor 1 (A), tonsil donor 2 (B) and tonsil donor 3 (C). Representative of two repeat experiments.

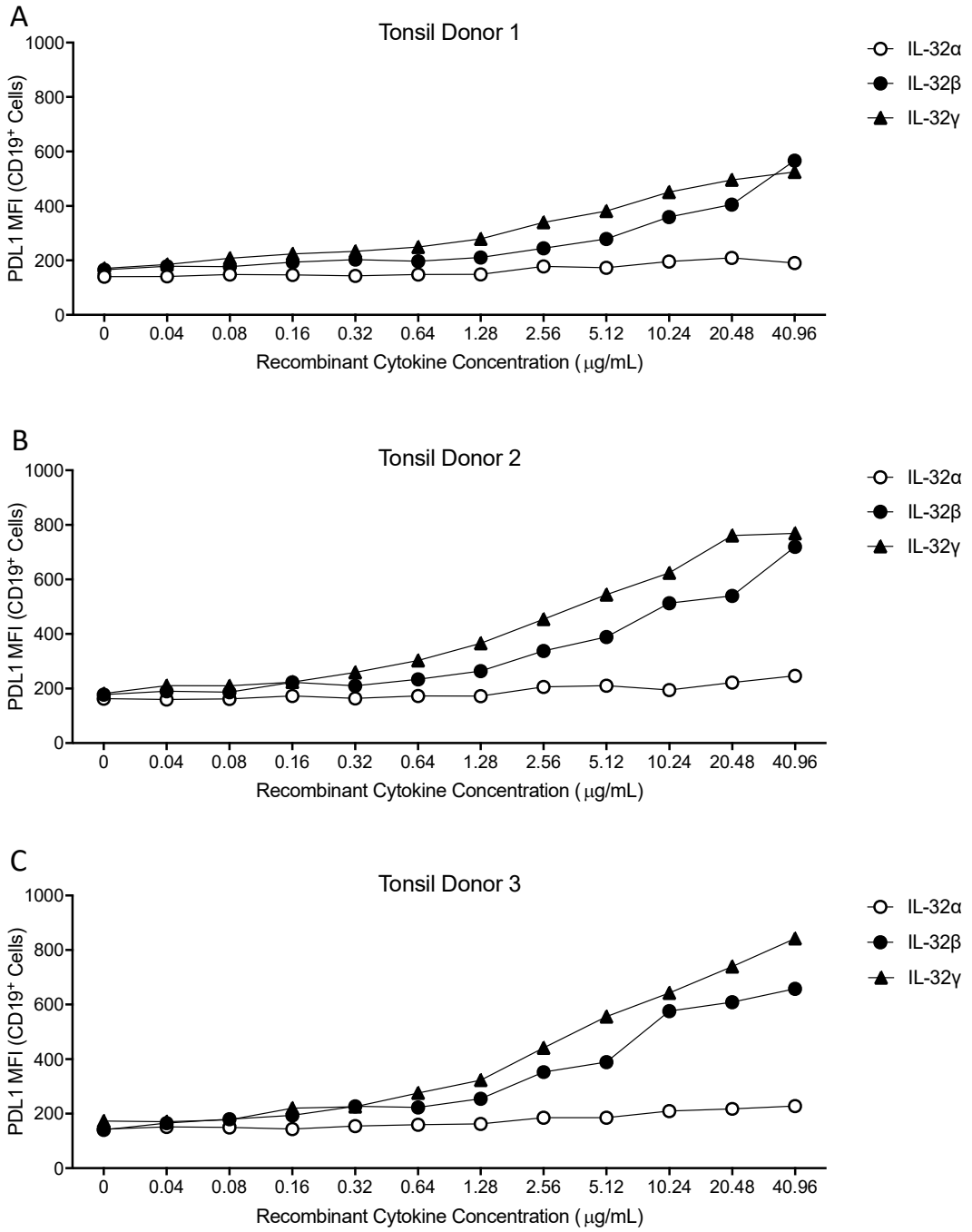


Figure 4.17. IL-32γ is the most potent isoform at inducing PD-L1 expression on the surface of human B cells.

$CD3^-$ $CD19^+$ B cells were MACS purified (Miltenyi Biotec) and B cells were gated as in Figure 4.5A to determine expression of PD-L1 on human B cells after stimulation with the indicated concentrations of rIL-32$\boldsymbol{\alpha}$, rIL-32$\boldsymbol{\beta}$ and rIL-32$\boldsymbol{\gamma}$ for 30 minutes, and surface expression of PD-L1 (as PD-L1 MFI on $CD3^-$ $CD19^+$ cells) was quantified for tonsil donor 1 (A), tonsil donor 2 (B) and tonsil donor 3 (C). Representative of two repeat experiments.

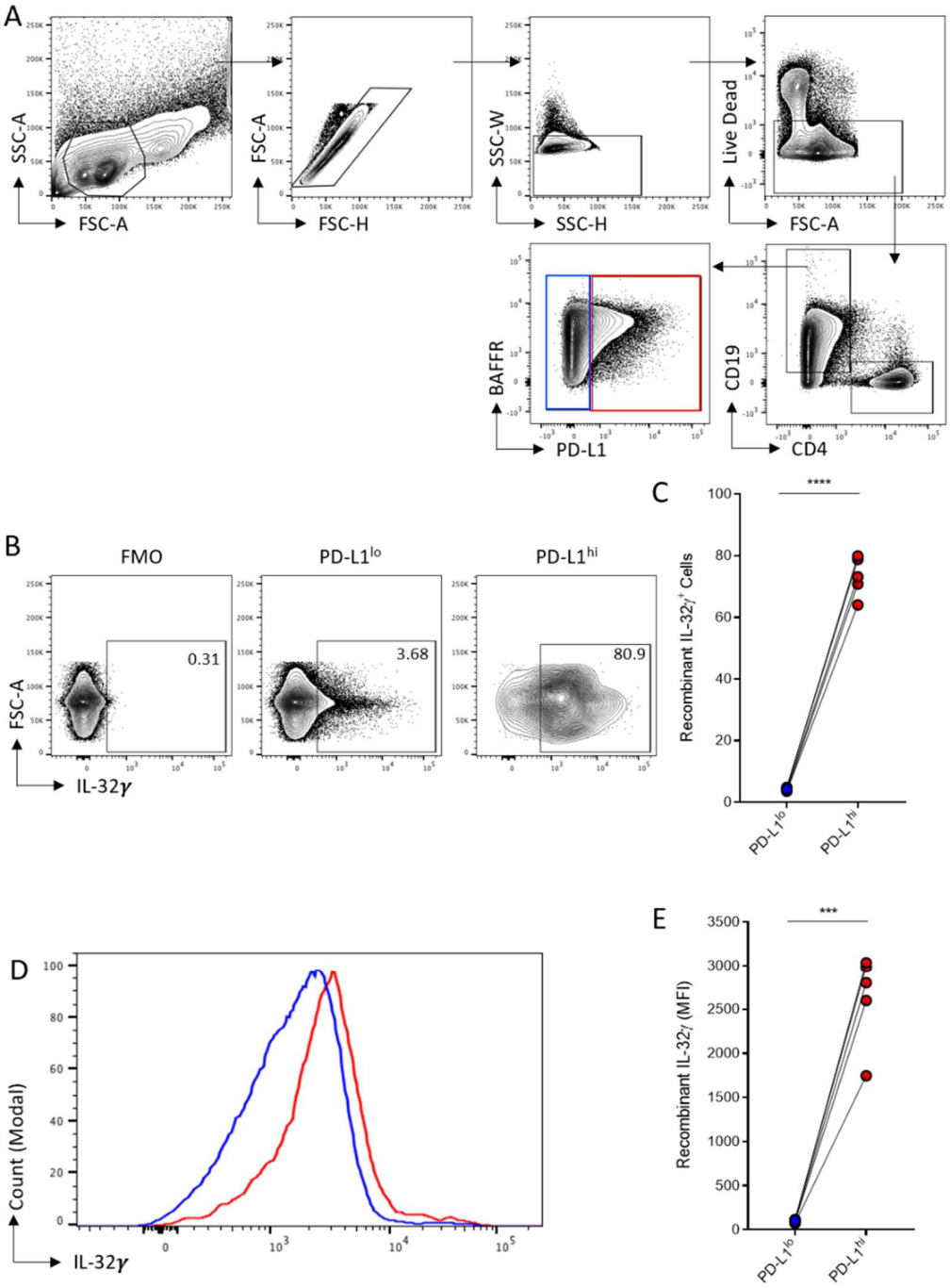


Figure 4.18. PD-L1^{hi} B cells bind more rIL-32γ than PD-L1^{lo} B cells.

Cells from total tonsil (five donors) were isolated and stimulated with 1000 ng/mL of conjugated (AF647) rIL-32γ for two hours. (A) Flow cytometric gating strategy to assess association of rIL-32γ on PD-L1^{hi} (red) and PD-L1^{lo} (blue) B cells. (B) Representative plots of the percentage of associated rIL-32γ-AF647 with fluorescence minus one (FMO) control (lacking rIL-32γ-AF647, PD-L1^{lo} B cells and PD-L1^{hi} B cells. (C) Column graph representing percentage of associated rIL-32γ-AF647 on PD-L1^{lo} and PD-L1^{hi} B cells. (D) Representative histogram of association of rIL-32γ on PD-L1^{hi} (red) and PD-L1^{lo} (blue) B cells. (E) Column graph representing MFI of associated rIL-32γ-AF647 on PD-L1^{lo} and PD-L1^{hi} B cells. Each dot represents one tonsil donor. (***p≤0.001) (****p≤0.0001) Paired t-test between PD-L1^{lo} and PD-L1^{hi}.

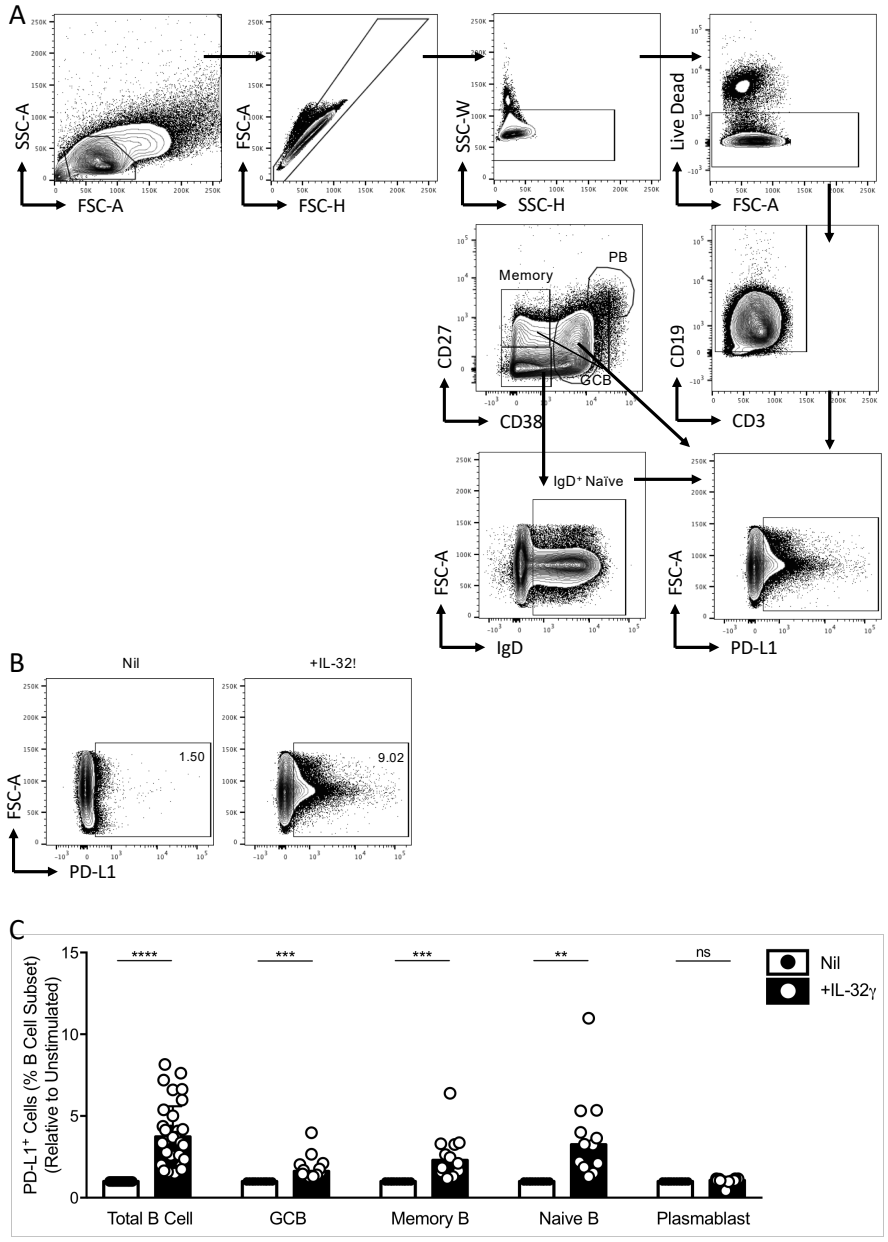


Figure 4.19. IL-32γ induces PD-L1 on most B cell subsets.

(A) Flow cytometric gating strategy used to sort-purify each cell subset total B cells ($CD3^-$ $CD19^+$), germinal centre B cells (GCB-$CD3^-$ $CD19^+$ $CD38^+$ $CD27^{int}$) memory B cells ($CD3^-$ $CD19^+$ $CD38^-$ $CD27^+$), naïve B cells ($CD3^-$ $CD19^+$ $CD27^-$ $CD38^-$ IgD^+) and plasmablasts ($CD3^-$ $CD19^+$ $CD38^{hi}$ $CD27^{hi}$) after MACS purification of total B cells (Miltenyi Biotec) and stimulation with 1500 ng/mL rIL-32γ to look at expression PD-L1 on the cell surface of each subset after 30 minutes. (B) Representative plots of $PD\text{-}L1^+$ cells from unstimulated (nil) and stimulated (rIL-32γ) B cells. (C) Column graph representing PD-L1 expression (as a percentage of the respective B cell subset) for both unstimulated and stimulated cells. (**p≤0.01) (***p≤0.001) (****p≤0.0001) (ns=not significant) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from more than ten experiments.

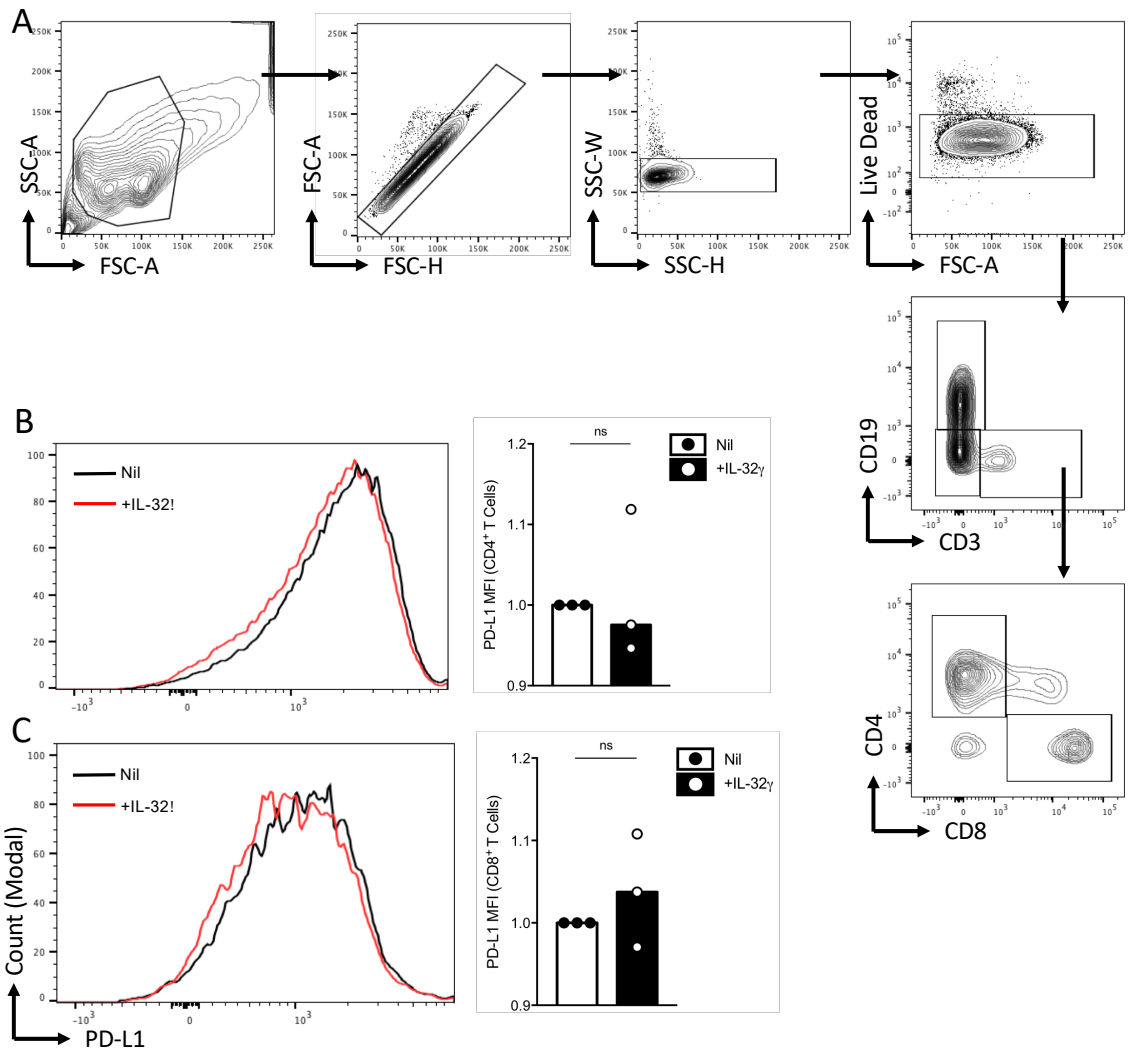


Figure 4.20. IL-32γ does not induce PD-L1 on CD4$^+$ or CD8$^+$ T cells.

(A) Flow cytometric gating strategy of CD3$^+$ CD4$^+$ T cells and CD3$^+$ CD8$^+$ T cells for stimulation with 1500 ng/mL rIL-32γ to look at expression PD-L1 on the cell surface after 30 minutes. (B-C) Representative histograms and corresponding column graph (medians) representing surface PD-L1 MFI on unstimulated (nil) and stimulated (rIL-32γ) (B) CD3$^+$ CD4$^+$ T cells and (C) CD3$^+$ CD8$^+$ T cells. (ns=not significant) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor (the mean of three technical replicates). Representative of pooled data from three experiments.

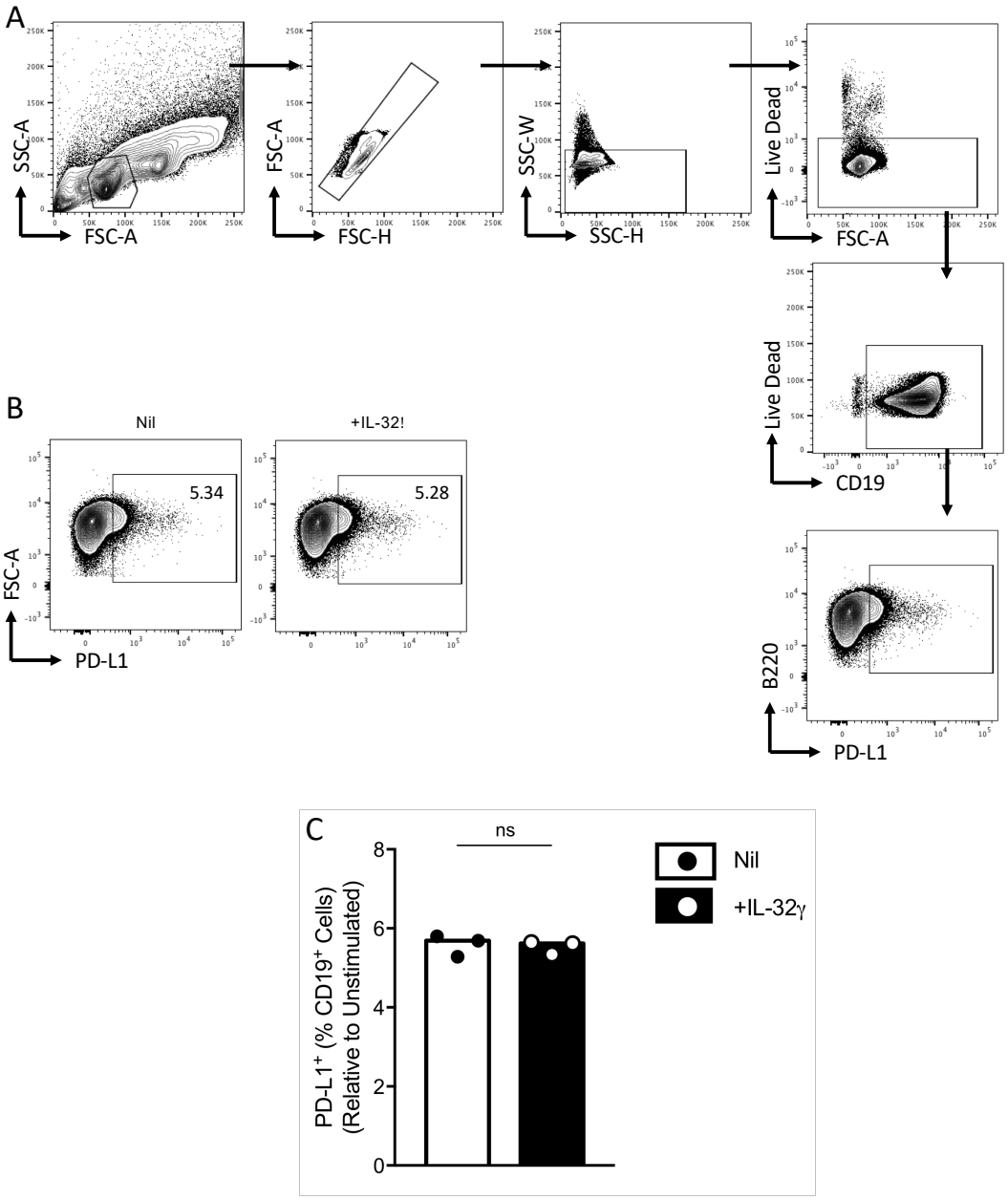


Figure 4.21. IL-32γ does not induce PD-L1 on mouse B cells.

(A) Flow cytometric gating strategy of $CD3^-$ $CD19^+$ B cells after MACS purification of mouse B cells (Miltenyi Biotec) and stimulation with 1500 ng/mL rIL-32γ for 30 minutes to look at expression of surface PD-L1 on mouse B cells. (B) Representative plots of PD-$L1^+$ cells from unstimulated (nil) and stimulated (rIL-32γ) B cells. (C) Column graph representing median PD-L1 expression (as a percentage of $CD19^+$ B cells) for both unstimulated and stimulated cells. (ns=not significant) Student's t-test between nil and IL-32γ. Each dot represents a wild-type mouse (the mean of three technical replicates). Representative of pooled data from three experiments.

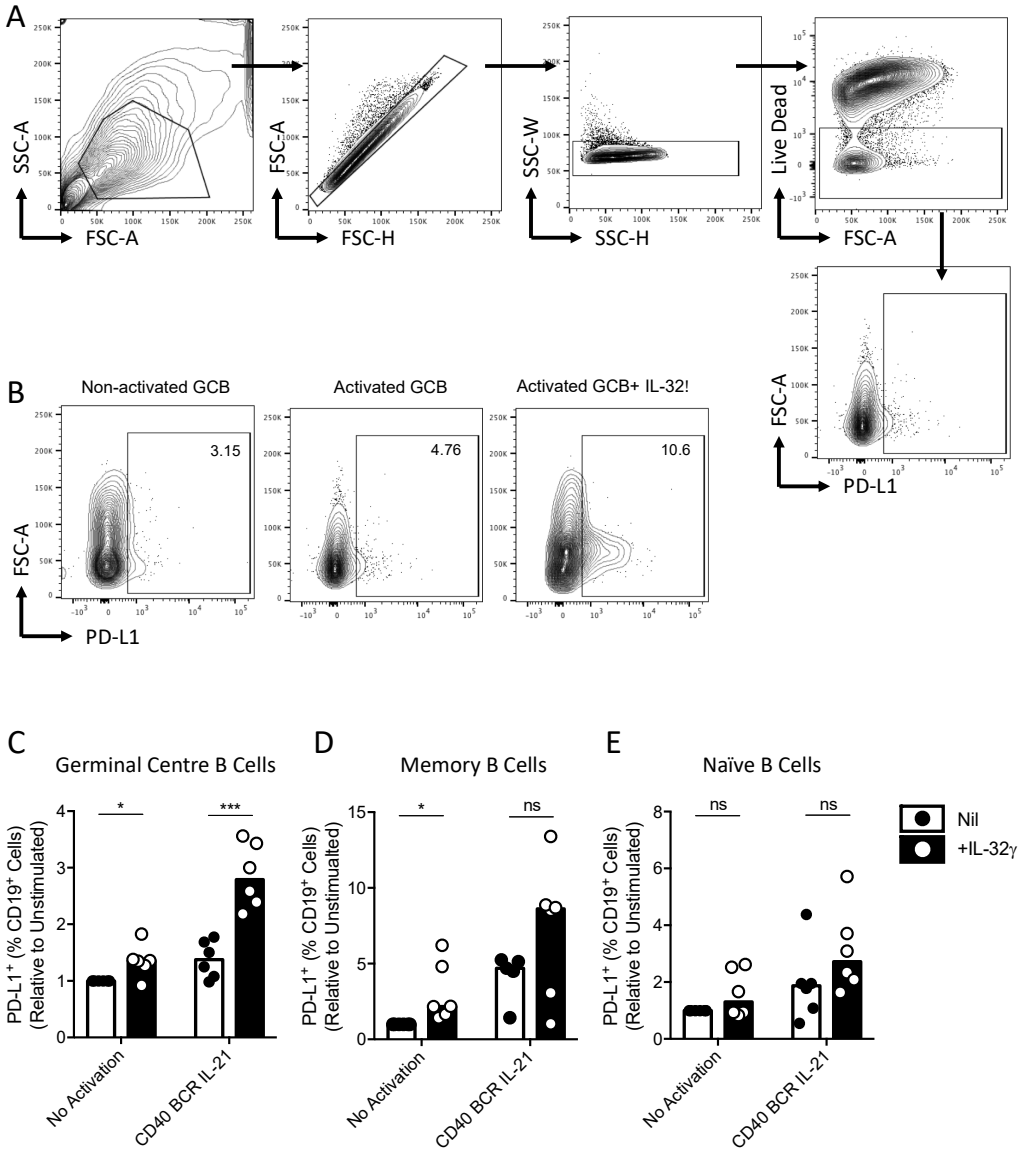


Figure 4.22. Induction of PD-L1 on the cell surface by IL-32γ is sustained for five days.

(A) Flow cytometric gating strategy of B cell subsets to quantify expression of surface PD-L1 on germinal centre B (GCB), memory B cells and naïve B cells following B cell activation (CD40, BCR, IL-21) and stimulation with 500 ng/mL rIL-32γ for five days. (B) Representative plots of PD-L1$^+$ cells from non-activated GCBs, activated GCBs and activated GCBs stimulated with rIL-32γ. (C-E) Column graphs representing median PD-L1$^+$ cells from non-activated and activated (C) germinal centre B cells, (D) memory B cells and (E) naïve B cells, with or without rIL-32γ. Stimulation. (*p≤0.05) (***p≤0.001) Student's t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from three experiments.

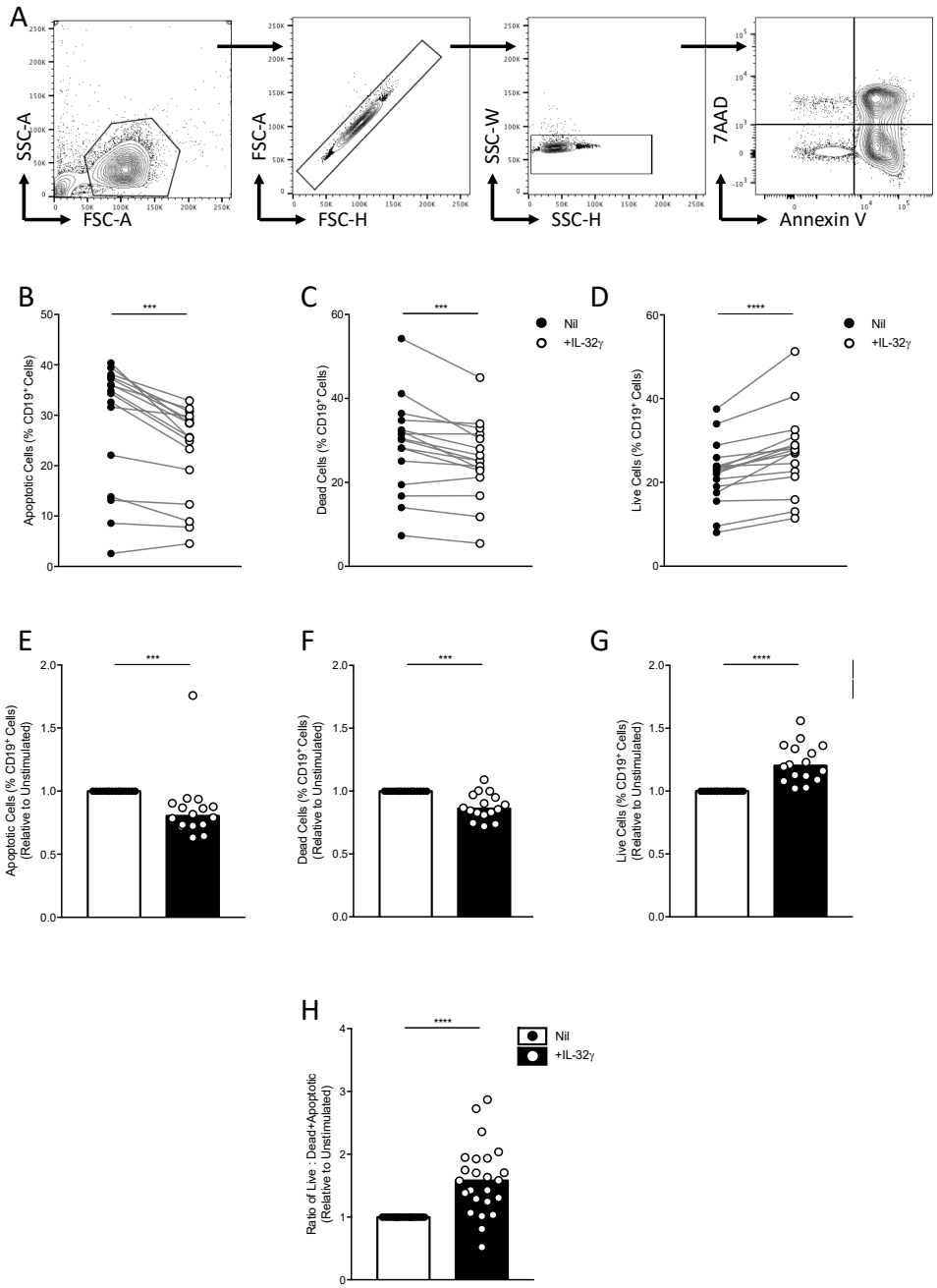


Figure 4.23. IL-32γ increases B cell survival.

CD3$^-$ CD19$^+$ cells were sort-purified according to the gating strategy in Figure 4.5A following MACS purification of B cells (Miltenyi Biotec) to enrich for B cells. (A) Flow cytometric gating strategy to quantify live, apoptotic and dead B cells following stimulation with 1500 ng/mL rIL-32γ for 24 hours. (B-D) Paired quantification of (B) apoptotic, (C) dead and (D) live cells as a percentage of CD19$^+$ B cells following rIL-32γ stimulation. (E-H) Column graphs representing median percentage of (E) apoptotic, (F) dead, (G) live and (H) the ratio of live/(dead+apoptotic) cells out of total CD19$^+$ B cells, following stimulation with IL-32γ, relative to unstimulated. (***p≤0.001) (****p≤0.0001) Paired t-test between nil and IL-32γ. Each dot represents a single tonsil donor. Representative of pooled data from three separate experiments.

4.3.3 IL-32 augments PD-L1 on a B cell derived cancer cell line

In addition to the role of PD-1:PD-L1 signalling in the germinal centre reaction, PD-L1 is overexpressed on tumour cells, contributing to evasion from the host immune system (Iwai et al., 2002, Chen and Huang, 2016, Juneja et al., 2017, Wang et al., 2018, Tang and Zheng, 2018). IL-32 itself has been associated with a number of cancers (Player et al., 2014, Zeng et al., 2014, Yousif et al., 2013, Yang et al., 2015, Seo et al., 2008, Plantinga et al., 2013, Nishida et al., 2009, Lee et al., 2011, Lee et al., 2012a, Kang et al., 2012b, Ishigami et al., 2013, Guenin et al., 2014, Erturk et al., 2016, Cui et al., 2016, Sorrentino and Di Carlo, 2009a) and has been shown to play a role in regulating the anti-tumour immune response (Yousif et al., 2013, Nishida et al., 2009, Lee et al., 2011, Kang et al., 2012c). We therefore considered the possibility that IL-32 upregulates PD-L1 not just on B cells but also perhaps on cancer cells. We tested nine different cancer cell lines: H2B-HeLa (adenocarcinoma), HOS (osteosarcoma), MNNG/HOS (osteosarcoma), SJSA-1 (osteosarcoma), HL-60 (acute promyelocytic leukemia), Jurkats (acute T cell leukemia), Raji (Burkitt's lymphoma), Ramos (Burkitt's lymphoma) and THP-1 (acute monocytic leukemia). Interestingly, many of these cells already expressed high amounts of surface PD-L1 before IL-32 stimulation. The only cell line that upregulated surface PD-L1 following rIL-32$\boldsymbol{\gamma}$ stimulation for 30 minutes was Raji cells, which is a B cell line derived from Burkitt's lymphoma (Figure 4.24). Interestingly, Ramos cells, which are also derived from Burkitt's lymphoma, did not upregulate surface PD-L1 following IL-32 stimulation.

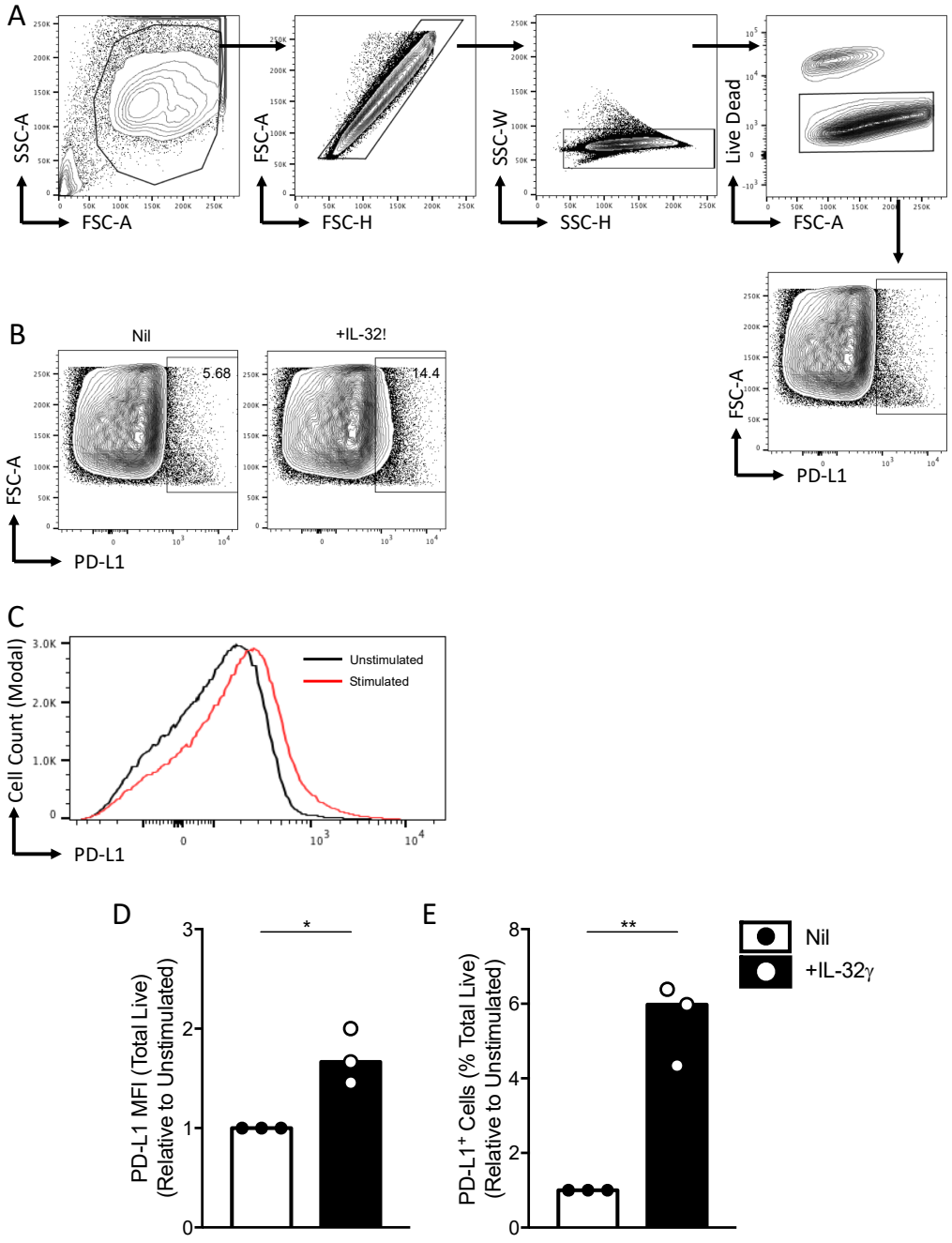


Figure 4.24. IL-32γ induces PD-L1 on the surface of Raji cells.

(A) Flow cytometric gating strategy of live Raji cells following stimulation with 1500 ng/mL rIL-32γ for 30 minutes to look at expression of surface PD-L1 on Raji cells. (B) Representative plots of PD-L1$^+$ cells from unstimulated (nil) and stimulated (rIL-32γ) Raji cells. (C) Representative histogram representing surface PD-L1 MFI Column graph representing PD-L1 expression (out of CD19$^+$ B cells) on unstimulated (nil) and stimulated (rIL-32γ) Raji cells. (D-E) column graphs representing PD-L1 expression on Raji cells as (D) MFI of total live Raji cells and (E) PD-L1$^+$ cells as a percentage of total live Raji cells for both unstimulated and stimulated cells. (*p≤0.05) (**p≤0.01) Student's t-test between nil and IL-32γ. Representative of data from two experiments.

4.3.4 IL-32 induces PD-L1^{hi} B regulatory cells

Regulatory B cells are a specific subset of B cells that have immunosuppressive capabilities, preventing the expansion of pathogenic T cells and pro-inflammatory lymphocytes, mainly through the production of IL-10, TGF$\boldsymbol{\beta}$ and IL-35 (Rosser and Mauri, 2015). The biology and phenotypes of these suppressive B cells is still incompletely understood. Whilst a variety of B cell subsets have been documented as regulatory B cells, no specific marker for regulatory B cells has been identified. Interestingly, several studies have demonstrated that some subsets of regulatory B cells express PD-L1 on their cell surface, with the expression of PD-L1 correlating to the immunosuppressive behaviour of the cell. Furthermore, Khan et al. demonstrated that there is a regulatory B cell population that express elevated levels of PD-L1 (Khan et al., 2015), which was subsequently supported by further studies (Gallego-Valle et al., 2018, Guan et al., 2016). They found that this subset of PD-L1^{hi} regulatory B cells were important in supressing humoral responses through preventing T cell activation and antibody production - independently of IL-10, and that the PD-L1^{hi} regulatory B cells expressed the highest level of BAFFR and acted as BAFF 'sinks' by sequestering BAFF (Khan et al., 2015). BAFF is an essential survival factor for immature B cells (Batten et al., 2000), and excess BAFF has been shown to cause aberrant B cell survival and lupus-like disease (Vincent et al., 2014). Furthermore, a BAFF neutralising monoclonal antibody (belimumab) has been approved for the treatment of SLE (Navarra et al., 2011, Furie et al., 2011).

To test the possibility that the PD-L1^{hi} cells induced by IL-32 correspond to the IL-10-independent B_{regs} described by Fallon and colleagues, we first set out to determine whether IL-32γ-stimulated B cells produce IL-10. Human B cells were stimulated with PMA and

Ionomycin in the presence or absence of IL-32γ, and the supernatant was collected after 18 hours of stimulation. Analysis of the supernatant by CBA revealed no significant increase in IL-10 production from stimulated total B cells. We then attempted to determine whether the IL-32-induced PD-L1^{hi} cells were producers of IL-10 by flow cytometry. Cells were stimulated with PMA and Ionomycin, with or without IL-32γ, in the presence of Brefeldin A for 6 hours. Flow cytometric analysis revealed that the PD-L1^{lo} B cells were the main producers of IL-10 as opposed to the PD-L1^{hi} cells (Figure 4.25), which did not appear to produce IL-10 ($p<0.0001$).

We next asked whether the PD-L1^{hi} cells also expressed high amounts of BAFFR. Investigation of the expression profile of BAFFR on IL-32-induced PD-L1^{hi} B cells revealed that almost all of the PD-L1^{hi} B cells were BAFFR positive ($p=0.0002$) (Figure 4.26), and that the PD-L1^{hi} B cells expressed significantly higher amounts of BAFFR compared to the PD-L1^{lo} B cells ($p<0.0001$) (Figure 4.26). These results suggest that these PD-L1^{hi} cells may have the ability to sequester BAFF, which would enhance their survival and deplete BAFF from the milieu of surrounding B cells.

To assess this, we performed the assay described previously to test the function of PD-L1^{hi} B_{regs} (Khan et al., 2015): we conjugated human BAFF to the fluorochrome AF647 (Thermo Fisher Scientific) and added it for two hours to human B cells that had been pre-cultured with or without IL-32γ for 30 minutes. PD-L1^{hi} B cells demonstrated the greatest association with conjugated BAFF compared to PD-L1^{lo} B cells ($p=0.0001$) (Figure 4.26), demonstrating that the PD-L1^{hi} B cells preferentially bind (and thus sequester) BAFF, consistent with their high expression of BAFFR and previous findings (Khan et al., 2015).

The findings in this chapter suggest that T_{FR}-derived IL-32 acts on B cells to convert them into PD-L1^{hi} BAFFR^{hi} B_{regs}, that may repress antibody responses potentially by delivering inhibitory signals to T_{FH} cells through PD-L1 and through sequestering BAFF to limit B cell survival. Whether this B_{reg} induction occurs as a result of cognate T_{FR}:B cell interaction and delivery of IL-32 across an immune synapse, and whether the target B cells are predominantly self-reactive remains to be determined.

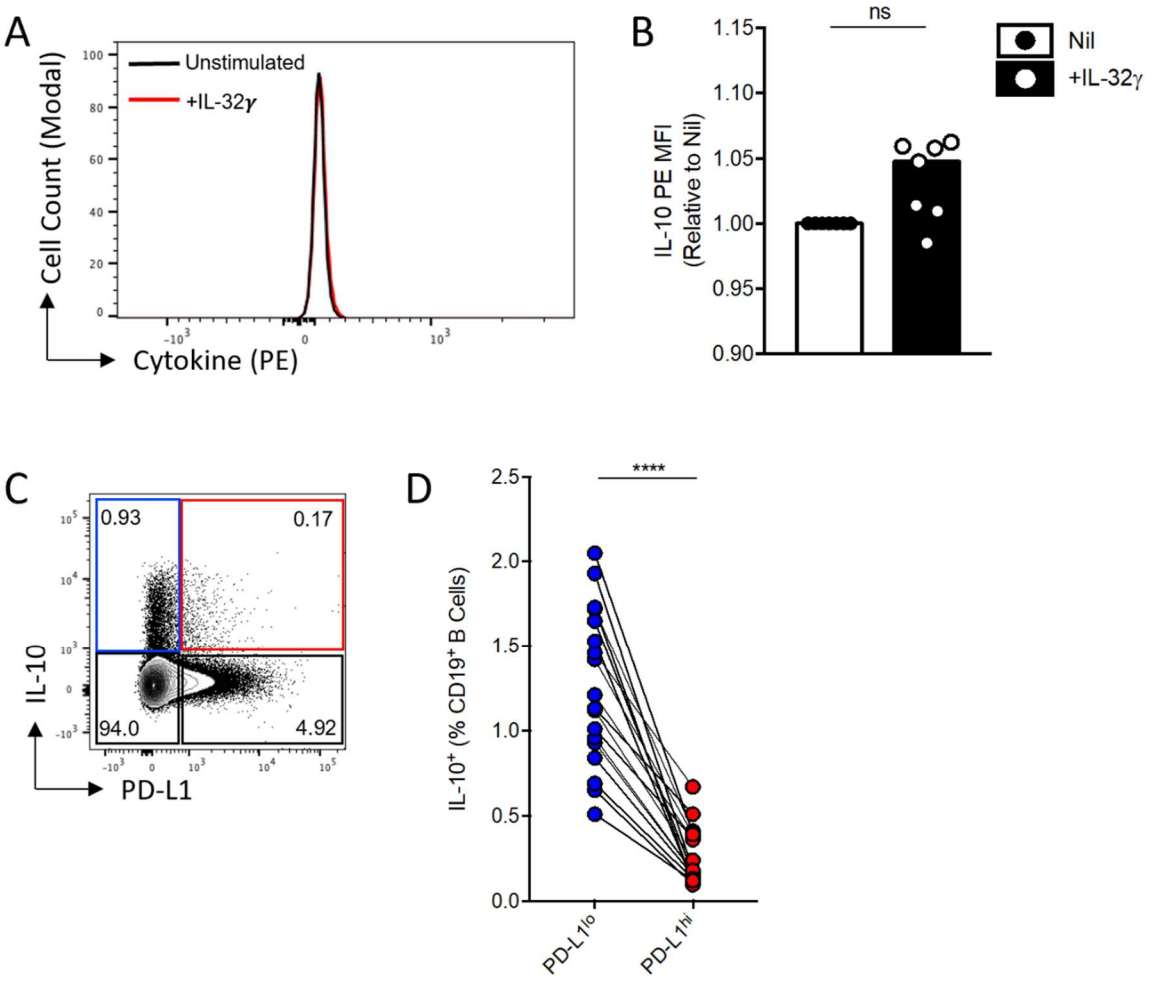


Figure 4.25. PD-L1^{hi} B cells induced by rIL-32γ do not produce IL-10.

(A) CD3^{-} CD19^{+} cells were sort-purified according to the gating strategy in Figure 4.5A following MACS purification of B cells (Miltenyi Biotec) to enrich for B cells and were stimulated with PMA and Ionomycin, with or without 1000 ng/mL rIL-32γ for 24 hours. Supernatant was isolated and a human inflammatory cytometric bead array was performed on the supernatant. Representative histogram of PE (cytokine) for unstimulated (nil) and stimulated (rIL-32γ) cells. (B) Column graph representing the median amount of IL-10 in the supernatant relative to unstimulated cells. (C) CD3^{-} CD19^{+} cells were sort-purified and stimulated with PMA and Ionomycin, with or without 1000 ng/mL rIL-32γ in the presence of Brefeldin A for 6 hours. IL-10 was measured by flow cytometry on the PD-L1^{hi} and PD-L1^{lo} B cells. (D) Column graph representing amount of IL-10 produced by PD-L1^{hi} and PD-L1^{lo} B cells. (***p<0.001) (****p<0.0001) (ns=not significant) Student's t-test (B) and Paired t-test (D). Each dot represents a tonsil donor, and represents pooled data from two separate experiment

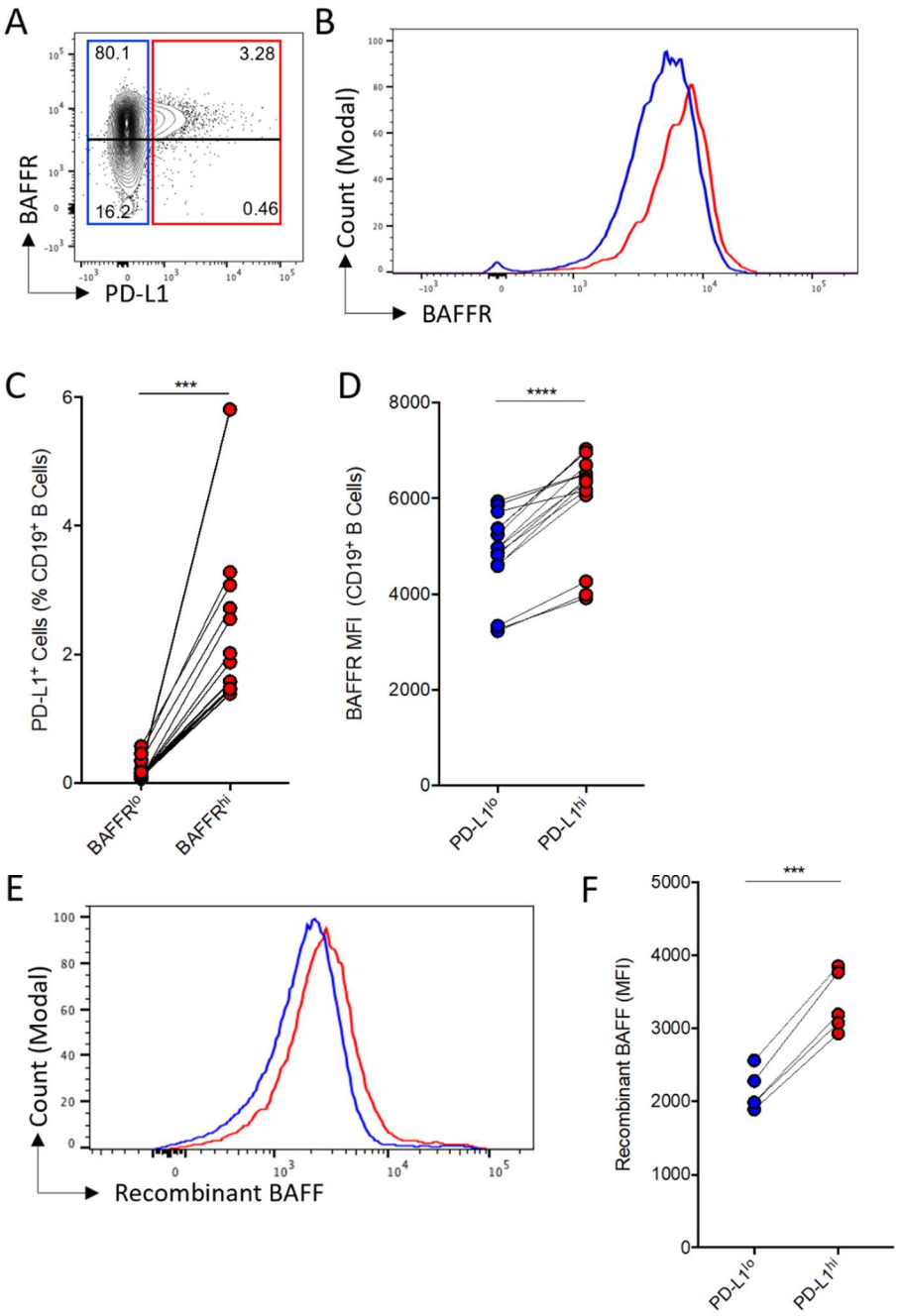


Figure 4.26. PD-L1^{hi} B cells express BAFFR and sequester BAFF.

(A) CD3^{-} CD19^{+} B cells were MACS purified (Miltenyi Biotec) and B cells were gated as in Figure 4.5A to determine co-expression of PD-L1 and BAFFR on human B cells after stimulation with 1000 ng/mL rIL-32γ for 30 minutes. Representative plot demonstrating co-expression of BAFFR and PD-L1 on human B cells, with blue indicating PD-L1^{lo} cells and red indicating PD-L1^{hi} cells. (B) Representative histogram demonstrating BAFFR expression on PD-L1^{lo} B cells (blue) and PD-L1^{hi} B cells (red). (C) Quantification of percentage of PD-L1^{+} cells on BAFFR^{lo} and BAFFR^{hi} cells, as a percentage of total CD19^{+} B cells, following rIL-32γ stimulation. (D) Quantification of BAFFR MFI on CD19^{+} B cells on PD-L1^{lo} and PD-L1^{hi} B cells following rIL-32γ stimulation. (E-F) Representative histogram and (F) column graph demonstrating association of recombinant BAFF (conjugated to AF647) with PD-L1^{lo} (blue) and PD-L1^{hi} (red) B cells pre-cultured with 1000 ng/mL rIL-32γ for 30 minutes. (***p≤0.001) (****p≤0.0001) Paired t-test. Each dot represents a tonsil donor and represents pooled data from two experiments.

4.4 Chapter Summary

This chapter investigates the role of interleukin-32 (IL-32), a novel cytokine that we found to be highly expressed in a T_{FR} cells, in the germinal centre reaction. IL-32 has been linked to cancers and has been described as a proinflammatory cytokine that induces cytokines such as TNFα, IL-1$\boldsymbol{\beta}$, IL-8 and IL-6, however its role in the germinal centre reaction has never been described.

Results from this study demonstrate that IL-32 mainly associates with B cells rather than T cells, and therefore we were interested in how this cytokine could affect B cell responses. Stimulation of B cells with recombinant IL-32$\boldsymbol{\gamma}$ rapidly induced and sustained PD-L1 expression on the surface of B cells within five minutes of stimulation. PD-L1 is one of the two ligands for PD-1, a surface molecule highly expressed on germinal centre T_{FH} and T_{FR} cells. The interaction between PD-1 and PD-L1 has been shown to have paradoxical roles in the germinal centre reaction, although studies show that it attenuates T cell activation, and supresses T_{FH} cell differentiation and help for immunoglobulin class switching. This study demonstrates that stimulation of tonsil-derived B cells (naïve B cells, memory B cells and germinal centre B cells) with rIL-32 increases their viability and induces the B cells to become a previously described subset of B_{regs} that express elevated PD-L1 and BAFFR. We show that, like the previously described B_{regs}, these IL-32 induced PD-L1^{hi} B cells act as a BAFF 'sink' and sequester BAFF. This study proposes a novel pathway by which IL-32 is produced by human T_{FR} cells and acts on tonsil-derived B cells, inducing them to become PD-L1^{hi} BAFF sequestering B_{regs}, which we hypothesise will sequester BAFF from the germinal centre milieu.

Chapter Five: Discussion

5.1 Overview

Work presented in this thesis explored the role of human T cell cytokines in follicular biology and autoimmunity through two independent projects, undertaken in parallel. The first project investigated the role of ZFR2, a poorly characterised protein with no known immune function, in T cell signalling and cytokine regulation, and how variants in this gene may be contributing to SLE pathogenesis in two families affected by SLE. This is the first demonstration to date that provides evidence for a role of ZFR2 in the immune system, and how variants in this gene may dysregulate pathways involved in cytokine regulation and perhaps contribute to autoimmune pathogenesis. Whilst Family A and Family B carried two different mutations, they were both in the same domain (the DZF domain). Interestingly, the patients from both families possessed similar cellular and serological phenotypes that were supported by *in vitro* and *in vivo* investigation of ZFR2 functions, providing evidence for how gain of function variants in ZFR2 can disrupt cytokine regulation and contribute to the phenotypes identified in the affected patients. Whether such phenotypes are key to lupus development in the families remains to be proven; here we discuss how this may occur. This project demonstrates the power of whole exome and genome sequencing to identify rare and novel gene variants that may predispose to SLE.

The second part of this thesis elucidates the role of the human cytokine IL-32 in B cell responses. We found that IL-32 is highly expressed by human T cells, specifically T_{FR}-like cells, and associates with tonsil-derived B cells rather than T cells. Stimulation with recombinant IL-32γ increases viability and rapidly translocates intracellular PD-L1 to the surface of human B cells within 5 minutes, inducing them to become a previously described subset of PD-L1^{hi}

regulatory-type B cells that express BAFFR and act as a BAFF 'sink' to sequester BAFF (Khan et al., 2015). Together, these projects provide new insights into the role of T cells and their cytokine products in the regulation of B cell responses, follicular biology and autoimmunity.

5.2 Project One: Investigating the role of ZFR2 in the regulation of T cell-derived IL-2 and lupus pathogenesis

5.2.1 The role of ZFR2 in the immune system, and the effect of gain of function variants

The patient from Family A carrying the ZFR2^{A771T} variant and the patient from Family B carrying the ZFR2^{A619W} both presented with an increase in plasma IL-2. Whilst a small number of studies have found an association between increased serum IL-2 and SLE disease activity (Sedighi et al., 2014, Cuadrado et al., 1993), classically SLE is associated with a relative lack in IL-2 production by T cells (Lieberman and Tsokos, 2010, Moulton and Tsokos, 2015, Ghiran et al., 2011, Crispin and Tsokos, 2010, Ohl and Tenbrock, 2015, von Spee-Mayer et al., 2016). Nevertheless, the studies that associate low IL-2 with SLE have not drawn their conclusions based on plasma IL-2. Instead, they reported decreased IL-2 after stimulating peripheral (blood) CD4^{+} T cells. It is plausible that these measurements are not truly reflective of the amount of total IL-2, as circulating T cells in SLE patients may be enriched for exhausted cells (McKinney et al., 2015). Alternatively, it is likely that SLE is a cluster of different diseases and pathogenic pathways. While low dose IL-2 therapy has been proposed to be effective for SLE in recent studies and clinical trials (Solomou et al., 2001, Schorle et al., 1991, He et al., 2016, Mizui and Tsokos, 2016, Humrich and Riemekasten, 2016a, Humrich and Riemekasten, 2016c, Humrich and Riemekasten, 2016b, Ye et al., 2018), it appears that it does not work effectively in all patients (Mizui and Tsokos, 2016, He et al., 2016). Thus, it is conceivable that increased

IL-2 may contribute to SLE pathogenesis in a small subset of patients, whereas low IL-2 may contribute to SLE in a larger group of patients through a different mechanism.

The increase in plasma IL-2 that we observed in the patients from Family A and Family B may explain some of the relatively unique cellular phenotypes observed in the patients, such as the increase in regulatory T cells. Again, this is an unusual phenotype in SLE patients as classically SLE is associated with a deficiency in regulatory T cells (Long and Buckner, 2011, Dejaco et al., 2006, van der Vliet and Nieuwenhuis, 2007, Chavele and Ehrenstein, 2011, Liu et al., 2014b). IL-2 however is essential for the differentiation, maintenance and survival of regulatory T cells (de la Rosa et al., 2004), with IL-2 therapy augmenting regulatory T cell numbers in vivo (Shevach, 2012), and therefore may explain the increased regulatory T cells observed in the patients carrying *ZFR2* variants.

Elevated plasma IL-2 may also explain the expanded cytotoxic $CD4^+$ $CD57^+$ T cell population observed in the patients, as stimulation of cells with IL-2 has been shown to upregulate CD57 expression on cells and their cytotoxic potential (Wu et al., 2012, Lopez-Verges et al., 2010a, Batista et al., 2013, Focosi et al., 2010). CD57 has most widely been explored as a marker for T cell cytotoxicity and granularity, clonal exhaustion and proliferative senescence (Lopez-Verges et al., 2010b, Brenchley et al., 2003, Strioga et al., 2011), and is associated with autoimmune diseases (Cencioni et al., 2017, Wang et al., 1997, Mikulkova et al., 2010). CD57 can also be used to identify a subset of NK cells, however further phenotyping of the patients demonstrated that this expanded $CD4^+$ $CD57^+$ T cell population was not a NK cell population. Furthermore, a recent study by Alshekaili et al. demonstrated that there is a population of

$CD57^+$ T_{FH} cells in tonsil and blood that are PD-1^{hi} and have a cytotoxic signature (Alshekaili et al., 2018b). Nevertheless, the $CD4^+$ $CD57^+$ population present in our patients was not uniformly PD-1^{hi}, suggesting that the population found in the patients is likely a cytotoxic $CD4^+$ T cell population that is induced by excess IL-2 and may be connected with the patient's autoimmune disease or severe end-organ damage. Indeed, the patients had been referred to us due to the severity of the organ manifestations (central nervous system, renal disease and arthritis). Cytotoxic $CD8^+$ T cells have been reported to be involved in the inflammation of target organs seen in lupus (Blanco et al., 2005, Manjarrez Orduño et al., 2017) and are often seen in increased numbers in kidneys from SLE patients. It is therefore not inconceivable that cytotoxic $CD4^+$ T cells may also contribute to, and exacerbate, end-organ inflammation (Daca et al., 2011).

Before this study, the function of ZFR2 in the immune system remained unclear. Based on the roles of the paralogs ILF2 and ILF3, we hypothesised that ZFR2 played important roles in the regulation of cytokines transcriptionally and post-transcriptionally. Co-immunoprecipitation of wild-type ZFR2 and subsequent mass spectrometry revealed several immune and non-immune protein interactions and networks. These included the NFAT/AP-1/ILF2/ILF3 protein network for transcriptional activation and expression of T cell cytokine genes (Shi et al., 2007a, Shi et al., 2007b), negative regulation of cytokines through JAK/STAT via the SOCS3/CUL5 proteins (O'Sullivan et al., 2007), and type I interferon expression through the RNA-editing enzymes of the ADAR family (Yang et al., 2014a). Several interactions with other RNA binding proteins important for alternative splicing and mRNA stabilisation, as well as proteins with unknown functions, were also identified. Co-immunoprecipitation and mass

spectrometry of ZFR2^{A771T} revealed that the variant affected the interactions with proteins from several pathways, of note the NFAT/AP-1/ILF2/ILF3 and SOCS3/CUL5 protein networks. Given the already described roles of the paralogs in cytokine regulation, this mass spectrometry data provided us with a good foundation for further investigation of specific cytokine-regulatory pathways, including IL-2 (NFAT and AP-1 activity) and IFN-β.

In vitro studies revealed that ZFR2 plays a role in mRNA splicing, although the A771T mutation did not alter the protein's splicing ability. Interestingly, deletion of the DZF domain of ZFR2 (ZFR2$^{\Delta DZF}$) did not appear to alter mRNA splicing, suggesting that this domain is not required for mRNA splicing and consistent with normal splicing of mutant ZFR2. It will be interesting to elucidate the mRNA targets regulated by ZFR2 at the level of splicing in T cells.

Whilst we demonstrated that the ZFR2 A771T variant leads to an increase in IL-2 *in vitro* using Jurkat T cells, this increase was not due to stabilisation of the IL-2 transcript via the 3'UTR, which is one of the ways that ILF2 and ILF3 regulate IL-2 (Pei et al., 2008, Shim et al., 2002b). IL-2 transcription in T cells is strongly regulated by NFAT/AP-1 (Chow et al., 1999, Rooney et al., 1995) and these transcription factors form a complex with, and are regulated by, the ZFR2 paralogs ILF2 and ILF3 (Shi et al., 2007a, Shi et al., 2007b, Zhao et al., 2005). Notably, this study demonstrated that ZFR2 plays a role in NFAT as well as AP-1 activity and IFN$\boldsymbol{\beta}$ expression. The A771T variant augments NFAT and AP-1 whereas it decreases IFN$\boldsymbol{\beta}$ activity, suggesting that this mutation is a gain of function (as the paralogs of ZFR2 are known to activate NFAT/AP-1 and supress IFN$\boldsymbol{\beta}$). Furthermore, the DZF domain appeared crucial for NFAT and AP-1 activity, as deletion of the DZF domain (ZFR2$^{\Delta DZF}$) reduced both NFAT and AP-1 activity. Together, our data suggest that the increase in IL-2 that we observed in cells

expressing ZFR2^{A771T} is likely a functional consequence of increased NFAT and AP-1 activity, as opposed to a defect in the stability or splicing of the IL-2 mRNA transcript, and may explain the increased plasma IL-2 we observed in the patients carrying the ZFR2 variants. The reduction in IFN$\boldsymbol{\beta}$ activity in cells expressing ZFR2^{A771T} is not in-line with the demonstrated increase in type I IFN in ~70-80% of SLE patients (Park et al., 2013), and we do not understand the functional consequence of this finding. It may be a consequence of the interactions between ZFR2 and the ADAR proteins that we identified using mass spectrometry. Again, this pathway may contribute to SLE pathogenesis in a subset of SLE patients, potentially the subset that does not present with elevated type I IFN.

We generated mice carrying the orthologous ZFR2 variant using CRISPR/Cas9 editing. Unimmunised mutant mice displayed some cellular phenotypes found in the patients, such as an increase in regulatory T cells at 6 months of age and subtle increase in serum IL-2 at 12-14 weeks. Nevertheless, they did not develop any autoimmune phenotypes such as anti-nuclear antibodies. We attempted to exacerbate disease by chronically infecting the mice with LCMV, and preliminary findings with small numbers of mice suggested trends of decreased T_{FH} and increased exhausted $CD8^+$ memory cells (T_{MEX}) that may be consistent with increased IL-2 production. Further investigation is currently underway to confirm these results with larger mouse numbers so statistics can be performed. These further investigations will include assessing the effect of exacerbating stressors on cytokine production by activated T cells, as well as T cell proliferation and expansion.

Overall, the findings from this project suggest that the ZFR2^{A771T} variant in Family A, and likely the ZFR2^{A619W} variant in Family B, are gain-of-function variants that lead to elevated production of IL-2 (and likely other proinflammatory cytokines) through a dysregulation in NFAT/AP-1 signalling (Figure 5.1). Whilst lupus is classically considered to be a B cell/autoantibody driven disease, the strong HLA association suggests an important contribution of T cells. It is possible that an aberrant production of IL-2 and other proinflammatory cytokines may be pathogenic and contribute to disease initiation, acceleration, or exacerbate end-organ damage. For example, IL-2 is critical for the differentiation and proliferation of effector CD4$^+$ and CD8$^+$ T cells (Liao et al., 2013, Liao et al., 2011, Cheng et al., 2011, Arenas-Ramirez et al., 2015, Boyman and Sprent, 2012) and increases T cell survival (Kelly et al., 2002) and clonal expansion (Kumar et al., 1996).

Given that IL-2 deprivation has been considered key to the induction of T cell anergy (Dure and Macian, 2009, Chikuma et al., 2009, Schwartz, 2003, Boussiotis et al., 1994), it is conceivable that IL-2 excess overcomes T cell anergy, leading to self-reactive T cells activating self-reactive B cells. IL-2 also promotes the development of cytotoxic T cells (Rollings et al., 2018, Rosenberg, 2014, Gillis and Smith, 1977, Gillis et al., 1978) which are damaging to end-organs in the context of lupus (Blanco et al., 2005, Manjarrez Orduño et al., 2017). IL-2 also activates NK and NK T cells (Lehmann et al., 2001, Bessoles et al., 2008) leading to large amounts of IFNγ production (Hervier et al., 2011). IL-2 induced excessive IFNγ, which can be derived from T_H1, cytotoxic CD8$^+$ T cells, NK or NKT cells, has also been shown to be pathogenic in lupus: leading to lupus autoantibodies in mice (Lee et al., 2012b) and preceding SLE development in humans (Munroe et al., 2016). Not surprisingly, high dose IL-2 therapy

has been shown to stimulate effector T cells and lead to insulitis and development of type I diabetes in predisposed mice (Baeyens et al., 2013, Tang et al., 2008, Tang, 2015, Rabinovitch et al., 2002).

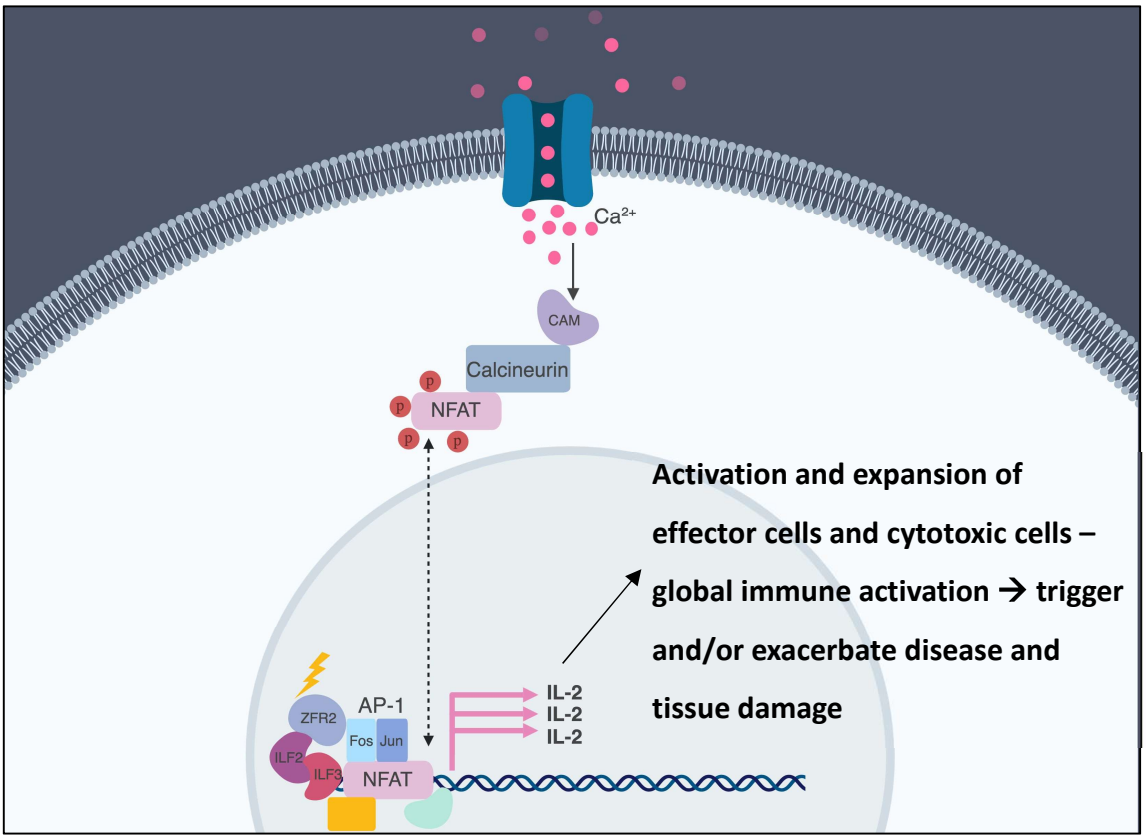


Figure 5.1. Proposed effect of gain-of-function ZFR2 mutations in SLE pathogenesis.

5.2.2 Implications for understanding the role of genetics in SLE

Although SLE is the result of a combination of environmental and intrinsic predispositions, genetic risk remains one of the most potent risk factors (Alarcon-Segovia et al., 2005). Genome Wide Association Studies (GWAS) have identified a number of common gene variants that that are associated with susceptibility to SLE and other complex diseases. Whilst these studies have uncovered important genes and disease pathways, they generally fail to illuminate or explain how the individual genes and variants contribute to disease mechanisms (van der Sijde et al., 2014, Moser et al., 2009). This is because variants identified by GWAS are typically found at high frequencies in the population and thus only modestly increase risk and often do not cause discernible changes in protein function. Thus, in general, whilst GWAS are capable of identifying important pathways, they are insufficiently powered to identify disease-causing variants that have a strong effect on protein function and can therefore throw light onto disease mechanisms (Cooper and Shendure, 2011, Moser et al., 2009). It has been postulated that it is the additive effect of many common variants that determines the susceptibility or risk of SLE. Although it has been argued that for some common GWAS variants, the association detected by GWAS may actually be that of rare damaging variants in linkage disequilibrium with the GWAS common variant. A large study concluded that the contribution of rare variants to autoimmunity is negligible, after failing to identify rare variants from 25 GWAS genes contributing to susceptibility to six different autoimmune diseases, in a large cohort of 24,892 autoimmune patients and 17,019 healthy controls (Hunt et al., 2013). Importantly, SLE was not included amongst the autoimmune diseases tested, and several genes which have been associated with autoimmunity by both GWAS and as rare variants were also not sequenced. An example is *BLK* and *BANK1*, two genes extensively associated to SLE by GWAS (Hom et al., 2008b, Lu et al., 2009). Our group has recently

demonstrated that many different rare and low frequency variants are found in a large proportion of SLE patients and healthy controls (Jiang et al., Nature Comm. 2019, in press). Intriguingly, BLK rare variants present in SLE patients fail to repress IRF5 and type I IFN, while those present in healthy controls repress normally.

In principle, using next-generation DNA sequencing to identify rare variants contributing to SLE should not be such a futile exercise given the growing list of monogenic causes described to date, identified in the past by familial linkage studies. These monogenic causes are enriched in gene variants that alter the function of DNA- and RNA-sensing or degrading enzymes, or complement factors (Lo, 2016a) and typically cause severe disease of childhood onset.

The two major theories for genetic risk in autoimmunity are therefore the polygenic *many common variants of weak effects acting together) and the monogenic/oligogenic (one or a few rare or novel variants of strong effects) hypotheses. There have been numerous debates in the literature between strongly-held viewpoints. As lupus is a complex disease with no clear pattern of Mendelian inheritance, it is plausible that lupus could be explained by monogenic, oligogenic and polygenic causes in different individuals/families, therefore indicating that the genetic risk of SLE is like a spectrum. Indeed, sporadic cases can often be monogenic due to de novo mutations, which are frequent causes of childhood-onset disease and have been found in SLE (Pullabhatla et al., 2018).

It is still challenging however to test the oligogenic hypotheses with functional studies, i.e. to study how two or more genes can act in functional epistasis to cause disease. This is relevant to the current study, in which all affected individuals in Family A had additional rare gene variants in *DHX58* and *SMG7*, genes which regulate RNA/DNA processing and have been shown to be relevant to SLE pathogenesis and associated with SLE by GWAS. Thus, it is possible that all three genes, *ZFR2, DHX58* and *SMG7* are involved in RNA and/or DNA regulatory pathways that when dysregulated contribute to SLE. The contribution of these or other variants may explain the milder phenotype in the CRISPR/Cas9 mice which only carry the ZFR2 variant. Thus, identification of rare variants in both SLE-associated and non-associated genes may be important to understand the pathways that contribute to this disease.

5.2.3 Implications for SLE diagnosis and treatment

The results in this study demonstrate the ability for whole exome and genome sequencing to identify potentially causative variants in individuals and families, forming the basis for personalised treatments for patients with complex autoimmune disorders such as SLE. In addition, the findings in this thesis contribute to the both the understanding of an uncharacterised protein in the immune system and its role in preventing autoimmunity, as well as the genetic architecture of complex autoimmunity.

As mentioned earlier, a formal diagnosis of SLE is based on eleven clinical and laboratory criteria, of which four are necessary for diagnosis. Furthermore, not only are the clinical manifestations of SLE diverse, which often makes it difficult to diagnose patients, but so are

the underlying molecular mechanisms and signalling pathways that contribute to SLE pathogenesis. This restricted definition of SLE and clinical heterogeneity may therefore partially explain the lack of success in SLE clinical trials (Rodriguez-Pinto et al., 2016). Identifying the underlying molecular mechanisms contributing to the pathogenesis of SLE and then stratifying and reclassifying patients based on the genetic lesions and molecular pathways affected (for example complement activation), rather than by their clinical symptoms, may enable patients to be treated with more specific, targeted and successful treatments. For example, patient III.3 is on Arcoxia (etoricoxib), which is an anti-inflammatory drug that reduces proinflammatory cytokine synthesis (Nadda et al., 2013), and this drug appears to lower the patients plasma proinflammatory cytokines, specifically IL-2, and thus may be a more effective treatment for patients with ZFR2 gain of function variants. Importantly, when considering the range of treatment options that may routinely become available, we suggest low dose IL-2 therapy should be avoided in these families, given that it could exacerbate some disease manifestations. Such adverse reactions to low-dose IL-2 have recently been described (Ye et al., 2018, Castela et al., 2014, Hartemann et al., 2013, Rosenzwajg et al., 2015). *ZFR2* mutations may explain some these paradoxical responses to therapy.

Intriguingly, the patient who is being treated with mycophenolic acid (patient II.3) displayed lower levels of plasma IL-2, as well as lower levels of T_{regs} and reduced $CD4^+$ $CD57^+$ cell population compared to other affected family members. Mycophenolic acid has been shown to inhibit the production of proinflammatory cytokines (Jonsson and Carlsten, 2002), and therefore this may serve as an effective medication for individuals with defects in IL-2 pathways that lead to excessive IL-2 production.

5.2.4 Limitations and technical challenges of this project

This project demonstrates the strength of whole exome and genome sequencing in identifying rare and novel variants that may contribute to SLE gene risk, and has initiated our understanding of a previously uncharacterised protein in the immune system and its role in regulation of IL-2 production and IFN-β regulation in human T cells. There were however significant factors that have limited this study and must be considered.

Like GWAS, whole exome/genome sequencing has its limitations, especially when it comes to complex diseases like SLE that do not follow classical Mendelian inheritance patterns. For complex diseases, carriers of rare/novel susceptibility variants can be unaffected, particularly when two or more gene variants are required for disease development. This differs from classical Mendelian inheritance, where almost all individuals who carry the risk variant have the disease (Manolio et al., 2009). Furthermore, the many thousands of variations in each individual means that the path to finding the major genetic contributors to disease is challenging. Furthermore, even when a disease is known to have monogenic inheritance in a particular proband, we are still limited by the fact that our genome analysis tools cannot properly interrogate the significance of variants in non-coding parts of the genome, and current analysis software still fails to identify large structural variants accurately (large deletions, insertions and translocations). This is illustrated by the findings from the Baylor University and the University of California Los Angeles (UCLA) which shows that whole exome sequencing identifies the underlying genetic defect in approximately 25% of patients with presumed Mendelian diseases (Lee et al., 2014a, Yang et al., 2014c, Yang et al., 2013). Furthermore, causative variants and novel targets can be missed as a result of false-negative variant calls due to inadequate coverage of a region containing the causative allele, and

coverage with both whole exome and genome sequencing has approximately a 10% and 5% false negative rate respectively. Additionally, if the disease-causing alleles are present in the reference genome they will also not be identified.

Secondly, the predictive frameworks PolyPhen2, SIFT and CADD that were used in the scoring algorithm are imperfect and are not definitive. These algorithms are based on evolutionary and sequence conservation, and available experimental data, and thus do not account for the biological context of the variant or protein-protein interactions. The scoring algorithm we developed also weighted genes known to have a role in the immune system, and whilst this can aid in identifying obvious candidate gene variants, variants in genes with no known function could easily be missed. Furthermore, the estimation of the frequency of a variant was obtained from the NCBI dbSNP database given that when this project commenced, improved tool such as ExAC and gNOMAD were not available. Frequency estimates using dpSNP are often based on small and biased studies (i.e. the estimation is specific for a certain demographic or ethic group) (Cooper and Shendure, 2011). In an attempt to overcome these limitations, heavier weighting was put on a candidate gene variant being novel or rare, compared to being damaging or having an established role in the immune system. Also, each pedigree has been analysed independently, multiple times, and the scoring algorithm is being continually improved and refined to be informative for a specific pedigree. For example, in Family A, the rarity of a gene variant and whether it segregated with disease were the two higher-scoring factors in the algorithm, which explains why a gene with unknown function was identified as one of the top candidates. Finally, the Centre for Personalised Immunology Variant Database was used to determine the frequency of gene variants in around 1700

sequenced healthy controls, autoimmune patients and patients with other diseases from multiple ethnicities, which is how we found the second ZFR2 gene variant in Family B.

Our scoring algorithm does not consider environmental, gender, infectious, epigenetic or other genetic factors that may be contributing to disease, however the role of these factors in predisposing to the development of SLE has been well documented. In addition, it did not consider common variants that may be in epistasis with the novel and rare variants. Therefore, it is likely that novel and rare variants predispose to the pathogenesis of disease in the two families studied, and act in combination with of other factors to modify the risks associated with these genetic variants. For example, all the individuals in Family A, across three generations, were diagnosed with SLE around the same time, suggesting a viral/infectious trigger. Unfortunately, we could not confirm or further investigate this. It is therefore evident that identifying genetic variants that contribute to complex diseases without clear Mendelian inheritance, such as SLE, is often challenging, which is why the aetiology of many complex diseases remains unknown.

Whilst this study has revealed a role for ZFR2 in the immune system, as well as suggested how variants in this gene may contribute to SLE gene risk, it does not entirely identify the mechanisms through which this variant leads to a break in tolerance or exacerbates disease. The ZFR2$^{wt/mut}$ mice did not recapitulate the autoimmune phenotypes observed in the SLE patients carrying the orthologous variant, and making the mouse homozygous for the *ZFR2* variant and aging them in an attempt to exacerbate the phenotypes only revealed a very mild cellular phenotype with no signs of autoimmunity. As mentioned above, one reason for this

could be that this study did not address the other candidate variants in *DHX58* and *SMG7* as these variants were identified later and there were delays with generating and breeding those mouse strains, however these gene variants (or other contributors already mentioned above) may be essential for disease pathogenesis. The failure to induce disease in these mice may also be due to the fact that there are significant differences between relevant aspects of the human and mouse immune systems, a growing concern that may account for many failures in translating human disease to valid mouse models (Mestas and Hughes, 2004, Seok et al., 2013, Shay et al., 2013, Mak et al., 2014, Rivera and Tessarollo, 2008, Payne and Crooks, 2007, von Herrath and Nepom, 2005, Takao and Miyakawa, 2015). Furthermore, multiple studies have suggested and demonstrated that laboratory mutant mice which are raised in specific pathogen-free conditions may not display the same phenotype as humans carrying orthologous variants, due to the lack of pathogens in the environment and their immature immune system (Cadwell et al., 2008, Cadwell et al., 2010, Tao and Reese, 2017, Reese et al., 2016, Beura et al., 2016). It is for this reason that we attempted to trigger and exacerbate disease in these Zfr2 mutant mice with LCMV infection, and whilst preliminary findings are promising, we are still in the process of performing these infections.

5.2.5 Future directions of this project

This study provides a foundation for the role of ZFR2 in the immune system and suggests how dysregulation in the pathways that this protein is involved in may contribute to disease. We demonstrated *in vitro* that ZFR2^{A771T} leads to an increase in the proinflammatory cytokine IL-2, likely via increased transcription as a result of activation of NFAT/AP-1 as opposed to post-transcriptionally (mRNA splicing or stabilising the *IL-2* transcript via its 3'UTR). In order to

confirm this finding *in vivo*, we could cross the ZFR2$^{mut/mut}$ mice to the NFAT-luc-transgenic mice which express an NFAT response element that drives the transcription of the luciferase reporter gene, emitting light following TCR activation. The mechanism of action by which this variant is affecting NFAT and AP-1 signalling could also be elucidated, for example by investigating whether it affects direct protein-protein interactions. Whilst investigation of ZFR2 protein-protein interactions through immunoprecipitation and mass spectrometry helped identify pathways in which ZFR2 is involved, ZFR2 is an RNA binding protein and therefore investigation into the RNA sequences that ZFR2 binds to, and whether the variant affects RNA binding, would help elucidate further the function of ZFR2 and the effect of the variant.

In this study we focused on IL-2 given the patient's phenotypes, however NFAT and AP-1 are also transcriptional regulators of GM-CSF, IL-3, IL-4, IL-5, IL-8, IL-13, IFN$\boldsymbol{\gamma}$, TNF$\boldsymbol{\alpha}$, CD40L, FasL, CD5, Ig$\boldsymbol{\kappa}$, CD25, MIP-1$\boldsymbol{\alpha}$ and cyclooxygenase 2 (Macian et al., 2001). Investigating whether other targets are altered by the ZFR2^{A771T} variant would give a better overview of how this variant may be leading to a breakdown in tolerance as IL-2 is unlikely to be the only contributing factor to the abnormal phenotypes observed in these patients. Additionally, although a connection between excess IL-2 (and other proinflammatory cytokines) and autoimmunity has been described in the literature, the mechanisms of exactly how this occurs remains to be fully elucidated. Determining the source of these proinflammatory cytokines (i.e. what cell type is producing them) may aid in elucidating this molecular mechanism.

Gene knockout and knockdown systems are powerful and versatile ways to dissect functional properties of proteins. Given the protein sequence and conservation of ZFR2, as well as its ubiquitous expression, a knockout system may disrupt normal cellular development and prevent investigation of its role in the immune system, especially as knocking out ILF2, ILF3 and ZFR in mice is perinatally or embryonically lethal (Kiesler et al., 2010, Shi et al., 2005, Meagher and Braun, 2001). We attempted to create a ZFR2 knockout mouse, however initial attempts failed due to problems with obtaining a structural variant (i.e. an insertion or deletion) that would lead to a stop codon. To circumvent the fact that knockout out ZFR2 would likely be lethal, we could create a conditional knockout system in mice, depleting ZFR2 in a specific cell lineage or tissue using the cre-lox system.

Finally, the ZFR2$^{wt/mut}$ mice did not completely recapitulate the phenotypes observed in the SLE patients, and we attempted to overcome this by investigating the phenotypes in mice homozygous for the variant, and through challenging the mice with LCMV. Additionally, given the sequence conservation between ZFR2 and the paralog ZFR, there may be some redundancy between these two proteins and thus crossing the ZFR2$^{mut/mut}$ mice to a mouse deficient in ZFR may exacerbate the phenotypes in these mice as well as further elucidate the roles of these intriguing proteins. Furthermore, exploration into the roles of the DHX58 and SMG7 variants as contributors to disease will also be crucial to determine whether the combined effect of these novel and rare variants triggers autoimmunity in mice. It would be ideal to generate mice carrying all three ZFR2^{A704T}, DHX58^{H314C} and SMG7^{D525Y} variants to determine potential epistatic interactions between the variants. It might also be informative to identify and introduce other genetic contributors in the patient from Family B, enabling us

to determine whether the combination of these variants recapitulates the autoimmune phenotype and dysregulated cytokine production we observed in the patients. Finally, investigating the effects of other immune stressors, for example other type of infections, may also be informative and help elucidate the role of these proteins in the immune system.

5.2.6 Broader implications of this study and concluding remarks

This study provides a foundation for the involvement of ZFR2 in the immune system, and how dysregulation in the pathways of this protein may contribute to disease pathogenesis. Although the connection between SLE pathogenesis and dysregulated NFAT/AP-1 signalling and IL-2 production remains elusive, the findings in this thesis open avenues to treat these patients with molecules that target this dysregulated pathway. This approach demonstrates how whole exome and genome sequencing can provide novel insights into gene function and novel pathways in human autoimmune diseases. Identifying more targeted therapies could minimise the off-target side-effects characteristic of broad immunosuppressants and corticosteroids. This multifaceted approach combines whole exome and genome sequencing with cellular phenotyping, and when successful, it has the potential to group patients with complex monogenic or polygenic disorders based on the pathogenic mechanism underlying disease, thus leading to significant improvements in disease management and treatment.

5.3 Investigating the role of human T_{FR}-derived IL-32 in B cell responses

5.3.1 Summary of findings in the context of the literature

In an RNA sequencing (RNAseq) experiment performed by Mr Pablo Fernandez De Canete from our laboratory on T follicular helper (T_{FH}), T follicular regulatory (T_{FR}) and naïve T cells, we discovered that interleukin-32 (IL-32) is highly expressed by human follicular regulatory-like cells (T_{FR}-like cells). This was confirmed by RT-PCR, which also revealed that B cells do not express IL-32. IL-32 is a recently described proinflammatory cytokine that is dissimilar to other cytokines and not expressed in rodents. Whilst its role in cancers and inflammatory diseases has been reported in the literature, a role in the germinal centre has not been described. Immunohistochemical analysis of human tonsils identified expression of IL-32 within the light-zone of the germinal centre, which is where the majority of germinal centre T cells localise (Vinuesa et al., 2005c, Breitfeld et al., 2000). Interestingly, all IL-32 positive cells were also CD4 positive, however the majority of the IL-32 positive cells localised in the middle of the light zone rather than in the outer zone (the part of the light zone close to the follicular mantle); the outer zone is where the majority of the $CD4^+$ T cells localise in the germinal centre (Kerfoot et al., 2011).

Given the fact that we found IL-32 preferentially bound to B cells rather than T cells, and the fact that T_{FR}-like cells influence B cell responses, we chose to focus on the effect of IL-32 on B cells. Stimulation of tonsil-derived B cells with recombinant IL-32γ (rIL-32γ) for 24 hours revealed no changes in PD-L2, BAFF-R, IgD, IL-21R, FAS-R, CD86, ICOSL, PD-1, BCL6 or FOXP3 expression on B cells. Interestingly however, we observed a significant upregulation of PD-L1 on the surface of B cells. Further investigation revealed that this upregulation of PD-L1 was

rapid, occurring with five minutes, and that PD-L1 is stored intracellularly, and that it is shuttled to the B cell surface upon stimulation, as demonstrated in previous studies on cancer cells (Herbst, 2014 and Hye Ryun Kim 2016).

We also investigated which IL-32 isoform (IL-32α, IL-32β or IL-32γ) was best at inducing PD-L1 using a dose titration of all isoforms, and identified IL-32γ as the most potent isoform, consistent with other studies which have looked at other aspects of IL-32 function (Choi et al., 2009b). Interestingly, we also found that fluorochrome-conjugated rIL-32γ associated more with PD-L1^{hi} B cells compared to PD-L1^{lo} B cells. Furthermore, using an optimal dose of IL-32γ determined by the dose titration, we found that IL-32 induced upregulation of PD-L1 occurred on all B cell subsets tested (naïve, memory and germinal centre B cells), except for plasmablasts. This was consistent with the fact that plasmablasts did not associate with fluorochrome-conjugated rIL-32γ, and the fact that plasmablasts already express high amounts of surface PD-L1 (Yousef et al., 2015, Liu et al., 2007).

No difference in PD-L1 expression on CD4$^+$ and CD8$^+$ T cells was observed following stimulation with rIL-32γ. Even though IL-32 is not expressed in mice, it has been demonstrated that murine macrophages may respond to IL-32 (although whether they respond because they possess the receptor, or they are responding to a foreign antigen remains unclear). Regardless, we tested murine B cells to see if they upregulated surface PD-L1 after stimulation with human rIL-32γ and found that they did not respond, failing to upregulate surface PD-L1.

IL-32 has been shown to induce differentiation of human monocytes and THP-1 leukemic cells into macrophage-like cells (Netea et al., 2008). We therefore investigated whether IL-32 affected B cell differentiation and proliferation in response to B cell activating factors (anti-BCR, IL-21 and anti-CD40) and did not observe any significant differences. Interestingly however, the increased PD-L1 on cells stimulated with rIL-32γ remained after five days in culture. No differences in the other cell markers (PD-L2, BAFF-R, IgD, IL-21R, FAS-R, CD86, ICOSL or PD-1) or transcription factors (BCL6 and FOXP3) were observed after five days.

PD-1/PD-L1 signalling also has been shown to be important for B cell survival (Hamel et al., 2010b, Good-Jacobson et al., 2010b), and IL-32 has been shown to promote macrophage survival (Osman et al., 2014), and increase cell viability in endometrial stromal cells (Lee et al., 2018). Interestingly, in this study we found that B cells stimulated with rIL-32γ overnight survived better than those that were unstimulated.

Finally, given the immune evasion mechanism that PD-1/PD-L1 plays in tumour cells, we also tested several cancer cell lines to determine if rIL-32γ upregulated surface PD-L1, however the only cell line that upregulated PD-L1 on its surface was Raji cells, a Burkitt's lymphoma B cell line, possibly because the majority of the other cells lines already expressed high amounts of surface PD-L1.

5.3.2 Induction of PD-L1 regulation B cells by IL-32

Regulatory T and B cells are essential for preserving immune homeostasis and self-tolerance. Regulatory B cells appear to be a subset of B cells that have immunosuppressive capabilities,

preventing the expansion of pathogenic T cells and pro-inflammatory lymphocytes, mainly through the production of IL-10, TGF$\boldsymbol{\beta}$ and IL-35 (Rosser and Mauri, 2015). We are still trying to understand the biology and phenotypes of these suppressive B cells, and whilst a variety of B cell subsets have been documented as regulatory B cells, no specific marker or unique transcription factor has been consistently identified. PD-L1 however has been reported as identifying subsets of regulatory B cells (Zhang et al., 2016, Guan et al., 2016, Khan et al., 2014, Schwartz et al., 2016, Zacca et al., 2018, Khan et al., 2015). Khan et al., 2015 demonstrated that there is a regulatory B cell population that express elevated levels of PD-L1 (Khan et al., 2015), which was subsequently supported by further studies (Gallego-Valle et al., 2018, Guan et al., 2016). This subset of PD-L1^{hi} regulatory B cells was found to be important in supressing humoral responses through preventing T cell activation and antibody production, independently of IL-10, and that the PD-L1^{hi} regulatory B cells expressed the highest level of BAFFR and acted as a BAFF 'sink' by sequestering BAFF (Khan et al., 2015). BAFF is an important B cell survival factor (Batten et al., 2000), and in excess causes autoimmunity (Vincent et al., 2014, Matsushita and Sato, 2005, Kalled, 2005, Stohl et al., 2005). Anergic self-reactive b cells are more dependent on BAFF compared to non-self-reactive counterparts (Lesley et al., 2004). Thus, sequestration of BAFF is potentially an important immunoregulatory mechanism to prevent survival and aberrant activation of self-reactive B cells.

Our analysis of the IL-32γ induced PD-L1^{hi} B cells demonstrated that these cells also express the highest amount of BAFFR and do not secrete IL-10 following stimulation with PMA + Ionomycin, consistent with findings by Khan et al., 2015 (Khan et al., 2015). Furthermore, the

IL-32-induced PD-L1^{hi} cells also highly associated with fluorescent-conjugated recombinant BAFF compared to the PD-L1^{lo} B cells, suggesting they take up BAFF from the environment. Not only would this enhance their own survival, but most importantly this is likely to curtail survival of surrounding self-reactive B cells that are more dependent on BAFF (Lesley et al., 2004).

Overall, we hypothesise that T_{FR}-like cells are inducing, possibly self-reactive B cells, to become PD-L1^{hi} BAFF sequestering regulatory B cells through the secretion of IL-32. This may occur through direct cognate interaction and recognition of self-peptide presented on MHC-class II by the B cells. At least in the case of thymic-derived T_{regs}, they are selected in the thymus on the basis of self-antigen recognition, however the ontogeny of T_{FR}-like cells in humans is currently unknown. These PD-L1^{hi} cells, which are induced by T_{FR}-like cell-derived IL-32, may sequester BAFF from the germinal centre milieu, taking survival factors away from other self-reactive B cells. Furthermore, as proposed by Khan et al., these PD-L1^{hi} B_{regs} may also bind PD-1 in T_{FH} cells to reduce BCL6 transcription, thus reducing T_{FH} cell help to self-reactive B cells (Figure 5.2).

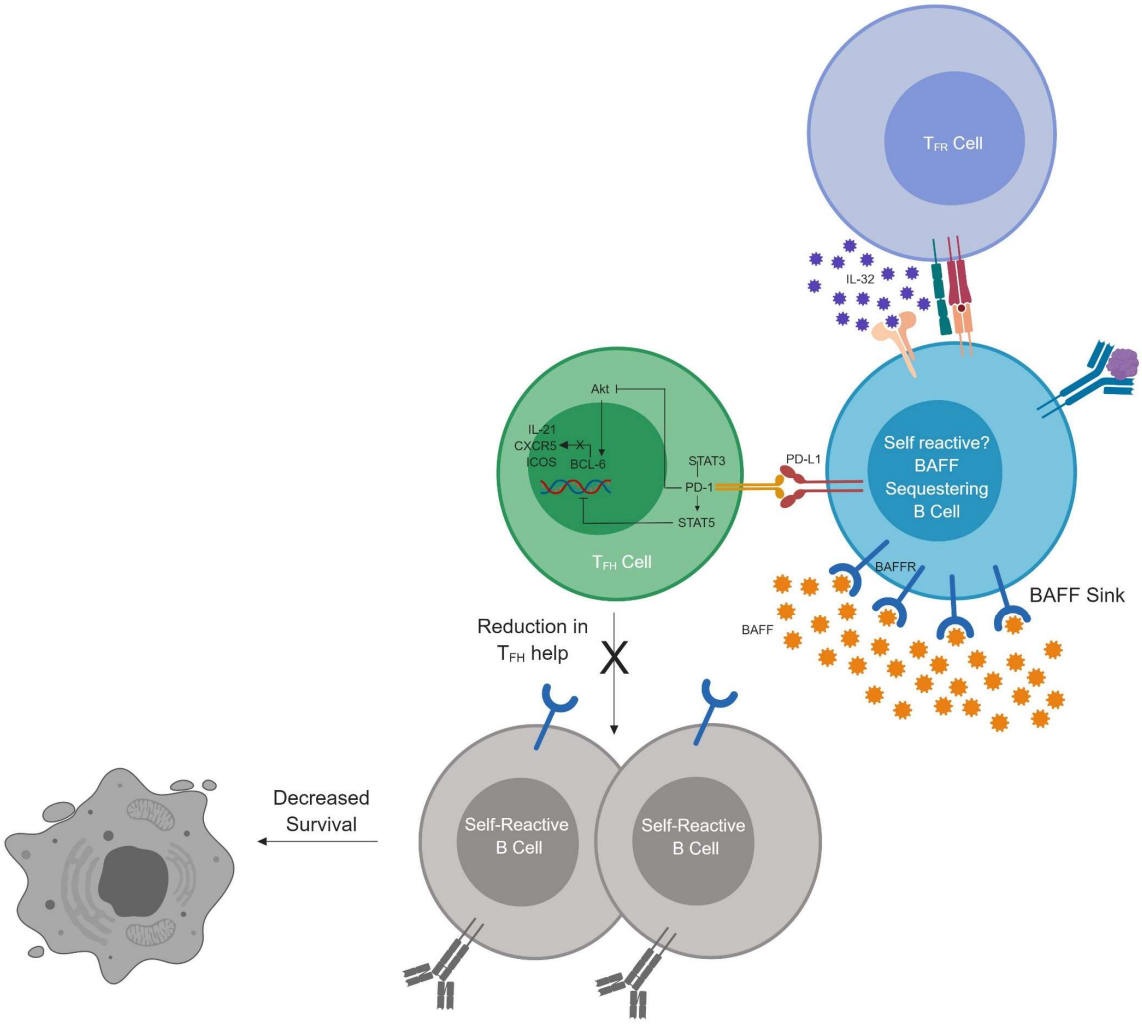


Figure 5.2. Proposed model for BAFF sequestering PD-L1^{hi} regulatory B cells (adapted from Khan et al., 2015).

5.3.3 Implications for B cell tolerance in germinal centres

Over the last decade, multiple studies have suggested that regulatory B cells play pivotal roles in human tolerance, however there is limited information regarding the immune mechanisms involved in the development and functions of these cells. Some studies have demonstrated that regulatory B cells cause expansion of T_{FR} cells, control maturation and development of T_{FH} cells, and inhibit B cell differentiation and immunoglobulin secretion (Achour et al., 2017, Yang et al., 2014b). Whilst the best-studied mechanism of regulatory B cell function is their ability to produce immunosuppressive IL-10, there are many IL-10-independent regulatory B cell functions, including production of transforming growth factor-$\boldsymbol{\beta}$ (Lee et al., 2014b), IL-35 (Shen et al., 2014), IgM (Kirkland et al., 2012, Shimomura et al., 2008) and the expression of PD-L1 (Bodhankar et al., 2011, Bodhankar et al., 2012, Bodhankar and Offner, 2013, Khan et al., 2015). It is not known whether these different mechanisms utilised by regulatory B cells correlate to specific subsets of B cells with regulatory properties, or whether the regulatory mechanisms are dependent upon the signals received by B cells during an immune response. Our study highlights that IL-32 selectively induces a regulatory B cell subset that does not produce IL-10: indeed, there was no overlap in IL-10 secreting PD-L1^{hi} B cell populations upon IL-32 treatment in the presence of PMA and ionomycin. It is therefore possible that different regulatory subsets are specialised in repressing specific aspects of the immune response.

In depth knowledge of the cellular and molecular events driving antibody affinity maturation and memory formation in germinal centres is critical to illuminate the design of potent and effective vaccines. Furthermore, understanding how immune tolerance is preserved in the face of extensive somatic hypermutation is important in preventing human diseases such as

autoimmune diseases. This study proposes a novel pathway by which IL-32 is produced by T_{FR}-like cells and induces potentially self-reactive B cells to become PD-L1^{hi} BAFF-sequestering regulatory B cells. Such B_{regs} act independently of IL-10 and operate through PD-1/PD-L1 signalling to dampen T_{FH} cell help, and BAFFR mediated BAFF sequestration to prevent self-reactive B cells (Khan et al., 2015). Khan et al. demonstrated that these PD-L1^{hi} regulatory B cells limited the differentiation and function of T_{FH} cells through PD-1/PD-L1 signalling, thus controlling the humoral immune response (Khan et al., 2015). This is supported by the fact that an absence of PD-L1 on B cells leads to an increase in T_{FH} cells and elevated antibody responses (Hams et al., 2011b).

In our study, these B_{regs} would be generated within the light zone of germinal centres, which is where the IL-32-producing T_{FR}-like cells are found. This would constitute a novel and important checkpoint in germinal centre tolerance. BAFF is most prominently secreted by follicular dendritic cells (Lesley et al., 2004, Kranich and Krautler, 2016), which are non-hemopoietic cells confined to the follicles, specifically to the light zones in germinal centres (secondary follicles). Light zone cells need to be selected by T cells, not only for their ability to bind antigen with high affinity, but also for their inability to bind self-antigen. How the latter may occur has remained elusive (Vinuesa et al., 2009). This check-point is important because not only can self-reactive B cells can enter germinal centres (Vinuesa et al., 2009, Sabouri et al., 2014); non-self-reactive B cells can also acquire self-reactivity through the process of somatic hypermutation in germinal centres (Jacobi and Diamond, 2005, Diamond et al., 1992, Rice et al., 2005). It therefore makes sense to have a dedicated regulatory

mechanism in the light zone, the environment where BAFF is produced and light zone cells are selected for survival, possibly to become long-lived memory B cells and plasma cells.

The role of regulatory B cells in autoimmune diseases, including SLE, RA, MS and psoriasis has been well characterised (Blair et al., 2010, Flores-Borja et al., 2013, Knippenberg et al., 2011, Bosma et al., 2012, Hayashi et al., 2016), with studies demonstrating an inverse correlation between regulatory B cell numbers and function, and disease activity. Furthermore, regulatory B cells have been shown to play important roles in bacteria, viral and parasitic infections (Shen and Fillatreau, 2015, Siewe et al., 2013), as well as allergies (Karnekura et al., 2015), cancer (Olkhanud et al., 2011, Wejksza et al., 2013, Han et al., 2014) and transplantation (Khoder et al., 2014). Therefore, the therapeutic potential of regulatory B cells is promising, especially in the context of autoimmunity, as the long-term use of steroids and immunosuppressive drugs increases the risks of infection, malignancy, cardiovascular disease and bone marrow suppression (Hsu and Katelaris, 2009). Whereas autoimmune diseases and transplantation would require an expansion of immunosuppressive regulatory B cells, other diseases such as chronic infections and cancers may benefit from a reduction in these cells. Therefore, the knowledge that IL-32 may be inducing regulatory B cells in the germinal centre could be used as a strategy to isolate, expand or deplete regulatory B cells, providing a novel opportunity to treat several immune-mediated disorders.

5.3.3 Limitations and technical challenges of this project

We have shown that T_{FR} cells express large amounts of IL-32, and that IL-32 upregulates PD-L1 on B cells and induces them to become BAFF sequestering regulatory B cells. It would however be useful to demonstrate that the IL-32 produced by the T_{FR}-like cells is inducing the

PD-L1^{hi} BAFF sequestering regulatory B cells. Since IL-32 is not expressed in mice and does not induce PD-L1 upregulation in mouse B cells, *in vivo* experiments were not possible. This made studying the effects of this cytokine quite challenging as it is difficult to recapitulate the processes that normally occur in the human germinal centre reaction *in vitro*. Furthermore, assessing antigen-specific interactions between T and B cells *in vitro* is challenging, and allogenic cell cultures between T and B cells from different tonsil donors produced variable and unreliable results, likely due to high cell death.

The fact that there is no known role for IL-32 in the germinal reaction was, whilst fascinating, also a challenge, and experiments were largely exploratory. Once we had found a robust effect of IL-32 in the upregulation of PD-L1 on B cells, we set out to perform RNA-seq on sorted germinal centre B cells and naïve B cells stimulated with/without IL-32γ. Unfortunately, repeated attempts failed due to low cell numbers after culture, poor RNA quality of cultured cells, and issues with obtaining enough fresh human tonsils on a given day. Furthermore, the lack of a good-quality anti-IL-32 antibodies for flow cytometry, and antibodies that recognise independent IL-32 isoforms, meant we were unable to assess IL-32 production by germinal centre T cells, and we had to rely on RNA to measure *IL-32.* Furthermore, we attempted to quantify each of the IL-32 isoforms in the germinal centre cell subsets by qPCR, however the primers for each of the isoforms had different amplification efficiencies, which is why we used a primer that identified all IL-32 isoforms. We did however investigate the RNA-seq data for T_{FR}-like cells, and whilst we were unable to accurately quantify the different isoforms due to variation in coverage and read depth, we did observe that all isoforms were present in T_{FR}-like cells. We used the gamma isoform for our *in vitro*

stimulation experiments initially as it has been reported to be the most potent isoform, and we knew from the RNA-seq data that T_{FR} cells expressed the gamma isoform of IL-32. We would have liked to quantify the expression of each isoform in each germinal centre T cell subsets, ideally looking at protein by flow cytometry, or by RNA-seq with better coverage and read depth.

5.3.4 Future directions

As mentioned above, whilst T_{FR}-like cells express the highest amount of IL-32 RNA, it would be valuable to confirm that it is the IL-32 from the T_{FR} cells that is inducing these PD-L1^{hi} B cells. Given the large amount of IL-32 RNA in $T_{FR\text{-}like}$ cells and their important regulatory and inhibitory functions, it is highly likely that this T cell subset is responsible for inducing PD-L1^{hi} regulatory B cells, however T_{FH} cells share many characteristics with T_{FR} cells and express some IL-32, although significantly less than T_{FR}-like cells. It would also be important to quantify each IL-32 isoform in germinal centre T cell subsets to get an idea of the isoforms that are most highly expressed, and whether this expression differs between T cell subsets. Whilst this could be done by standard RNA-seq analysis, which depends on averaging the signal from hundreds of thousands of cells, we would prefer to perform single-cell RNA-seq (scRNA-seq) as this would control for the heterogeneity of cell subsets within the germinal centre. As mentioned earlier, performing RNA-seq on B cells stimulated with IL-32 would also provide us with a robust starting point to discover additional functions of IL-32.

IL-32 is not expressed in mice, however transgenic mice have been previously generated to study the effect of this cytokine in human infections and inflammatory diseases, showing that

IL-32 is an immunoregulatory and suppressive cytokine that is protective against infections (Bai et al., 2015b), reducing the production of proinflammatory cytokines and increasing the production of IL-10 in certain inflammatory conditions (Choi et al., 2010). It would therefore be interesting to perform cellular phenotyping of the secondary lymphoid organs of these transgenic mice to see what subsets of cells are altered, and to see if these mice have increased regulatory B cells (although findings from our study showed that human IL-32γ did not upregulate PD-L1 on mouse splenic B cells). Subcutaneous xenografting of human tonsil into SCID mice, with or without administration of IL-32 into the graft site (as has been done by Duchosal et al., 2000), would allow us to study the human immune response to IL-32 *in vivo* (Duchosal et al., 2000). Finally, the receptor and targets of IL-32 are yet to be identified; identification of the receptor and the cells that express this receptor will help understand how IL-32 signals.

Finally, in order to confirm the functional roles of the IL-32γ induced PD-L1^{hi} BAFFR^{hi} regulatory B cells, we could isolate them and co-culture them with other germinal centre cell populations to evaluate the consequences of their suppressive activity. This would also tell us what cells these induced regulatory B cells are targeting, and potentially provide new avenues for the development of better therapies for several immune-mediated disorders.

5.3.5 Concluding remarks

Together, this study proposes a novel pathway by which T_{FR}-derived IL-32 increases B cell viability and induces B cells to become a previously described subset of regulatory B cells that acts independently of IL-10 and operates through PD-1/PD-L1 signalling and an elevated

expression of BAFFR, which acts to sequester BAFF from the germinal centre milieu. If further investigations support this proposal, IL-32 could be used as a strategy to isolate, expand or deplete regulatory B cells, providing a novel opportunity to treat several immune-mediated disorders. Overall, this study has provided a novel insight into the suppressive functions human TFR-like cells and identified a new cytokine signalling pathway in human germinal centre reactions.

Chapter Six: References

ACHOUR, A., SIMON, Q., MOHR, A., SEITE, J. F., YOUINOU, P., BENDAOUD, B., GHEDIRA, I., PERS, J. O. & JAMIN, C. 2017. Human regulatory B cells control the T-FH cell response. *Journal of Allergy and Clinical Immunology,* 140**,** 215-222.

ADACHI, O., KAWAI, T., TAKEDA, K., MATSUMOTO, M., TSUTSUI, H., SAKAGAMI, M., NAKANISHI, K. & AKIRA, S. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity,* 9**,** 143-50.

AKEY, J. M., EBERLE, M. A., RIEDER, M. J., CARLSON, C. S., SHRIVER, M. D., NICKERSON, D. A. & KRUGLYAK, L. 2004. Population history and natural selection shape patterns of genetic variation in 132 genes. *PLoS Biol,* 2**,** e286.

AL-MAYOUF, S. M., SUNKER, A., ABDWANI, R., ABRAWI, S. A., ALMURSHEDI, F., ALHASHMI, N., AL SONBUL, A., SEWAIRI, W., QARI, A., ABDALLAH, E., AL-OWAIN, M., AL MOTYWEE, S., AL-RAYES, H., HASHEM, M., KHALAK, H., AL-JEBALI, L. & ALKURAYA, F. S. 2011. Loss-of-function variant in DNASE1L3 causes a familial form of systemic lupus erythematosus. *Nat Genet,* 43**,** 1186-8.

ALARCON-SEGOVIA, D., ALARCON-RIQUELME, M. E., CARDIEL, M. H., CAEIRO, F., MASSARDO, L., VILLA, A. R., PONS-ESTEL, B. A. & GLADEL 2005. Familial aggregation of systemic lupus erythematosus, rheumatoid arthritis, and other autoimmune diseases in 1,177 lupus patients from the GLADEL cohort. *Arthritis and Rheumatism,* 52**,** 1138-1147.

ALLEN, C. D., ANSEL, K. M., LOW, C., LESLEY, R., TAMAMURA, H., FUJII, N. & CYSTER, J. G. 2004. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol,* 5**,** 943-52.

ALLEN, C. D., OKADA, T. & CYSTER, J. G. 2007. Germinal-center organization and cellular dynamics. *Immunity,* 27**,** 190-202.

ALOULOU, M., CARR, E. J., GADOR, M., BIGNON, A., LIBLAU, R. S., FAZILLEAU, N. & LINTERMAN, M. A. 2016. Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells. *Nat Commun,* 7**,** 10579.

ALSHEKAILI, J., CHAND, R., LEE, C. E., CORLEY, S., KWONG, K., PAPA, I., FULCHER, D. A., RANDALL, K. L., LEIDING, J. W., MA, C. S., WILKINS, M. R., UZEL, G., GOODNOW, C. C., VINUESA, C. G., TANGYE, S. G. & COOK, M. C. 2018a. STAT3 regulates cytotoxicity of human CD57+ CD4+ T cells in blood and lymphoid follicles. *Sci Rep,* 8**,** 3529.

ALSHEKAILI, J., CHAND, R., LEE, C. E., CORLEY, S., KWONG, K., PAPA, I., FULCHER, D. A., RANDALL, K. L., LEIDING, J. W., MA, C. S., WILKINS, M. R., UZEL, G., GOODNOW, C. C., VINUESA, C. G., TANGYE, S. G. & COOK, M. C. 2018b. STAT3 regulates cytotoxicity of human CD57+CD4+T cells in blood and lymphoid follicles. *Scientific Reports,* 8.

AN, J., BRIGGS, T. A., DUMAX-VORZET, A., ALARCON-RIQUELME, M. E., BELOT, A., BERESFORD, M., BRUCE, I. N., CARVALHO, C., CHAPEROT, L., FROSTEGARD, J., PLUMAS, J., RICE, G. I., VYSE, T. J., WIEDEMAN, A., CROW, Y. J. & ELKON, K. B. 2017. Tartrate-Resistant Acid Phosphatase Deficiency in the Predisposition to Systemic Lupus Erythematosus. *Arthritis Rheumatol,* 69**,** 131-142.

ANDERSON, G., MOORE, N. C., OWEN, J. J. & JENKINSON, E. J. 1996. Cellular interactions in thymocyte development. *Annu Rev Immunol,* 14**,** 73-99.

ANDOH, A., MASUDA, A., YAMAKAWA, M., KUMAZAWA, Y. & KASAJIMA, T. 2000. Absence of interleukin-4 enhances germinal center reaction in secondary immune response. *Immunol Lett,* 73**,** 35-41.

ANGELOSANTO, J. M., BLACKBURN, S. D., CRAWFORD, A. & WHERRY, E. J. 2012. Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. *J Virol,* 86**,** 8161-70.

ARBUCKLE, M. R., MCCLAIN, M. T., RUBERTONE, M. V., SCOFIELD, R. H., DENNIS, G. J., JAMES, J. A. & HARLEY, J. B. 2003. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med,* 349**,** 1526-33.

ARENAS-RAMIREZ, N., WOYTSCHAK, J. & BOYMAN, O. 2015. Interleukin-2: Biology, Design and Application. *Trends Immunol,* 36**,** 763-777.

AVERY, D. T., BRYANT, V. L., MA, C. S., DE WAAL MALEFYT, R. & TANGYE, S. G. 2008. IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *J Immunol,* 181**,** 1767-79.

BACHL, J., STEINBERG, C. & WABL, M. 1997. Critical test of hot spot motifs for immunoglobulin hypermutation. *European Journal of Immunology,* 27**,** 3398-3403.

BAEYENS, A., PEROL, L., FOURCADE, G., CAGNARD, N., CARPENTIER, W., WOYTSCHAK, J., BOYMAN, O., HARTEMANN, A. & PIAGGIO, E. 2013. Limitations of IL-2 and rapamycin in immunotherapy of type 1 diabetes. *Diabetes,* 62**,** 3120-31.

BAI, X., KIM, S. H., AZAM, T., MCGIBNEY, M. T., HUANG, H., DINARELLO, C. A. & CHAN, E. D. 2010. IL-32 is a host protective cytokine against Mycobacterium tuberculosis in differentiated THP-1 human macrophages. *J Immunol,* 184**,** 3830-40.

BAI, X., SHANG, S., HENAO-TAMAYO, M., BASARABA, R. J., OVRUTSKY, A. R., MATSUDA, J. L., TAKEDA, K., CHAN, M. M., DAKHAMA, A., KINNEY, W. H., TROSTEL, J., BAI, A., HONDA, J. R., ACHCAR, R., HARTNEY, J., JOOSTEN, L. A., KIM, S. H., ORME, I., DINARELLO, C. A., ORDWAY, D. J. & CHAN, E. D. 2015a. Human IL-32 expression protects mice against a hypervirulent strain of Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A,* 112**,** 5111-6.

BAI, X. Y., SHANG, S. B., HENAO-TAMAYO, M., BASARABA, R. J., OVRUTSKY, A. R., MATSUDA, J. L., TAKEDA, K., CHAN, M. M., DAKHAMA, A., KINNEY, W. H., TROSTEL, J., BAI, A., HONDA, J. R., ACHCAR, R., HARTNEY, J., JOOSTEN, L. A. B., KIM, S. H., ORME, I., DINARELLO, C. A., ORDWAY, D. J. & CHAN, E. D. 2015b. Human IL-32 expression protects mice against a hypervirulent strain of Mycobacterium tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America,* 112**,** 5111-5116.

BALACHANDRAN, S., ROBERTS, P. C., BROWN, L. E., TRUONG, H., PATTNAIK, A. K., ARCHER, D. R. & BARBER, G. N. 2000. Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity,* 13**,** 129-41.

BANCHEREAU, J. & PASCUAL, V. 2006. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. *Immunity,* 25**,** 383-92.

BARATH, S., ALEKSZA, M., TARR, T., SIPKA, S., SZEGEDI, G. & KISS, E. 2007. Measurement of natural (CD4+CD25high) and inducible (CD4+IL-10+) regulatory T cells in patients with systemic lupus erythematosus. *Lupus,* 16**,** 489-96.

BARBER, D. L., WHERRY, E. J., MASOPUST, D., ZHU, B., ALLISON, J. P., SHARPE, A. H., FREEMAN, G. J. & AHMED, R. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature,* 439**,** 682-7.

BATISTA, M. D., TINCATI, C., MILUSH, J. M., HO, E. L., NDHLOVU, L. C., YORK, V. A., KALLAS, E. G., KALIL, J., KEATING, S. M., NORRIS, P. J., CHANG, D., UNEMORI, P., LESLIE, K. S.,

MAURER, T., LIAO, W. & NIXON, D. F. 2013. CD57 Expression and Cytokine Production by T Cells in Lesional and Unaffected Skin from Patients with Psoriasis. *Plos One,* 8.

BATTEN, M., GROOM, J., CACHERO, T. G., QIAN, F., SCHNEIDER, P., TSCHOPP, J., BROWNING, J. L. & MACKAY, F. 2000. BAFF mediates survival of peripheral immature B lymphocytes. *J Exp Med,* 192**,** 1453-66.

BELOT, A. & CIMAZ, R. 2012. Monogenic forms of systemic lupus erythematosus: new insights into SLE pathogenesis. *Pediatr Rheumatol Online J,* 10**,** 21.

BELOT, A., KASHER, P. R., TROTTER, E. W., FORAY, A. P., DEBAUD, A. L., RICE, G. I., SZYNKIEWICZ, M., ZABOT, M. T., ROUVET, I., BHASKAR, S. S., DALY, S. B., DICKERSON, J. E., MAYER, J., O'SULLIVAN, J., JUILLARD, L., URQUHART, J. E., FAWDAR, S., MARUSIAK, A. A., STEPHENSON, N., WASZKOWYCZ, B., M, W. B., BIESECKER, L. G., G, C. M. B., RENE, C., ELIAOU, J. F., FABIEN, N., RANCHIN, B., COCHAT, P., GAFFNEY, P. M., ROZENBERG, F., LEBON, P., MALCUS, C., CROW, Y. J., BROGNARD, J. & BONNEFOY, N. 2013. Protein kinase cdelta deficiency causes mendelian systemic lupus erythematosus with B cell-defective apoptosis and hyperproliferation. *Arthritis Rheum,* 65**,** 2161-71.

BENOIST, C. & MATHIS, D. 1989. Positive and negative selection of the T cell repertoire in MHC class II transgenic mice. *Semin Immunol,* 1**,** 117-24.

BENOIST, C. & MATHIS, D. 1999. T-cell development: a new marker of differentiation state. *Curr Biol,* 9**,** R59-61.

BENOIST, C. & MATHIS, D. 2012. Treg cells, life history, and diversity. *Cold Spring Harb Perspect Biol,* 4**,** a007021.

BESSOLES, S., FOURET, F., DUDAL, S., BESRA, G. S., SANCHEZ, F. & LAFONT, V. 2008. IL-2 triggers specific signaling pathways in human NKT cells leading to the production of pro- and anti-inflammatory cytokines. *J Leukoc Biol,* 84**,** 224-33.

BEURA, L. K., HAMILTON, S. E., BI, K., SCHENKEL, J. M., ODUMADE, O. A., CASEY, K. A., THOMPSON, E. A., FRASER, K. A., ROSATO, P. C., FILALI-MOUHIM, A., SEKALY, R. P., JENKINS, M. K., VEZYS, V., HAINING, W. N., JAMESON, S. C. & MASOPUST, D. 2016. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature,* 532**,** 512-+.

BILLINGHAM, R. E., BRENT, L. & MEDAWAR, P. B. 1953. Actively acquired tolerance of foreign cells. *Nature,* 172**,** 603-6.

BLACKBURN, S. D., SHIN, H., HAINING, W. N., ZOU, T., WORKMAN, C. J., POLLEY, A., BETTS, M. R., FREEMAN, G. J., VIGNALI, D. A. & WHERRY, E. J. 2009. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol,* 10**,** 29-37.

BLAIR, P. A., NORENA, L. Y., FLORES-BORJA, F., RAWLINGS, D. J., ISENBERG, D. A., EHRENSTEIN, M. R. & MAURI, C. 2010. CD19(+)CD24(hi)CD38(hi) B Cells Exhibit Regulatory Capacity in Healthy Individuals but Are Functionally Impaired in Systemic Lupus Erythematosus Patients. *Immunity,* 32**,** 129-140.

BLANCO, P., PITARD, V., VIALLARD, J. F., TAUPIN, J. L., PELLEGRIN, J. L. & MOREAU, J. F. 2005. Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates with disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum,* 52**,** 201-11.

BODHANKAR, S. & OFFNER, H. 2013. PD-1 interaction with PD-L1 but not PD-L2 on B cells mediates protective effects of estrogen against EAE. *Journal of Immunology,* 190.

BODHANKAR, S., VANDENBARK, A. A. & OFFNER, H. 2012. Oestrogen treatment of experimental autoimmune encephalomyelitis requires 17 beta-oestradiol-receptor-positive B cells that up-regulate PD-1 on CD4(+) Foxp3(+) regulatory T cells. *Immunology,* 137**,** 282-293.

BODHANKAR, S., WANG, C. H., VANDENBARK, A. A. & OFFNER, H. 2011. Estrogen-induced protection against experimental autoimmune encephalomyelitis is abrogated in the absence of B cells. *European Journal of Immunology,* 41**,** 1165-1175.

BORCHERS, A. T., KEEN, C. L., SHOENFELD, Y. & GERSHWIN, M. E. 2004. Surviving the butterfly and the wolf: mortality trends in systemic lupus erythematosus. *Autoimmun Rev,* 3**,** 423-53.

BOSMA, A., ABDEL-GADIR, A., ISENBERG, D. A., JURY, E. C. & MAURI, C. 2012. Lipid-Antigen Presentation by CD1d(+) B Cells Is Essential for the Maintenance of Invariant Natural Killer T Cells. *Immunity,* 36**,** 477-490.

BOUSSIOTIS, V. A., BARBER, D. L., NAKARAI, T., FREEMAN, G. J., GRIBBEN, J. G., BERNSTEIN, G. M., D'ANDREA, A. D., RITZ, J. & NADLER, L. M. 1994. Prevention of T cell anergy by signaling through the gamma c chain of the IL-2 receptor. *Science,* 266**,** 1039-42.

BOYMAN, O. & SPRENT, J. 2012. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol,* 12**,** 180-90.

BREITFELD, D., OHL, L., KREMMER, E., ELLWART, J., SALLUSTO, F., LIPP, M. & FORSTER, R. 2000. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med,* 192**,** 1545-52.

BRENCHLEY, J. M., KARANDIKAR, N. J., BETTS, M. R., AMBROZAK, D. R., HILL, B. J., CROTTY, L. E., CASAZZA, J. P., KURUPPU, J., MIGUELES, S. A., CONNORS, M., ROEDERER, M., DOUEK, D. C. & KOUP, R. A. 2003. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8(+) T cells. *Blood,* 101**,** 2711-2720.

BRIGGS, T. A., RICE, G. I., DALY, S., URQUHART, J., GORNALL, H., BADER-MEUNIER, B., BASKAR, K., BASKAR, S., BAUDOUIN, V., BERESFORD, M. W., BLACK, G. C., DEARMAN, R. J., DE ZEGHER, F., FOSTER, E. S., FRANCES, C., HAYMAN, A. R., HILTON, E., JOB-DESLANDRE, C., KULKARNI, M. L., LE MERRER, M., LINGLART, A., LOVELL, S. C., MAURER, K., MUSSET, L., NAVARRO, V., PICARD, C., PUEL, A., RIEUX-LAUCAT, F., ROIFMAN, C. M., SCHOLL-BURGI, S., SMITH, N., SZYNKIEWICZ, M., WIEDEMAN, A., WOUTERS, C., ZEEF, L. A., CASANOVA, J. L., ELKON, K. B., JANCKILA, A., LEBON, P. & CROW, Y. J. 2011. Tartrate-resistant acid phosphatase deficiency causes a bone dysplasia with autoimmunity and a type I interferon expression signature. *Nat Genet,* 43**,** 127-31.

BRYAN, A. R. & WU, E. Y. 2014. Complement deficiencies in systemic lupus erythematosus. *Curr Allergy Asthma Rep,* 14**,** 448.

BRYANT, V. L., MA, C. S., AVERY, D. T., LI, Y., GOOD, K. L., CORCORAN, L. M., DE WAAL MALEFYT, R. & TANGYE, S. G. 2007. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol,* 179**,** 8180-90.

BUJAN, S., ORDI-ROS, J., PAREDES, J., MAURI, M., MATAS, L., CORTES, J. & VILARDELL, M. 2003. Contribution of the initial features of systemic lupus erythematosus to the clinical evolution and survival of a cohort of Mediterranean patients. *Ann Rheum Dis,* 62**,** 859-65.

BURGOS, P. I., MCGWIN, G., JR., PONS-ESTEL, G. J., REVEILLE, J. D., ALARCON, G. S. & VILA, L. M. 2011. US patients of Hispanic and African ancestry develop lupus nephritis early in

the disease course: data from LUMINA, a multiethnic US cohort (LUMINA LXXIV). *Ann Rheum Dis,* 70**,** 393-4.

BURNET, F. M. 1976. A modification of Jerne's theory of antibody production using the concept of clonal selection. *CA Cancer J Clin,* 26**,** 119-21.

BUTLER, N. S., MOEBIUS, J., PEWE, L. L., TRAORE, B., DOUMBO, O. K., TYGRETT, L. T., WALDSCHMIDT, T. J., CROMPTON, P. D. & HARTY, J. T. 2011. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage Plasmodium infection. *Nat Immunol,* 13**,** 188-95.

CADWELL, K., LIU, J. Y., BROWN, S. L., MIYOSHI, H., LOH, J., LENNERZ, J. K., KISHI, C., KC, W., CARRERO, J. A., HUNT, S., STONE, C. D., BRUNT, E. M., XAVIER, R. J., SLECKMAN, B. P., LI, E., MIZUSHIMA, N., STAPPENBECK, T. S. & VIRGIN, H. W. 2008. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature,* 456**,** 259-U62.

CADWELL, K., PATEL, K. K., MALONEY, N. S., LIU, T. C., NG, A. C. Y., STORER, C. E., HEAD, R. D., XAVIER, R., STAPPENBECK, T. S. & VIRGIN, H. W. 2010. Virus-Plus-Susceptibility Gene Interaction Determines Crohn's Disease Gene Atg16L1 Phenotypes in Intestine. *Cell,* 141**,** 1135-U64.

CALABRESE, F., BARALDO, S., BAZZAN, E., LUNARDI, F., REA, F., MAESTRELLI, P., TURATO, G., LOKAR-OLIANI, K., PAPI, A., ZUIN, R., SFRISO, P., BALESTRO, E., DINARELLO, C. A. & SAETTA, M. 2008. IL-32, a novel proinflammatory cytokine in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 178**,** 894-901.

CAMACHO-GARCIA, R. J., HERVAS, A., TOMA, C., BALMANA, N., CORMAND, B., MARTINEZ-MIR, A. & SCHOLL, F. G. 2013. Rare variants analysis of neurexin-1beta in autism reveals a novel start codon mutation affecting protein levels at synapses. *Psychiatr Genet,* 23**,** 262-6.

CARRERAS, J., LOPEZ-GUILLERMO, A., FOX, B. C., COLOMO, L., MARTINEZ, A., RONCADOR, G., MONTSERRAT, E., CAMPO, E. & BANHAM, A. H. 2006. High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. *Blood,* 108**,** 2957-2964.

CASELLAS, R., SHIH, T. A., KLEINEWIETFELD, M., RAKONJAC, J., NEMAZEE, D., RAJEWSKY, K. & NUSSENZWEIG, M. C. 2001. Contribution of receptor editing to the antibody repertoire. *Science,* 291**,** 1541-4.

CASTELA, E., LE DUFF, F., BUTORI, C., TICCHIONI, M., HOFMAN, P., BAHADORAN, P., LACOUR, J. P. & PASSERON, T. 2014. Effects of low-dose recombinant interleukin 2 to promote T-regulatory cells in alopecia areata. *JAMA Dermatol,* 150**,** 748-51.

CAZENAVE, P. L. A. E., SCHEDEL, H. E., BIETT, BURGESS, T. H. & BULKLEY, H. D. 1852. *Manual of diseases of the skin,* New York,, S. S. & W. Wood.

CECCARELLI, F., AGMON-LEVIN, N. & PERRICONE, C. 2016. Genetic Factors of Autoimmune Diseases. *J Immunol Res,* 2016**,** 3476023.

CENCIONI, M. T., MAGLIOZZI, R., NICHOLAS, R., ALI, R., MALIK, O., REYNOLDS, R., BORSELLINO, G., BATTISTINI, L. & MURARO, P. A. 2017. Programmed death 1 is highly expressed on CD8(+) CD57(+) T cells in patients with stable multiple sclerosis and inhibits their cytotoxic response to Epstein-Barr virus. *Immunology,* 152**,** 660-676.

CEROVEC, M., ANIC, B., PADJEN, I. & CIKES, N. 2012. Prevalence of the American College of Rheumatology classification criteria in a group of 162 systemic lupus erythematosus patients from Croatia. *Croat Med J,* 53**,** 149-54.

CHANG, P. P., BARRAL, P., FITCH, J., PRATAMA, A., MA, C. S., KALLIES, A., HOGAN, J. J., CERUNDOLO, V., TANGYE, S. G., BITTMAN, R., NUTT, S. L., BRINK, R., GODFREY, D. I., BATISTA, F. D. & VINUESA, C. G. 2011. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat Immunol,* 13**,** 35-43.

CHANG, Y. K., YANG, W., ZHAO, M., MOK, C. C., CHAN, T. M., WONG, R. W., LEE, K. W., MOK, M. Y., WONG, S. N., NG, I. O., LEE, T. L., HO, M. H., LEE, P. P., WONG, W. H., LAU, C. S., SHAM, P. C. & LAU, Y. L. 2009. Association of BANK1 and TNFSF4 with systemic lupus erythematosus in Hong Kong Chinese. *Genes Immun,* 10**,** 414-20.

CHAVELE, K. M. & EHRENSTEIN, M. R. 2011. Regulatory T-cells in systemic lupus erythematosus and rheumatoid arthritis. *Febs Letters,* 585**,** 3603-3610.

CHEN, C. & HUANG, J. A. 2016. New insights into the anti-PD-L1 and anti-PD-1 reagents in cancer therapy. *European Journal of Inflammation,* 14**,** 61-65.

CHENG, G., YU, A. & MALEK, T. R. 2011. T-cell tolerance and the multi-functional role of IL-2R signaling in T-regulatory cells. *Immunol Rev,* 241**,** 63-76.

CHIKUMA, S., TERAWAKI, S., HAYASHI, T., NABESHIMA, R., YOSHIDA, T., SHIBAYAMA, S., OKAZAKI, T. & HONJO, T. 2009. PD-1-mediated suppression of IL-2 production induces CD8+ T cell anergy in vivo. *J Immunol,* 182**,** 6682-9.

CHOI, J., BAE, S., HONG, J., RYOO, S., JHUN, H., HONG, K., YOON, D., LEE, S., HER, E., CHOI, W., KIM, J., AZAM, T., DINARELLO, C. A. & KIM, S. 2010. Paradoxical effects of constitutive human IL-32 gamma in transgenic mice during experimental colitis. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 21082-21086.

CHOI, J. D., BAE, S. Y., HONG, J. W., AZAM, T., DINARELLO, C. A., HER, E., CHOI, W. S., KIM, B. K., LEE, C. K., YOON, D. Y., KIM, S. J. & KIM, S. H. 2009a. Identification of the most active interleukin-32 isoform. *Immunology,* 126**,** 535-42.

CHOI, J. D., BAE, S. Y., HONG, J. W., AZAM, T., DINARELLO, C. A., HER, E., CHOI, W. S., KIM, B. K., LEE, C. K., YOON, D. Y., KIM, S. J. & KIM, S. H. 2009b. Identification of the most active interleukin-32 isoform. *Immunology,* 126**,** 535-542.

CHOI, J. Y., HO, J. H., PASOTO, S. G., BUNIN, V., KIM, S. T., CARRASCO, S., BORBA, E. F., GONCALVES, C. R., COSTA, P. R., KALLAS, E. G., BONFA, E. & CRAFT, J. 2015. Circulating follicular helper-like T cells in systemic lupus erythematosus: association with disease activity. *Arthritis Rheumatol,* 67**,** 988-99.

CHOW, C. W., RINCON, M. & DAVIS, R. J. 1999. Requirement for transcription factor NFAT in interleukin-2 expression. *Mol Cell Biol,* 19**,** 2300-7.

CHUNG, J. B., SILVERMAN, M. & MONROE, J. G. 2003. Transitional B cells: step by step towards immune competence. *Trends in Immunology,* 24**,** 343-349.

CHUNG, Y., TANAKA, S., CHU, F., NURIEVA, R. I., MARTINEZ, G. J., RAWAL, S., WANG, Y. H., LIM, H., REYNOLDS, J. M., ZHOU, X. H., FAN, H. M., LIU, Z. M., NEELAPU, S. S. & DONG, C. 2011a. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med,* 17**,** 983-8.

CHUNG, Y., TANAKA, S., CHU, F. L., NURIEVA, R. I., MARTINEZ, G. J., RAWAL, S., WANG, Y. H., LIM, H., REYNOLDS, J. M., ZHOU, X. H., FAN, H. M., LIU, Z. M., NEELAPU, S. S. & DONG, C. 2011b. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nature Medicine,* 17**,** 983-U102.

CLARK, R. A. 2015. Resident memory T cells in human health and disease. *Sci Transl Med,* 7**,** 269rv1.

CLEARY, A. M., FORTUNE, S. M., YELLIN, M. J., CHESS, L. & LEDERMAN, S. 1995. Opposing roles of CD95 (Fas/APO-1) and CD40 in the death and rescue of human low density tonsillar B cells. *J Immunol,* 155**,** 3329-37.

CONTI, P., KEMPURAJ, D., FRYDAS, S., KANDERE, K., BOUCHER, W., LETOURNEAU, R., MADHAPPAN, B., SAGIMOTO, K., CHRISTODOULOU, S. & THEOHARIDES, T. C. 2003a. IL-10 subfamily members: IL-19, IL-20, IL-22, IL-24 and IL-26. *Immunol Lett,* 88**,** 171-4.

CONTI, P., KEMPURAJ, D., KANDERE, K., DI GIOACCHINO, M., BARBACANE, R. C., CASTELLANI, M. L., FELACO, M., BOUCHER, W., LETOURNEAU, R. & THEOHARIDES, T. C. 2003b. IL-10, an inflammatory/inhibitory cytokine, but not always. *Immunol Lett,* 86**,** 123-9.

COOPER, G. M. & SHENDURE, J. 2011. Needles in stacks of needles: finding disease-causal variants in a wealth of genomic data. *Nature Reviews Genetics,* 12**,** 628-640.

CORRIONERO, A., RAKER, V. A., IZQUIERDO, J. M. & VALCARCEL, J. 2011. Strict 3' splice site sequence requirements for U2 snRNP recruitment after U2AF binding underlie a genetic defect leading to autoimmune disease. *RNA,* 17**,** 401-11.

CRISPIN, J. C., OUKKA, M., BAYLISS, G., COHEN, R. A., VAN BEEK, C. A., STILLMAN, I. E., KYTTARIS, V. C., JUANG, Y. T. & TSOKOS, G. C. 2008. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J Immunol,* 181**,** 8761-6.

CRISPIN, J. C. & TSOKOS, G. C. 2010. IL-17 in systemic lupus erythematosus. *J Biomed Biotechnol,* 2010**,** 943254.

CROTTY, S. 2011. Follicular Helper CD4 T Cells (T-FH). *Annual Review of Immunology, Vol 29,* 29**,** 621-663.

CROTTY, S. 2014. T follicular helper cell differentiation, function, and roles in disease. *Immunity,* 41**,** 529-42.

CUADRADO, M. J., MARUBAYASHI, M., ORTEGA, C., FERNANDEZ-ARCAS, N., GARCIA-COZAR, F., PENA, J., SANCHEZ-GUIJO, P. & SANTAMARIA, M. 1993. Relationship of IL-2, IL-2R (CD25+), soluble IL-2R and IL-4 with disease activity in SLE patients. *Lupus,* 2**,** 257-60.

CUI, Y. Y., SUN, Z. C., LI, X., LENG, C. S., ZHANG, L., FU, X. R., LI, L., ZHANG, X. D., CHANG, Y., NAN, F. F., LI, Z. M., YAN, J. Q., ZHANG, M. Z., LI, W. C., WANG, G. N., ZHANG, D. D. & MA, Y. Z. 2016. Expression and clinical significance of cyclooxygenase-2 and interleukin-32 in primary gastric B-cell lymphoma. *Oncology Letters,* 11**,** 693-698.

D'SOUZA, W. N. & LEFRANCOIS, L. 2003. IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion. *J Immunol,* 171**,** 5727-35.

D'SOUZA, W. N., SCHLUNS, K. S., MASOPUST, D. & LEFRANCOIS, L. 2002. Essential role for IL-2 in the regulation of antiviral extralymphoid CD8 T cell responses. *J Immunol,* 168**,** 5566-72.

DACA, A., CZUSZYNSKA, Z., SMOLENSKA, Z., ZDROJEWSKI, Z., WITKOWSKI, J. M. & BRYL, E. 2011. Two systemic lupus erythematosus (SLE) global disease activity indexes--the SLE Disease Activity Index and the Systemic Lupus Activity Measure--demonstrate different correlations with activation of peripheral blood CD4+ T cells. *Hum Immunol,* 72**,** 1160-7.

DAHL, C. A., SCHALL, R. P., HE, H. L. & CAIRNS, J. S. 1992. Identification of a novel gene expressed in activated natural killer cells and T cells. *J Immunol,* 148**,** 597-603.

DAMEN, M., DOS SANTOS, J. C., HERMSEN, R., ADAM VAN DER VLIET, J., NETEA, M. G., RIKSEN, N. P., DINARELLO, C. A., JOOSTEN, L. A. B. & HEINHUIS, B. 2018. Interleukin-32

upregulates the expression of ABCA1 and ABCG1 resulting in reduced intracellular lipid concentrations in primary human hepatocytes. *Atherosclerosis,* 271**,** 193-202.

DAMEN, M., POPA, C. D., NETEA, M. G., DINARELLO, C. A. & JOOSTEN, L. A. B. 2017. Interleukin-32 in chronic inflammatory conditions is associated with a higher risk of cardiovascular diseases. *Atherosclerosis,* 264**,** 83-91.

DANSKA, J. S., LIVINGSTONE, A. M., PARAGAS, V., ISHIHARA, T. & FATHMAN, C. G. 1990. The presumptive CDR3 regions of both T cell receptor alpha and beta chains determine T cell specificity for myoglobin peptides. *J Exp Med,* 172**,** 27-33.

DAVIS, L. S., HUTCHESON, J. & MOHAN, C. 2011. The role of cytokines in the pathogenesis and treatment of systemic lupus erythematosus. *J Interferon Cytokine Res,* 31**,** 781-9.

DE LA ROSA, M., RUTZ, S., DORNINGER, H. & SCHEFFOLD, A. 2004. Interleukin-2 is essential for CD4(+)CD25(+) regulatory T cell function. *European Journal of Immunology,* 34**,** 2480-2488.

DEJACO, C., DUFTNER, C., GRUBECK-LOEBENSTEIN, B. & SCHIRMER, M. 2006. Imbalance of regulatory T cells in human autoimmune diseases. *Immunology,* 117**,** 289-300.

DENG, Y. & TSAO, B. P. 2010. Genetic susceptibility to systemic lupus erythematosus in the genomic era. *Nature Reviews Rheumatology,* 6**,** 683-692.

DENG, Y., ZHAO, J., SAKURAI, D., SESTAK, A. L., OSADCHIY, V., LANGEFELD, C. D., KAUFMAN, K. M., KELLY, J. A., JAMES, J. A., PETRI, M. A., BAE, S. C., ALARCON-RIQUELME, M. E., ALARCON, G. S., ANAYA, J. M., CRISWELL, L. A., FREEDMAN, B. I., KAMEN, D. L., GILKESON, G. S., JACOB, C. O., MERRILL, J. T., GAFFNEY, P. M., SIVILS, K. M., NIEWOLD, T. B., RAMSEY-GOLDMAN, R., REVEILLE, J. D., SCOFIELD, R. H., STEVENS, A. M., BOACKLE, S. A., VILA, L. M., SOHN, II, LEE, S., CHANG, D. M., SONG, Y. W., VYSE, T. J., HARLEY, J. B., BROWN, E. E., EDBERG, J. C., KIMBERLY, R. P., CANTOR, R. M., HAHN, B. H., GROSSMAN, J. M. & TSAO, B. P. 2016. Decreased SMG7 expression associates with lupus-risk variants and elevated antinuclear antibody production. *Ann Rheum Dis,* 75**,** 2007-2013.

DI MASO, V., MEDIAVILLA, M. G., VASCOTTO, C., LUPO, F., BACCARANI, U., AVELLINI, C., TELL, G., TIRIBELLI, C. & CROCE, L. S. 2015. Transcriptional Up-Regulation of APE1/Ref-1 in Hepatic Tumor: Role in Hepatocytes Resistance to Oxidative Stress and Apoptosis. *Plos One,* 10.

DIAMOND, B., KATZ, J. B., PAUL, E., ARANOW, C., LUSTGARTEN, D. & SCHARFF, M. D. 1992. The role of somatic mutation in the pathogenic anti-DNA response. *Annu Rev Immunol,* 10**,** 731-57.

DOMEIER, P. P., SCHELL, S. L. & RAHMAN, Z. S. 2017. Spontaneous germinal centers and autoimmunity. *Autoimmunity,* 50**,** 4-18.

DOS SANTOS, J. C., HEINHUIS, B., GOMES, R. S., DAMEN, M. S., REAL, F., MORTARA, R. A., KEATING, S. T., DINARELLO, C. A., JOOSTEN, L. A. & RIBEIRO-DIAS, F. 2017. Cytokines and microbicidal molecules regulated by IL-32 in THP-1-derived human macrophages infected with New World Leishmania species. *PLoS Negl Trop Dis,* 11**,** e0005413.

DREES, C., VAHL, J. C., BORTOLUZZI, S., HEGER, K. D., FISCHER, J. C., WUNDERLICH, F. T., PESCHEL, C. & SCHMIDT-SUPPRIAN, M. 2017. Roquin Paralogs Differentially Regulate Functional NKT Cell Subsets. *J Immunol,* 198**,** 2747-2759.

DUCHOSAL, M. A., FUZZATI-ARMENTERO, M. T., BACCALA, R., LAYER, A., GONZALEZ-QUINTIAL, R., LETURCQ, D., RUEGG, M., TROUILLET, P., MAURAY, S., TISSOT, J. D. & SCHAPIRA, M. 2000. Human adult tonsil xenotransplantation into SCID mice for

studying human immune responses and B cell lymphomagenesis. *Experimental Hematology,* 28**,** 177-192.

DURE, M. & MACIAN, F. 2009. IL-2 signaling prevents T cell anergy by inhibiting the expression of anergy-inducing genes. *Mol Immunol,* 46**,** 999-1006.

DUSTIN, M. L. 2014. The immunological synapse. *Cancer Immunol Res,* 2**,** 1023-33.

ECKL-DORNA, J., PREE, I., REISINGER, J., MARTH, K., CHEN, K. W., VRTALA, S., SPITZAUER, S., VALENTA, R. & NIEDERBERGER, V. 2012. The majority of allergen-specific IgE in the blood of allergic patients does not originate from blood-derived B cells or plasma cells. *Clin Exp Allergy,* 42**,** 1347-55.

EFFROS, R. B. 2000. Long-term immunological memory against viruses. *Mechanisms of Ageing and Development,* 121**,** 161-171.

EL-FAR, M., KOUASSI, P., SYLLA, M., ZHANG, Y., FOUDA, A., FABRE, T., GOULET, J. P., VAN GREVENYNGHE, J., LEE, T., SINGER, J., HARRIS, M., BARIL, J. G., TROTTIER, B., ANCUTA, P., ROUTY, J. P., BERNARD, N., TREMBLAY, C. L. & INVESTIGATORS OF THE CANADIAN, H. I. V. S. P. C. 2016. Proinflammatory isoforms of IL-32 as novel and robust biomarkers for control failure in HIV-infected slow progressors. *Sci Rep,* 6**,** 22902.

ELLYARD, J. I., JERJEN, R., MARTIN, J. L., LEE, A. Y., FIELD, M. A., JIANG, S. H., CAPPELLO, J., NAUMANN, S. K., ANDREWS, T. D., SCOTT, H. S., CASAROTTO, M. G., GOODNOW, C. C., CHAITOW, J., PASCUAL, V., HERTZOG, P., ALEXANDER, S. I., COOK, M. C. & VINUESA, C. G. 2014. Identification of a pathogenic variant in TREX1 in early-onset cerebral systemic lupus erythematosus by Whole-exome sequencing. *Arthritis Rheumatol,* 66**,** 3382-6.

ELVIRA, G., MASSIE, B. & DESGROSEILLERS, L. 2006. The zinc-finger protein ZFR is critical for Staufen 2 isoform specific nucleocytoplasmic shuttling in neurons. *J Neurochem,* 96**,** 105-17.

ERTURK, K., TASTEKIN, D., SERILMEZ, M., BILGIN, E., BOZBEY, H. U. & VATANSEVER, S. 2016. Clinical significance of serum interleukin-29, interleukin-32, and tumor necrosis factor alpha levels in patients with gastric cancer. *Tumor Biology,* 37**,** 405-412.

ETTINGER, R., SIMS, G. P., FAIRHURST, A. M., ROBBINS, R., DA SILVA, Y. S., SPOLSKI, R., LEONARD, W. J. & LIPSKY, P. E. 2005. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J Immunol,* 175**,** 7867-79.

EZKURDIA, I., JUAN, D., RODRIGUEZ, J. M., FRANKISH, A., DIEKHANS, M., HARROW, J., VAZQUEZ, J., VALENCIA, A. & TRESS, M. L. 2014. Multiple evidence strands suggest that there may be as few as 19,000 human protein-coding genes. *Hum Mol Genet,* 23**,** 5866-78.

FALCONE, M. & SARVETNICK, N. 1999. Cytokines that regulate autoimmune responses. *Curr Opin Immunol,* 11**,** 670-6.

FARH, K. K., MARSON, A., ZHU, J., KLEINEWIETFELD, M., HOUSLEY, W. J., BEIK, S., SHORESH, N., WHITTON, H., RYAN, R. J., SHISHKIN, A. A., HATAN, M., CARRASCO-ALFONSO, M. J., MAYER, D., LUCKEY, C. J., PATSOPOULOS, N. A., DE JAGER, P. L., KUCHROO, V. K., EPSTEIN, C. B., DALY, M. J., HAFLER, D. A. & BERNSTEIN, B. E. 2015. Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature,* 518**,** 337-43.

FEINSTEIN, A., RICHARDSON, N. & TAUSSIG, M. I. 1986. Immunoglobulin flexibility in complement activation. *Immunol Today,* 7**,** 169-74.

FIELDS, M. L., HONDOWICZ, B. D., WHARTON, G. N., ADAIR, B. S., METZGAR, M. H., ALEXANDER, S. T., CATON, A. J. & ERIKSON, J. 2005. The regulation and activation of lupus-associated B cells. *Immunol Rev,* 204**,** 165-83.

FLANAGAN, J. M., COCCIARDI, S., WADDELL, N., JOHNSTONE, C. N., MARSH, A., HENDERSON, S., SIMPSON, P., DA SILVA, L., KCONFAB, I., KHANNA, K., LAKHANI, S., BOSHOFF, C. & CHENEVIX-TRENCH, G. 2010. DNA methylome of familial breast cancer identifies distinct profiles defined by mutation status. *Am J Hum Genet,* 86**,** 420-33.

FLORES-BORJA, F., BOSMA, A., NG, D., REDDY, V., EHRENSTEIN, M. R., ISENBERG, D. A. & MAURI, C. 2013. CD19(+)CD24(hi)CD38(hi) B Cells Maintain Regulatory T Cells While Limiting T(H)1 and T(H)17 Differentiation. *Science Translational Medicine,* 5.

FOCOSI, D., BESTAGNO, M., BURRONE, O. & PETRINI, M. 2010. CD57(+) T lymphocytes and functional immune deficiency (vol 87, pg 111, 2010). *Journal of Leukocyte Biology,* 87**,** 1167-1167.

FONSECA, V. R., AGUA-DOCE, A., MACEIRAS, A. R., PIERSON, W., RIBEIRO, F., ROMAO, V. C., PIRES, A. R., DA SILVA, S. L., FONSECA, J. E., SOUSA, A. E., LINTERMAN, M. A. & GRACA, L. 2017. Human blood T-fr cells are indicators of ongoing humoral activity not fully licensed with suppressive function. *Science Immunology,* 2.

FORSTER, R., SCHUBEL, A., BREITFELD, D., KREMMER, E., RENNER-MULLER, I., WOLF, E. & LIPP, M. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell,* 99**,** 23-33.

FRIERI, M. 2012. Accelerated atherosclerosis in systemic lupus erythematosus: role of proinflammatory cytokines and therapeutic approaches. *Curr Allergy Asthma Rep,* 12**,** 25-32.

FU, S. M., WINCHESTER, R. J. & KUNKEL, H. G. 1974. Occurrence of surface IgM, IgD, and free light chains of human lymphocytes. *J Exp Med,* 139**,** 451-6.

FU, W. W., LIU, X. D., LIN, X., FENG, H., SUN, L., LI, S. R., CHEN, H. R., TANG, H., LU, L. W., JIN, W. & DONG, C. 2018. Deficiency in T follicular regulatory cells promotes autoimmunity. *Journal of Experimental Medicine,* 215**,** 815-825.

FURIE, R., PETRI, M., ZAMANI, O., CERVERA, R., WALLACE, D. J., TEGZOVA, D., SANCHEZ-GUERRERO, J., SCHWARTING, A., MERRILL, J. T., CHATHAM, W. W., STOHL, W., GINZLER, E. M., HOUGH, D. R., ZHONG, Z. J., FREIMUTH, W., VAN VOLLENHOVEN, R. F. & GROUP, B.-S. 2011. A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. *Arthritis Rheum,* 63**,** 3918-30.

GALLEGO-VALLE, J., PEREZ-FERNANDEZ, V. A., CORREA-ROCHA, R. & PION, M. 2018. Generation of Human Breg-Like Phenotype with Regulatory Function In Vitro with Bacteria-Derived Oligodeoxynucleotides. *International Journal of Molecular Sciences,* 19.

GARRONE, P., NEIDHARDT, E. M., GARCIA, E., GALIBERT, L., VAN KOOTEN, C. & BANCHEREAU, J. 1995. Fas ligation induces apoptosis of CD40-activated human B lymphocytes. *J Exp Med,* 182**,** 1265-73.

GARSIDE, P., INGULLI, E., MERICA, R. R., JOHNSON, J. G., NOELLE, R. J. & JENKINS, M. K. 1998. Visualization of specific B and T lymphocyte interactions in the lymph node. *Science,* 281**,** 96-9.

GAUSS, G. H. & LIEBER, M. R. 1996. Mechanistic constraints on diversity in human V(D)J recombination. *Molecular and Cellular Biology,* 16**,** 258-269.

GAY, D., SAUNDERS, T., CAMPER, S. & WEIGERT, M. 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J Exp Med,* 177**,** 999-1008.

GHIRAN, I. C., ZEIDEL, M. L., SHEVKOPLYAS, S. S., BURNS, J. M., TSOKOS, G. C. & KYTTARIS, V. C. 2011. Systemic lupus erythematosus serum deposits C4d on red blood cells, decreases red blood cell membrane deformability, and promotes nitric oxide production. *Arthritis Rheum,* 63**,** 503-12.

GILLIS, S., BAKER, P. E., RUSCETTI, F. W. & SMITH, K. A. 1978. Long-term culture of human antigen-specific cytotoxic T-cell lines. *J Exp Med,* 148**,** 1093-8.

GILLIS, S. & SMITH, K. A. 1977. Long term culture of tumour-specific cytotoxic T cells. *Nature,* 268**,** 154-6.

GIRARD, S. L., DION, P. A., BOURASSA, C. V., GEOFFROY, S., LACHANCE-TOUCHETTE, P., BARHDADI, A., LANGLOIS, M., JOOBER, R., KREBS, M. O., DUBE, M. P. & ROULEAU, G. A. 2015. Mutation burden of rare variants in schizophrenia candidate genes. *PLoS One,* 10**,** e0128988.

GO, N. F., CASTLE, B. E., BARRETT, R., KASTELEIN, R., DANG, W., MOSMANN, T. R., MOORE, K. W. & HOWARD, M. 1990. Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells. *J Exp Med,* 172**,** 1625-31.

GODA, C., KANAJI, T., KANAJI, S., TANAKA, G., ARIMA, K., OHNO, S. & IZUHARA, K. 2006a. Involvement of IL-32 in activation-induced cell death in T cells. *International Immunology,* 18**,** 233-240.

GODA, C., KANAJI, T., KANAJI, S., TANAKA, G., ARIMA, K., OHNO, S. & IZUHARA, K. 2006b. Involvement of IL-32 in activation-induced cell death in T cells. *Int Immunol,* 18**,** 233-40.

GOENKA, R., BARNETT, L. G., SILVER, J. S., O'NEILL, P. J., HUNTER, C. A., CANCRO, M. P. & LAUFER, T. M. 2011. Cutting Edge: Dendritic Cell-Restricted Antigen Presentation Initiates the Follicular Helper T Cell Program but Cannot Complete Ultimate Effector Differentiation. *Journal of Immunology,* 187**,** 1091-1095.

GOLDRATH, A. W. & BEVAN, M. J. 1999. Selecting and maintaining a diverse T-cell repertoire. *Nature,* 402**,** 255-62.

GOLDSTEIN, M. D., DEBENEDETTE, M. A., HOLLENBAUGH, D. & WATTS, T. H. 1996. Induction of costimulatory molecules B7-1 and B7-2 in murine B cells. the CBA/N mouse reveals a role for Bruton's tyrosine kinase in CD40-mediated B7 induction. *Mol Immunol,* 33**,** 541-52.

GONSKY, R., DEEM, R. L., HUGHES, C. C. W. & TARGAN, S. R. 1998. Activation of the CD2 pathway in lamina propria T cells up-regulates functionally active AP-1 binding to the IL-2 promoter, resulting in messenger RNA transcription and IL-2 secretion. *Journal of Immunology,* 160**,** 4914-4922.

GOOD-JACOBSON, K. L. & SHLOMCHIK, M. J. 2010. Plasticity and Heterogeneity in the Generation of Memory B Cells and Long-Lived Plasma Cells: The Influence of Germinal Center Interactions and Dynamics. *Journal of Immunology,* 185**,** 3117-3125.

GOOD-JACOBSON, K. L., SZUMILAS, C. G., CHEN, L., SHARPE, A. H., TOMAYKO, M. M. & SHLOMCHIK, M. J. 2010a. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol,* 11**,** 535-42.

GOOD-JACOBSON, K. L., SZUMILAS, C. G., CHEN, L. P., SHARPE, A. H., TOMAYKO, M. M. & SHLOMCHIK, M. J. 2010b. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nature Immunology,* 11**,** 535-U107.

GOODNOW, C. C. & BASTEN, A. 1989. Self-tolerance in B lymphocytes. *Semin Immunol,* 1**,** 125-35.

GOODNOW, C. C., CROSBIE, J., JORGENSEN, H., BRINK, R. A. & BASTEN, A. 1989. Induction of self-tolerance in mature peripheral B lymphocytes. *Nature,* 342**,** 385-91.

GOODNOW, C. C., SPRENT, J., FAZEKAS DE ST GROTH, B. & VINUESA, C. G. 2005. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature,* 435**,** 590-7.

GRAMMER, A. C. & LIPSKY, P. E. 2003. B cell abnormalities in systemic lupus erythematosus. *Arthritis Research & Therapy,* 5**,** S22-S27.

GRAVANO, D. M. & HOYER, K. K. 2013. Promotion and prevention of autoimmune disease by CD8+ T cells. *J Autoimmun,* 45**,** 68-79.

GRIMM, W. A., MESSER, J. S., MURPHY, S. F., NERO, T., LODOLCE, J. P., WEBER, C. R., LOGSDON, M. F., BARTULIS, S., SYLVESTER, B. E., SPRINGER, A., DOUGHERTY, U., NIEWOLD, T. B., KUPFER, S. S., ELLIS, N., HUO, D., BISSONNETTE, M. & BOONE, D. L. 2016. The Thr300Ala variant in ATG16L1 is associated with improved survival in human colorectal cancer and enhanced production of type I interferon. *Gut,* 65**,** 456-64.

GRINDEL, A., GUGGENBERGER, B., EICHBERGER, L., POPPELMEYER, C., GSCHAIDER, M., TOSEVSKA, A., MARE, G., BRISKEY, D., BRATH, H. & WAGNER, K. H. 2016. Oxidative Stress, DNA Damage and DNA Repair in Female Patients with Diabetes Mellitus Type 2. *Plos One,* 11.

GU, T., RAO, Y., SU, J., YANG, C., CHEN, X., CHEN, L. & YAN, N. 2015. Functions of MDA5 and its domains in response to GCRV or bacterial PAMPs. *Fish Shellfish Immunol,* 46**,** 693-702.

GUAN, H. G., WAN, Y. Q., LAN, J., WANG, Q., WANG, Z. Y., LI, Y. C., ZHENG, J. Q., ZHANG, X. G., WANG, Z. M., SHEN, Y. P. & XIE, F. 2016. PD-L1 is a critical mediator of regulatory B cells and T cells in invasive breast cancer. *Scientific Reports,* 6.

GUENIN, S., MOUALLIF, M., HUBERT, P., JACOBS, N., KRUSY, N., DURAY, A., ENNAJI, M. M., SAUSSEZ, S. & DELVENNE, P. 2014. Interleukin-32 Expression Is Associated With a Poorer Prognosis in Head and Neck Squamous Cell Carcinoma. *Molecular Carcinogenesis,* 53**,** 667-673.

GUNTHER, C., MEURER, M., STEIN, A., VIEHWEG, A. & LEE-KIRSCH, M. A. 2009. Familial chilblain lupus--a monogenic form of cutaneous lupus erythematosus due to a heterozygous mutation in TREX1. *Dermatology,* 219**,** 162-6.

HAMEL, K. M., CAO, Y., WANG, Y., RODEGHERO, R., KOBEZDA, T., CHEN, L. & FINNEGAN, A. 2010a. B7-H1 expression on non-B and non-T cells promotes distinct effects on T- and B-cell responses in autoimmune arthritis. *Eur J Immunol,* 40**,** 3117-27.

HAMEL, K. M., CAO, Y. X., WANG, Y. M., RODEGHERO, R., KOBEZDA, T., CHEN, L. P. & FINNEGAN, A. 2010b. B7-H1 expression on non-B and non-T cells promotes distinct effects on T- and B-cell responses in autoimmune arthritis. *European Journal of Immunology,* 40**,** 3117-3127.

HAMS, E., MCCARRON, M. J., AMU, S., YAGITA, H., AZUMA, M., CHEN, L. & FALLON, P. G. 2011a. Blockade of B7-H1 (programmed death ligand 1) enhances humoral immunity by positively regulating the generation of T follicular helper cells. *J Immunol,* 186**,** 5648-55.

HAMS, E., MCCARRON, M. J., AMU, S., YAGITA, H., AZUMA, M., CHEN, L. P. & FALLON, P. G. 2011b. Blockade of B7-H1 (Programmed Death Ligand 1) Enhances Humoral Immunity by Positively Regulating the Generation of T Follicular Helper Cells. *Journal of Immunology,* 186**,** 5648-5655.

HAN, S., FENG, S. Z., REN, M. L., MA, E. L., WANG, X. N., XU, L. S. & XU, M. H. 2014. Glioma cell-derived placental growth factor induces regulatory B cells. *International Journal of Biochemistry & Cell Biology,* 57**,** 63-68.

HAO, Q. L., GEORGE, A. A., ZHU, J., BARSKY, L., ZIELINSKA, E., WANG, X., PRICE, M., GE, S. & CROOKS, G. M. 2008. Human intrathymic lineage commitment is marked by differential CD7 expression: identification of CD7- lympho-myeloid thymic progenitors. *Blood,* 111**,** 1318-26.

HAQUE, N., OUDA, R., CHEN, C., OZATO, K. & HOGG, J. R. 2018. ZFR coordinates crosstalk between RNA decay and transcription in innate immunity. *Nat Commun,* 9**,** 1145.

HARTEMANN, A., BENSIMON, G., PAYAN, C. A., JACQUEMINET, S., BOURRON, O., NICOLAS, N., FONFREDE, M., ROSENZWAJG, M., BERNARD, C. & KLATZMANN, D. 2013. Low-dose interleukin 2 in patients with type 1 diabetes: a phase 1/2 randomised, double-blind, placebo-controlled trial. *Lancet Diabetes Endocrinol,* 1**,** 295-305.

HAYASHI, S., HAMADA, T., ITOI, K. & KADOWAKI, M. 2016. PI3 Kinase p85 alpha Subunit-Deficient Macrophages Suppress the Development of DSS-Induced Acute Colitis Due to the Enhancement of IL-10 Production. *Gastroenterology,* 150**,** S964-S964.

HAYNES, N. M., ALLEN, C. D., LESLEY, R., ANSEL, K. M., KILLEEN, N. & CYSTER, J. G. 2007. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. *J Immunol,* 179**,** 5099-108.

HE, J., ZHANG, X., WEI, Y., SUN, X., CHEN, Y., DENG, J., JIN, Y., GAN, Y., HU, X., JIA, R., XU, C., HOU, Z., LEONG, Y. A., ZHU, L., FENG, J., AN, Y., JIA, Y., LI, C., LIU, X., YE, H., REN, L., LI, R., YAO, H., LI, Y., CHEN, S., ZHANG, X., SU, Y., GUO, J., SHEN, N., MORAND, E. F., YU, D. & LI, Z. 2016. Low-dose interleukin-2 treatment selectively modulates CD4(+) T cell subsets in patients with systemic lupus erythematosus. *Nat Med,* 22**,** 991-3.

HEATH, W. R. & CARBONE, F. R. 2001. Cross-presentation in viral immunity and self-tolerance. *Nat Rev Immunol,* 1**,** 126-34.

HEINHUIS, B., KOENDERS, M. I., VAN DE LOO, F. A., NETEA, M. G., VAN DEN BERG, W. B. & JOOSTEN, L. A. 2011a. Inflammation-dependent secretion and splicing of IL-32{gamma} in rheumatoid arthritis. *Proc Natl Acad Sci U S A,* 108**,** 4962-7.

HEINHUIS, B., KOENDERS, M. I., VAN DEN BERG, W. B., NETEA, M. G., DINARELLO, C. A. & JOOSTEN, L. A. 2012. Interleukin 32 (IL-32) contains a typical alpha-helix bundle structure that resembles focal adhesion targeting region of focal adhesion kinase-1. *J Biol Chem,* 287**,** 5733-43.

HEINHUIS, B., KOENDERS, M. I., VAN RIEL, P. L., VAN DE LOO, F. A., DINARELLO, C. A., NETEA, M. G., VAN DEN BERG, W. B. & JOOSTEN, L. A. 2011b. Tumour necrosis factor alpha-driven IL-32 expression in rheumatoid arthritis synovial tissue amplifies an inflammatory cascade. *Ann Rheum Dis,* 70**,** 660-7.

HEINHUIS, B., PLANTINGA, T. S., SEMANGO, G., KUSTERS, B., NETEA, M. G., DINARELLO, C. A., SMIT, J. W., NETEA-MAIER, R. T. & JOOSTEN, L. A. 2016a. Alternatively spliced isoforms of IL-32 differentially influence cell death pathways in cancer cell lines. *Carcinogenesis,* 37**,** 197-205.

HEINHUIS, B., PLANTINGA, T. S., SEMANGO, G., KUSTERS, B., NETEA, M. G., DINARELLO, C. A., SMIT, J. W. A., NETEA-MAIER, R. T. & JOOSTEN, L. A. B. 2016b. Alternatively spliced isoforms of IL-32 differentially influence cell death pathways in cancer cell lines. *Carcinogenesis,* 37**,** 197-205.

HEISSMEYER, V. & VOGEL, K. U. 2013. Molecular control of Tfh-cell differentiation by Roquin family proteins. *Immunol Rev,* 253**,** 273-89.

HERVIER, B., BEZIAT, V., HAROCHE, J., MATHIAN, A., LEBON, P., GHILLANI-DALBIN, P., MUSSET, L., DEBRE, P., AMOURA, Z. & VIEILLARD, V. 2011. Phenotype and function of natural killer cells in systemic lupus erythematosus: excess interferon-gamma production in patients with active disease. *Arthritis Rheum,* 63**,** 1698-706.

HOM, G., GRAHAM, R. R., MODREK, B., TAYLOR, K. E., ORTMANN, W., GARNIER, S., LEE, A. T., CHUNG, S. A., FERREIRA, R. C., PANT, P. V., BALLINGER, D. G., KOSOY, R., DEMIRCI, F. Y., KAMBOH, M. I., KAO, A. H., TIAN, C., GUNNARSSON, I., BENGTSSON, A. A., RANTAPAA-DAHLQVIST, S., PETRI, M., MANZI, S., SELDIN, M. F., RONNBLOM, L., SYVANEN, A. C., CRISWELL, L. A., GREGERSEN, P. K. & BEHRENS, T. W. 2008a. Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. *N Engl J Med,* 358**,** 900-9.

HOM, G., GRAHAM, R. R., MODREK, B., TAYLOR, K. E., ORTMANN, W., GARNIER, S., LEE, A. T., CHUNG, S. A., FERREIRA, R. C., PANT, P. V. K., BALLINGER, D. G., KOSOY, R., DEMIRCI, F. Y., KAMBOH, M. I., KAO, A. H., TIAN, C., GUNNARSSON, I., BENGTSSON, A. A., RANTAPAA-DAHLQVIST, S., PETRI, M., MANZI, S., SELDIN, M. F., RONNBLOM, L., SYVANEN, A. C., CRISWELL, L. A., GREGERSEN, P. K. & BEHRENS, T. W. 2008b. Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. *New England Journal of Medicine,* 358**,** 900-909.

HONDA, K., YANAI, H., NEGISHI, H., ASAGIRI, M., SATO, M., MIZUTANI, T., SHIMADA, N., OHBA, Y., TAKAOKA, A., YOSHIDA, N. & TANIGUCHI, T. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature,* 434**,** 772-7.

HONG, J. T., SON, D. J., LEE, C. K., YOON, D. Y., LEE, D. H. & PARK, M. H. 2017. Interleukin 32, inflammation and cancer. *Pharmacol Ther,* 174**,** 127-137.

HSU, D. C. & KATELARIS, C. H. 2009. Long-term management of patients taking immunosuppressive drugs. *Australian Prescriber,* 32**,** 68-71.

HUANG, S., DEERINCK, T. J., ELLISMAN, M. H. & SPECTOR, D. L. 1997. The dynamic organization of the perinucleolar compartment in the cell nucleus. *J Cell Biol,* 137**,** 965-74.

HUMRICH, J. Y. & RIEMEKASTEN, G. 2016a. Clinical trials: The rise of IL-2 therapy - a novel biologic treatment for SLE. *Nat Rev Rheumatol,* 12**,** 695-696.

HUMRICH, J. Y. & RIEMEKASTEN, G. 2016b. [IL-2-Therapy in SLE- Selective reconstitution of immunological tolerance]. *Z Rheumatol,* 75**,** 490-2.

HUMRICH, J. Y. & RIEMEKASTEN, G. 2016c. Restoring regulation - IL-2 therapy in systemic lupus erythematosus. *Expert Rev Clin Immunol,* 12**,** 1153-1160.

HUNT, K. A., MISTRY, V., BOCKETT, N. A., AHMAD, T., BAN, M., BARKER, J. N., BARRETT, J. C., BLACKBURN, H., BRAND, O., BURREN, O., CAPON, F., COMPSTON, A., GOUGH, S. C. L., JOSTINS, L., KONG, Y., LEE, J. C., LEK, M., MACARTHUR, D. G., MANSFIELD, J. C., MATHEW, C. G., MEIN, C. A., MIRZA, M., NUTLAND, S., ONENGUT-GUMUSCU, S., PAPOULI, E., PARKES, M., RICH, S. S., SAWCER, S., SATSANGI, J., SIMMONDS, M. J., TREMBATH, R. C., WALKER, N. M., WOZNIAK, E., TODD, J. A., SIMPSON, M. A.,

PLAGNOL, V. & VAN HEEL, D. A. 2013. Negligible impact of rare autoimmune-locus coding-region variants on missing heritability. *Nature,* 498**,** 232-+.

HUSE, M., LILLEMEIER, B. F., KUHNS, M. S., CHEN, D. S. & DAVIS, M. M. 2006. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol,* 7**,** 247-55.

INOUE, M., SHODA, H., SERI, Y., KUBO, K., KANDA, H., FUJIO, K. & YAMAMOTO, K. 2014. Three cases of lupus nephritis patients with serum interleukin-32gamma detection. *Lupus,* 23**,** 1187-91.

INTERNATIONAL CONSORTIUM FOR SYSTEMIC LUPUS ERYTHEMATOSUS, G., HARLEY, J. B., ALARCON-RIQUELME, M. E., CRISWELL, L. A., JACOB, C. O., KIMBERLY, R. P., MOSER, K. L., TSAO, B. P., VYSE, T. J., LANGEFELD, C. D., NATH, S. K., GUTHRIDGE, J. M., COBB, B. L., MIREL, D. B., MARION, M. C., WILLIAMS, A. H., DIVERS, J., WANG, W., FRANK, S. G., NAMJOU, B., GABRIEL, S. B., LEE, A. T., GREGERSEN, P. K., BEHRENS, T. W., TAYLOR, K. E., FERNANDO, M., ZIDOVETZKI, R., GAFFNEY, P. M., EDBERG, J. C., RIOUX, J. D., OJWANG, J. O., JAMES, J. A., MERRILL, J. T., GILKESON, G. S., SELDIN, M. F., YIN, H., BAECHLER, E. C., LI, Q. Z., WAKELAND, E. K., BRUNER, G. R., KAUFMAN, K. M. & KELLY, J. A. 2008. Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. *Nat Genet,* 40**,** 204-10.

ISAKOV, N. 1997. Immunoreceptor tyrosine-based activation motif (ITAM), a unique module linking antigen and Fc receptors to their signaling cascades. *J Leukoc Biol,* 61**,** 6-16.

ISHIGAMI, S., ARIGAMI, T., UCHIKADO, Y., SETOYAMA, T., KITA, Y., SASAKI, K., OKUMURA, H., KURAHARA, H., KIJIMA, Y., HARADA, A., UENO, S. & NATSUGOE, S. 2013. IL-32 expression is an independent prognostic marker for gastric cancer. *Medical Oncology,* 30.

IVANOV, P. & ANDERSON, P. 2013. Post-transcriptional regulatory networks in immunity. *Immunol Rev,* 253**,** 253-72.

IWAI, Y., ISHIDA, M., TANAKA, Y., OKAZAKI, T., HONJO, T. & MINATO, N. 2002. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 12293-12297.

IWASAKI, A. & MEDZHITOV, R. 2015. Control of adaptive immunity by the innate immune system. *Nat Immunol,* 16**,** 343-53.

IWATA, Y., FURUICHI, K., KANEKO, S. & WADA, T. 2011. The role of cytokine in the lupus nephritis. *J Biomed Biotechnol,* 2011**,** 594809.

JACOBI, A. M. & DIAMOND, B. 2005. Balancing diversity and tolerance: lessons from patients with systemic lupus erythematosus. *J Exp Med,* 202**,** 341-4.

JACOBS, P. A., BROWNE, C., GREGSON, N., JOYCE, C. & WHITE, H. 1992. Estimates of the frequency of chromosome abnormalities detectable in unselected newborns using moderate levels of banding. *J Med Genet,* 29**,** 103-8.

JAIN, A. & PASARE, C. 2017. Innate Control of Adaptive Immunity: Beyond the Three-Signal Paradigm. *J Immunol,* 198**,** 3791-3800.

JAIN, J., VALGEARCHER, V. E. & RAO, A. 1992. Analysis of the Ap-1 Sites in the Il-2 Promoter. *Journal of Immunology,* 148**,** 1240-1250.

JANEWAY, C. A., JR. & MEDZHITOV, R. 2002. Innate immune recognition. *Annu Rev Immunol,* 20**,** 197-216.

JARUKITSOPA, S., HOGANSON, D. D., CROWSON, C. S., SOKUMBI, O., DAVIS, M. D., MICHET, C. J., JR., MATTESON, E. L., MARADIT KREMERS, H. & CHOWDHARY, V. R. 2015. Epidemiology of systemic lupus erythematosus and cutaneous lupus erythematosus in a predominantly white population in the United States. *Arthritis Care Res (Hoboken),* 67**,** 817-28.

JAYACHANDRAN, U., GREY, H. & COOK, A. G. 2016. Nuclear factor 90 uses an ADAR2-like binding mode to recognize specific bases in dsRNA. *Nucleic Acids Res,* 44**,** 1924-36.

JIANG, S. H., SHEN, N. & VINUESA, C. G. 2015. Posttranscriptional T cell gene regulation to limit Tfh cells and autoimmunity. *Curr Opin Immunol,* 37**,** 21-27.

JO, Y. S., SONG, S. Y., KIM, M. S., YOO, N. J. & LEE, S. H. 2017. Frameshift Mutations of SMG7 Essential for Nonsense-Mediated mRNA Decay in Gastric and Colorectal Cancers. *Pathol Oncol Res,* 23**,** 221-222.

JOHNSON, B. J., COSTELLOE, E. O., FITZPATRICK, D. R., HAANEN, J. B., SCHUMACHER, T. N., BROWN, L. E. & KELSO, A. 2003. Single-cell perforin and granzyme expression reveals the anatomical localization of effector CD8+ T cells in influenza virus-infected mice. *Proc Natl Acad Sci U S A,* 100**,** 2657-62.

JONES, B. M., LIU, T. & WONG, R. W. 1999. Reduced in vitro production of interferon-gamma, interleukin-4 and interleukin-12 and increased production of interleukin-6, interleukin-10 and tumour necrosis factor-alpha in systemic lupus erythematosus. Weak correlations of cytokine production with disease activity. *Autoimmunity,* 31**,** 117-24.

JONSSON, C. A. & CARLSTEN, H. 2002. Mycophenolic acid inhibits inosine 5'-monophosphate dehydrogenase and suppresses production of pro-inflammatory cytokines, nitric oxide, and LDH in macrophages. *Cell Immunol,* 216**,** 93-101.

JOOSTEN, L. A., HEINHUIS, B., NETEA, M. G. & DINARELLO, C. A. 2013. Novel insights into the biology of interleukin-32. *Cell Mol Life Sci,* 70**,** 3883-92.

JOOSTEN, L. A., NETEA, M. G., KIM, S. H., YOON, D. Y., OPPERS-WALGREEN, B., RADSTAKE, T. R., BARRERA, P., VAN DE LOO, F. A., DINARELLO, C. A. & VAN DEN BERG, W. B. 2006. IL-32, a proinflammatory cytokine in rheumatoid arthritis. *Proc Natl Acad Sci U S A,* 103**,** 3298-303.

JUNEJA, V. R., MCGUIRE, K. A., MANGUSO, R. T., LAFLEUR, M. W., COLLINS, N., HAINING, W. N., FREEMAN, G. J. & SHARPE, A. H. 2017. PD-L1 on tumor cells is sufficient for immune evasion in immunogenic tumors and inhibits CD8 T cell cytotoxicity. *Journal of Experimental Medicine,* 214**,** 895-904.

KAFASLA, P., SKLIRIS, A. & KONTOYIANNIS, D. L. 2014. Post-transcriptional coordination of immunological responses by RNA-binding proteins. *Nat Immunol,* 15**,** 492-502.

KAIKO, G. E., HORVAT, J. C., BEAGLEY, K. W. & HANSBRO, P. M. 2008. Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology,* 123**,** 326-38.

KALIA, V. & SARKAR, S. 2018. Regulation of Effector and Memory CD8 T Cell Differentiation by IL-2-A Balancing Act. *Front Immunol,* 9**,** 2987.

KALLED, S. L. 2005. The role of BAFF in immune function and implications for autoimmunity. *Immunol Rev,* 204**,** 43-54.

KANEKO, Y., HIROSE, S., ABE, M., YAGITA, H., OKUMURA, K. & SHIRAI, T. 1996. CD40-mediated stimulation of B1 and B2 cells: implication in autoantibody production in murine lupus. *Eur J Immunol,* 26**,** 3061-5.

KANG, J. W., CHOI, S. C., CHO, M. C., KIM, H. J., KIM, J. H., LIM, J. S., KIM, S. H., HAN, J. Y. & YOON, D. Y. 2009. A proinflammatory cytokine interleukin-32beta promotes the production of an anti-inflammatory cytokine interleukin-10. *Immunology,* 128**,** e532-40.

KANG, J. W., PARK, Y. S., KIM, M. S., LEE, D. H., BAK, Y., HAM, S. Y., PARK, S. H., HONG, J. T. & YOON, D. Y. 2013a. Interleukin (IL)-32beta-mediated CCAAT/enhancer-binding protein alpha (C/EBPalpha) phosphorylation by protein kinase Cdelta (PKCdelta) abrogates the inhibitory effect of C/EBPalpha on IL-10 production. *J Biol Chem,* 288**,** 23650-8.

KANG, J. W., PARK, Y. S., LEE, D. H., KIM, J. H., KIM, M. S., BAK, Y., HONG, J. & YOON, D. Y. 2012a. Intracellular interaction of interleukin (IL)-32alpha with protein kinase Cepsilon (PKCepsilon) and STAT3 protein augments IL-6 production in THP-1 promonocytic cells. *J Biol Chem,* 287**,** 35556-64.

KANG, J. W., PARK, Y. S., LEE, D. H., KIM, M. S., BAK, Y., HAM, S. Y., PARK, S. H., KIM, H., AHN, J. H., HONG, J. T. & YOON, D. Y. 2014a. Interaction network mapping among IL-32 isoforms. *Biochimie,* 101**,** 248-251.

KANG, J. W., PARK, Y. S., LEE, D. H., KIM, M. S., BAK, Y., HAM, S. Y., PARK, S. H., KIM, H., AHN, J. H., HONG, J. T. & YOON, D. Y. 2014b. Interaction network mapping among IL-32 isoforms. *Biochimie,* 101**,** 248-51.

KANG, J. W., PARK, Y. S., LEE, D. H., KIM, M. S., BAK, Y., PARK, S. H., HAM, S. Y., YANG, Y., HONG, J. T. & YOON, D. Y. 2013b. Interleukin-32delta interacts with IL-32beta and inhibits IL-32beta-mediated IL-10 production. *FEBS Lett*.

KANG, Y. H., PARK, M. Y., YOON, D. Y., HAN, S. R., LEE, C. I., JI, N. Y., MYUNG, P. K., LEE, H. G., KIM, J. W., YEOM, Y. I., JANG, Y. J., AHN, D. K., KIM, J. W. & SONG, E. Y. 2012b. Dysregulation of overexpressed IL-32 alpha in hepatocellular carcinoma suppresses cell growth and induces apoptosis through inactivation of NF-kappa B and Bcl-2. *Cancer Letters,* 318**,** 226-233.

KANG, Y. H., PARK, M. Y., YOON, D. Y., HAN, S. R., LEE, C. I., JI, N. Y., MYUNG, P. K., LEE, H. G., KIM, J. W., YEOM, Y. I., JANG, Y. J., AHN, D. K., KIM, J. W. & SONG, E. Y. 2012c. Dysregulation of overexpressed IL-32alpha in hepatocellular carcinoma suppresses cell growth and induces apoptosis through inactivation of NF-kappaB and Bcl-2. *Cancer Lett,* 318**,** 226-33.

KAO, P. N., CHEN, L., BROCK, G., NG, J., KENNY, J., SMITH, A. J. & CORTHESY, B. 1994. Cloning and Expression of Cyclosporine-a-Sensitive and Fk506-Sensitive Nuclear Factor of Activated T-Cells - Nf45 and Nf90. *Journal of Biological Chemistry,* 269**,** 20691-20699.

KARAGEORGAS, T. P., TSERONIS, D. D. & MAVRAGANI, C. P. 2011. Activation of type I interferon pathway in systemic lupus erythematosus: association with distinct clinical phenotypes. *J Biomed Biotechnol,* 2011**,** 273907.

KARNEKURA, R., SHIGEHARA, K., MIYAJIMA, S., JITSUKAWA, S., KAWATA, K., YAMASHITA, K., NAGAYA, T., KUMAGAI, A., SATO, A., MATSUMIYA, H., OGASAWARA, N., SEKI, N., TAKANO, K., KOKAI, Y., TAKAHASHI, H., HIMI, T. & ICHIMIYA, S. 2015. Alteration of circulating type 2 follicular helper T cells and regulatory B cells underlies the comorbid association of allergic rhinitis with bronchial asthma. *Clinical Immunology,* 158**,** 204-211.

KAROUSIS, E. D., NASIF, S. & MUHLEMANN, O. 2016. Nonsense-mediated mRNA decay: novel mechanistic insights and biological impact. *Wiley Interdiscip Rev RNA,* 7**,** 661-82.

KASAIAN, M. T., WHITTERS, M. J., CARTER, L. L., LOWE, L. D., JUSSIF, J. M., DENG, B., JOHNSON, K. A., WITEK, J. S., SENICES, M., KONZ, R. F., WURSTER, A. L., DONALDSON, D. D., COLLINS, M., YOUNG, D. A. & GRUSBY, M. J. 2002. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. *Immunity,* 16**,** 559-69.

KASLOW, R. A. 1982. High rate of death caused by systemic lupus erythematosus among U. S. residents of Asian descent. *Arthritis Rheum,* 25**,** 414-8.

KATO, H. & FUJITA, T. 2015. RIG-I-like receptors and autoimmune diseases. *Curr Opin Immunol,* 37**,** 40-5.

KAUL, A., GORDON, C., CROW, M. K., TOUMA, Z., UROWITZ, M. B., VAN VOLLENHOVEN, R., RUIZ-IRASTORZA, G. & HUGHES, G. 2016. Systemic lupus erythematosus. *Nature Reviews Disease Primers,* 2.

KAWAMOTO, S., TRAN, T. H., MARUYA, M., SUZUKI, K., DOI, Y., TSUTSUI, Y., KATO, L. M. & FAGARASAN, S. 2012. The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. *Science,* 336**,** 485-9.

KAWAMURA, S., SAITOU, N. & UEDA, S. 1992. Concerted evolution of the primate immunoglobulin alpha-gene through gene conversion. *J Biol Chem,* 267**,** 7359-67.

KEARNEY, J. F., COOPER, M. D., KLEIN, J., ABNEY, E. R., PARKHOUSE, R. M. & LAWTON, A. R. 1977. Ontogeny of Ia and IgD on IgM-bearing B lymphocytes in mice. *J Exp Med,* 146**,** 297-301.

KELLY, E., WON, A., REFAELI, Y. & VAN PARIJS, L. 2002. IL-2 and related cytokines can promote T cell survival by activating AKT. *J Immunol,* 168**,** 597-603.

KERFOOT, S. M., YAARI, G., PATEL, J. R., JOHNSON, K. L., GONZALEZ, D. G., KLEINSTEIN, S. H. & HABERMAN, A. M. 2011. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity,* 34**,** 947-60.

KESWANI, A., CHUSTZ, R. T., SUH, L., CARTER, R., PETERS, A. T., TAN, B. K., CHANDRA, R., KIM, S. H., AZAM, T., DINARELLO, C. A., KERN, R. C., SCHLEIMER, R. P. & KATO, A. 2012. Differential expression of interleukin-32 in chronic rhinosinusitis with and without nasal polyps. *Allergy,* 67**,** 25-32.

KHAN, A. R., HAMS, E., FLOUDAS, A., SPARWASSER, T., WEAVER, C. T. & FALLON, P. G. 2015. PD-L1(hi) B cells are critical regulators of humoral immunity. *Nature Communications,* 6.

KHAN, A. R., HAMS, E., SPARWASSER, T., WEAVER, C. T. & FALLON, P. G. 2014. PD-L1(hi) B regulatory cells are critical for control of the humoral immune response. *Cytokine,* 70**,** 50-51.

KHAWAR, M. B., ABBASI, M. H. & SHEIKH, N. 2016. IL-32: A Novel Pluripotent Inflammatory Interleukin, towards Gastric Inflammation, Gastric Cancer, and Chronic Rhino Sinusitis. *Mediators Inflamm,* 2016**,** 8413768.

KHODER, A., SARVARIA, A., ALSULIMAN, A., CHEW, C., SEKINE, T., COOPER, N., MIELKE, S., DE LAVALLADE, H., MUFTUOGLU, M., CURBELO, I. F., LIU, E. L., MURARO, P. A., ALOUSI, A., STRINGARIS, K., PARMAR, S., SHAH, N., SHAIM, H., YVON, E., MOLLDREM, J., ROUCE, R., CHAMPLIN, R., MCNIECE, I., MAURI, C., SHPALL, E. J. & REZVANI, K. 2014. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. *Blood,* 124**,** 2034-2045.

KIESLER, P., HAYNES, P. A., SHI, L., KAO, P. N., WYSOCKI, V. H. & VERCELLI, D. 2010. NF45 and NF90 regulate HS4-dependent interleukin-13 transcription in T cells. *J Biol Chem,* 285**,** 8256-67.

KIM, S. 2014. Interleukin-32 in inflammatory autoimmune diseases. *Immune Netw,* 14**,** 123-7.

KIM, S. H., HAN, S. Y., AZAM, T., YOON, D. Y. & DINARELLO, C. A. 2005. Interleukin-32: a cytokine and inducer of TNFalpha. *Immunity,* 22**,** 131-42.

KING, I. L. & MOHRS, M. 2009. IL-4-producing CD4(+) T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *Journal of Experimental Medicine,* 206**,** 1001-1007.

KIRKLAND, D., BENSON, A., MIRPURI, J., PIFER, R., HOU, B. D., DEFRANCO, A. L. & YAROVINSKY, F. 2012. B Cell-Intrinsic MyD88 Signaling Prevents the Lethal Dissemination of Commensal Bacteria during Colonic Damage. *Immunity,* 36**,** 228-238.

KLEIN, U. & DALLA-FAVERA, R. 2008. Germinal centres: role in B-cell physiology and malignancy. *Nature Reviews Immunology,* 8**,** 22-33.

KNIPPENBERG, S., PEELEN, E., SMOLDERS, J., THEWISSEN, M., MENHEERE, P., TERVAERT, J. W. C., HUPPERTS, R. & DAMOISEAUX, J. 2011. Reduction in IL-10 producing B cells (Breg) in multiple sclerosis is accompanied by a reduced naive/memory Breg ratio during a relapse but not in remission. *Journal of Neuroimmunology,* 239**,** 80-86.

KOBAYASHI, H., HUANG, J., YE, F., SHYR, Y., BLACKWELL, T. S. & LIN, P. C. 2010. Interleukin-32beta propagates vascular inflammation and exacerbates sepsis in a mouse model. *PLoS One,* 5**,** e9458.

KOBAYASHI, H. & LIN, P. C. 2009. Molecular characterization of IL-32 in human endothelial cells. *Cytokine,* 46**,** 351-8.

KRANICH, J. & KRAUTLER, N. J. 2016. How Follicular Dendritic Cells Shape the B-Cell Antigenome. *Front Immunol,* 7**,** 225.

KRASNOSELSKAYA-RIZ, I., SPRUILL, A., CHEN, Y. W., SCHUSTER, D., TESLOVICH, T., BAKER, C., KUMAR, A. & STEPHAN, D. A. 2002. Nuclear factor 90 mediates activation of the cellular antiviral expression cascade. *AIDS Res Hum Retroviruses,* 18**,** 591-604.

KRAWCZAK, M., BALL, E. V., FENTON, I., STENSON, P. D., ABEYSINGHE, S., THOMAS, N. & COOPER, D. N. 2000. Human gene mutation database-a biomedical information and research resource. *Hum Mutat,* 15**,** 45-51.

KUCHEN, S., ROBBINS, R., SIMS, G. P., SHENG, C., PHILLIPS, T. M., LIPSKY, P. E. & ETTINGER, R. 2007. Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration. *J Immunol,* 179**,** 5886-96.

KUMAR, A., FARACE, F., GAUDIN, C. & TRIEBEL, F. 1996. Clonal T cell expansion induced by interleukin 2 therapy in blood and tumors. *J Clin Invest,* 97**,** 1219-26.

KYTTARIS, V. C., WANG, Y., JUANG, Y. T., WEINSTEIN, A. & TSOKOS, G. C. 2007. Increased levels of NF-ATc2 differentially regulate CD154 and IL-2 genes in T cells from patients with systemic lupus erythematosus. *J Immunol,* 178**,** 1960-6.

LAHITA, R. G. 1987. *Systemic Lupus Erythematosus,* New York, John Wiley and Sons.

LAIDLAW, B. J., LU, Y., AMEZQUITA, R. A., WEINSTEIN, J. S., VANDER HEIDEN, J. A., GUPTA, N. T., KLEINSTEIN, S. H., KAECH, S. M. & CRAFT, J. 2017. Interleukin-10 from CD4(+) follicular regulatory T cells promotes the germinal center response. *Sci Immunol,* 2.

LEDERBERG, J. 1959. Genes and antibodies. *Science,* 129**,** 1649-53.

LEDERMAN, S., YELLIN, M. J., CLEARY, A. M., PERNIS, A., INGHIRAMI, G., COHN, L. E., COVEY, L. R., LEE, J. J., ROTHMAN, P. & CHESS, L. 1994. T-BAM/CD40-L on helper T lymphocytes augments lymphokine-induced B cell Ig isotype switch recombination and rescues B cells from programmed cell death. *J Immunol,* 152**,** 2163-71.

LEE, H., DEIGNAN, J. L., DORRANI, N., STROM, S. P., KANTARCI, S., QUINTERO-RIVERA, F., DAS, K., TOY, T., HARRY, B., YOURSHAW, M., FOX, M., FOGEL, B. L., MARTINEZ-AGOSTO, J. A., WONG, D. A., CHANG, V. Y., SHIEH, P. B., PALMER, C. G., DIPPLE, K. M., GRODY, W. W., VILAIN, E. & NELSON, S. F. 2014a. Clinical exome sequencing for genetic identification of rare Mendelian disorders. *JAMA,* 312**,** 1880-7.

LEE, H. J., LEE, H. J., LIANG, Z. L., LIM, J. S. & KIM, J. M. 2012a. Overexpression of IL-32 as a novel prognostic factor in patients with clear cell renal cell carcinoma after a nephrectomy. *Journal of Clinical Oncology,* 30.

LEE, J., KIM, K. E., CHEON, S., SONG, J. H., HOUH, Y., KIM, T. S., GIL, M., LEE, K. J., KIM, S., KIM, D., HUR, D. Y., YANG, Y., BANG, S. I., PARK, H. J. & CHO, D. 2016. Interleukin-32alpha induces migration of human melanoma cells through downregulation of E-cadherin. *Oncotarget,* 7**,** 65825-65836.

LEE, K. M., STOTT, R. T., ZHAO, G. P., SOOHOO, J., XIONG, W., LIAN, M. M., FITZGERALD, L., SHI, S., AKRAWI, E., LEI, J., DENG, S. P., YEH, H., MARKMANN, J. F. & KIM, J. I. 2014b. TGF-beta-producing regulatory B cells induce regulatory T cells and promote transplantation tolerance. *European Journal of Immunology,* 44**,** 1728-1736.

LEE, M. Y., KIM, S. H., OH, Y. S., HEO, S. H., KIM, K. H., CHAE, H. D., KIM, C. H. & KANG, B. M. 2018. Role of interleukin-32 in the pathogenesis of endometriosis: in vitro, human and transgenic mouse data. *Hum Reprod,* 33**,** 807-816.

LEE, S., KIM, J. H., KIM, H., KANG, J. W., KIM, S. H., YANG, Y., KIM, J., PARK, J., PARK, S., HONG, J. & YOON, D. Y. 2011. Activation of the interleukin-32 pro-inflammatory pathway in response to human papillomavirus infection and over-expressionof interleukin-32 controls the expression of the humanpapillomavirus oncogene. *Immunology,* 132**,** 410-420.

LEE, S. K., SILVA, D. G., MARTIN, J. L., PRATAMA, A., HU, X., CHANG, P. P., WALTERS, G. & VINUESA, C. G. 2012b. Interferon-gamma excess leads to pathogenic accumulation of follicular helper T cells and germinal centers. *Immunity,* 37**,** 880-92.

LEFFLER, J., BENGTSSON, A. A. & BLOM, A. M. 2014. The complement system in systemic lupus erythematosus: an update. *Annals of the Rheumatic Diseases,* 73**,** 1601-1606.

LEHMANN, C., ZEIS, M. & UHAREK, L. 2001. Activation of natural killer cells with interleukin 2 (IL-2) and IL-12 increases perforin binding and subsequent lysis of tumour cells. *Br J Haematol,* 114**,** 660-5.

LESLEY, R., XU, Y., KALLED, S. L., HESS, D. M., SCHWAB, S. R., SHU, H. B. & CYSTER, J. G. 2004. Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity,* 20**,** 441-53.

LI, W., LIU, Y., MUKHTAR, M. M., GONG, R., PAN, Y., RASOOL, S. T., GAO, Y., KANG, L., HAO, Q., PENG, G., CHEN, Y., CHEN, X., WU, J. & ZHU, Y. 2008. Activation of interleukin-32 pro-inflammatory pathway in response to influenza A virus infection. *PLoS One,* 3**,** e1985.

LI, W., SUN, W., LIU, L., YANG, F., LI, Y., CHEN, Y., FANG, J., ZHANG, W., WU, J. & ZHU, Y. 2010. IL-32: a host proinflammatory factor against influenza viral replication is upregulated

by aberrant epigenetic modifications during influenza A virus infection. *J Immunol,* 185**,** 5056-65.

LI, Y. & BELSHAN, M. 2016. NF45 and NF90 Bind HIV-1 RNA and Modulate HIV Gene Expression. *Viruses,* 8.

LIAO, W., LIN, J. X. & LEONARD, W. J. 2011. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. *Curr Opin Immunol,* 23**,** 598-604.

LIAO, W., LIN, J. X. & LEONARD, W. J. 2013. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. *Immunity,* 38**,** 13-25.

LIEBERMAN, J., SHANKAR, P., MANJUNATH, N. & ANDERSSON, J. 2001. Dressed to kill? A review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection. *Blood,* 98**,** 1667-77.

LIEBERMAN, L. A. & TSOKOS, G. C. 2010. The IL-2 defect in systemic lupus erythematosus disease has an expansive effect on host immunity. *J Biomed Biotechnol,* 2010**,** 740619.

LIM, H. W., HILLSAMER, P. & KIM, C. H. 2004. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *Journal of Clinical Investigation,* 114**,** 1640-1649.

LINKER-ISRAELI, M., BAKKE, A. C., KITRIDOU, R. C., GENDLER, S., GILLIS, S. & HORWITZ, D. A. 1983. Defective production of interleukin 1 and interleukin 2 in patients with systemic lupus erythematosus (SLE). *J Immunol,* 130**,** 2651-5.

LINSLEY, P. S., BRADSHAW, J., URNES, M., GROSMAIRE, L. & LEDBETTER, J. A. 1993. CD28 engagement by B7/BB-1 induces transient down-regulation of CD28 synthesis and prolonged unresponsiveness to CD28 signaling. *J Immunol,* 150**,** 3161-9.

LINSLEY, P. S., GREENE, J. L., TAN, P., BRADSHAW, J., LEDBETTER, J. A., ANASETTI, C. & DAMLE, N. K. 1992. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J Exp Med,* 176**,** 1595-604.

LINSLEY, P. S. & LEDBETTER, J. A. 1993. The role of the CD28 receptor during T cell responses to antigen. *Annu Rev Immunol,* 11**,** 191-212.

LINTERMAN, M., PIERSON, W., LEE, C., KALLIES, A., RAYNER, T., KAWAMOTO, S., FAGARASAN, S., LISTON, A., SMITH, K. G. C. & VINUESA, C. 2011a. Foxp3(+) follicular regulatory T cells control T follicular helper cells and the germinal centre response. *Immunology,* 135**,** 39-39.

LINTERMAN, M. A., PIERSON, W., LEE, S. K., KALLIES, A., KAWAMOTO, S., RAYNER, T. F., SRIVASTAVA, M., DIVEKAR, D. P., BEATON, L., HOGAN, J. J., FAGARASAN, S., LISTON, A., SMITH, K. G. & VINUESA, C. G. 2011b. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med,* 17**,** 975-82.

LINTERMAN, M. A., PIERSON, W., LEE, S. K., KALLIES, A., KAWAMOTO, S., RAYNER, T. F., SRIVASTAVA, M., DIVEKAR, D. P., BEATON, L., HOGAN, J. J., FAGARASAN, S., LISTON, A., SMITH, K. G. C. & VINUESA, C. G. 2011c. Foxp3(+) follicular regulatory T cells control the germinal center response. *Nature Medicine,* 17**,** 975-U95.

LINTERMAN, M. A., RIGBY, R. J., WONG, R. K., YU, D., BRINK, R., CANNONS, J. L., SCHWARTZBERG, P. L., COOK, M. C., WALTERS, G. D. & VINUESA, C. G. 2009. Follicular helper T cells are required for systemic autoimmunity. *Journal of Experimental Medicine,* 206**,** 561-576.

LIU, D., XU, H., SHIH, C., WAN, Z., MA, X., MA, W., LUO, D. & QI, H. 2015a. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature,* 517**,** 214-8.

LIU, D. Y., SUCHARD, S. J., NADLER, S. G. & FORD, M. L. 2015b. Inhibition of Donor-Reactive CD8+T Cell Responses by Selective CD28 Blockade Is Independent of Reduced ICOS Expression. *Plos One,* 10.

LIU, J. Z., HAMROUNI, A., WOLOWIEC, D., COITEUX, V., KULICZKOWSKI, K., HETUIN, D., SAUDEMONT, A. & QUESNEL, B. 2007. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-gamma and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. *Blood,* 110**,** 296-304.

LIU, Q., WANG, Z., WU, Y., CAO, L., TANG, Q., XING, X., MA, H., ZHANG, S. & LUO, Y. 2014a. Five novel mutations in the ADAR1 gene associated with dyschromatosis symmetrica hereditaria. *BMC Med Genet,* 15**,** 69.

LIU, Y. Y., LIU, A. J., IIKUNI, N., XU, H. J., SHI, F. D. & LA CAVA, A. 2014b. Regulatory CD4(+) T Cells Promote B Cell Anergy in Murine Lupus. *Journal of Immunology,* 192**,** 4069-4073.

LIU, Z., LEE, J., KRUMMEY, S., LU, W., CAI, H. & LENARDO, M. J. 2011. The kinase LRRK2 is a regulator of the transcription factor NFAT that modulates the severity of inflammatory bowel disease. *Nat Immunol,* 12**,** 1063-70.

LO GIUDICE, T., LOMBARDI, F., SANTORELLI, F. M., KAWARAI, T. & ORLACCHIO, A. 2014. Hereditary spastic paraplegia: clinical-genetic characteristics and evolving molecular mechanisms. *Exp Neurol,* 261**,** 518-39.

LO, M. S. 2016a. Monogenic Lupus. *Current Rheumatology Reports,* 18.

LO, M. S. 2016b. Monogenic Lupus. *Curr Rheumatol Rep,* 18**,** 71.

LONG, S. A. & BUCKNER, J. H. 2011. CD4(+)FOXP3(+) T Regulatory Cells in Human Autoimmunity: More Than a Numbers Game. *Journal of Immunology,* 187**,** 2061-2066.

LOPEZ-VERGES, S., MILUSH, J. M., PANDEY, S., YORK, V. A., ARAKAWA-HOYT, J., PIRCHER, H., NORRIS, P. J., NIXON, D. F. & LANIER, L. L. 2010a. CD57 defines a functionally distinct population of mature NK cells in the human CD56(dim)CD16(+) NK-cell subset. *Blood,* 116**,** 3865-3874.

LOPEZ-VERGES, S., MILUSH, J. M., PANDEY, S., YORK, V. A., ARAKAWA-HOYT, J., PIRCHER, H., NORRIS, P. J., NIXON, D. F. & LANIER, L. L. 2010b. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood,* 116**,** 3865-74.

LOURENCO, E. V. & LA CAVA, A. 2009. Cytokines in systemic lupus erythematosus. *Curr Mol Med,* 9**,** 242-54.

LU, R., VIDAL, G. S., KELLY, J. A., DELGADO-VEGA, A. M., HOWARD, X. K., MACWANA, S. R., DOMINGUEZ, N., KLEIN, W., BURRELL, C., HARLEY, I. T., KAUFMAN, K. M., BRUNER, G. R., MOSER, K. L., GAFFNEY, P. M., GILKESON, G. S., WAKELAND, E. K., LI, Q. Z., LANGEFELD, C. D., MARION, M. C., DIVERS, J., ALARCON, G. S., BROWN, E. E., KIMBERLY, R. P., EDBERG, J. C., RAMSEY-GOLDMAN, R., REVEILLE, J. D., MCGWIN, G., VILA, L. M., PETRI, M. A., BAE, S. C., CHO, S. K., BANG, S. Y., KIM, I., CHOI, C. B., MARTIN, J., VYSE, T. J., MERRILL, J. T., HARLEY, J. B., ALARCON-RIQUELME, M. E., NATH, S. K., JAMES, J. A., GUTHRIDGE, J. M., COLLABORATIONS, B. M. & COLLABORATION, G. M. 2009. Genetic associations of LYN with systemic lupus erythematosus. *Genes and Immunity,* 10**,** 397-403.

LUO, H., COWEN, L., YU, G., JIANG, W. & TANG, Y. 2016. SMG7 is a critical regulator of p53 stability and function in DNA damage stress response. *Cell Discov,* 2**,** 15042.

LUZINA, I. G., ATAMAS, S. P., STORRER, C. E., DASILVA, L. C., KELSOE, G., PAPADIMITRIOU, J. C. & HANDWERGER, B. S. 2001. Spontaneous formation of germinal centers in autoimmune mice. *J Leukoc Biol,* 70**,** 578-84.

LYSSUK, E. Y., TORGASHINA, A. V., SOLOVIEV, S. K., NASSONOV, E. L. & BYKOVSKAIA, S. N. 2007. Reduced number and function of CD4+CD25highFoxP3+ regulatory T cells in patients with systemic lupus erythematosus. *Adv Exp Med Biol,* 601**,** 113-9.

MACIAN, F. 2005. NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol,* 5**,** 472-84.

MACIAN, F., LOPEZ-RODRIGUEZ, C. & RAO, A. 2001. Partners in transcription: NFAT and AP-1. *Oncogene,* 20**,** 2476-2489.

MACKAY, I. R., LARKIN, L. & BURNET, F. M. 1957. Failure of Autoimmune Antibody to React with Antigen Prepared from the Individuals Own Tissues. *Lancet,* 2**,** 122-123.

MACLENNAN, I. C. 1994. Germinal centers. *Annu Rev Immunol,* 12**,** 117-39.

MAK, I. W. Y., EVANIEW, N. & GHERT, M. 2014. Lost in translation: animal models and clinical trials in cancer treatment. *American Journal of Translational Research,* 6**,** 114-118.

MALISAN, F., BRIERE, F., BRIDON, J. M., HARINDRANATH, N., MILLS, F. C., MAX, E. E., BANCHEREAU, J. & MARTINEZ-VALDEZ, H. 1996. Interleukin-10 induces immunoglobulin G isotype switch recombination in human CD40-activated naive B lymphocytes. *J Exp Med,* 183**,** 937-47.

MANGER, K., MANGER, B., REPP, R., GEISSELBRECHT, M., GEIGER, A., PFAHLBERG, A., HARRER, T. & KALDEN, J. R. 2002. Definition of risk factors for death, end stage renal disease, and thromboembolic events in a monocentric cohort of 338 patients with systemic lupus erythematosus. *Ann Rheum Dis,* 61**,** 1065-70.

MANJARREZ ORDUÑO, N., C. MENARD, L., CARMAN, J., J. SUCHARD, S., CASANO, F., LEE, D., DAOUTI, S., HABTE, S., KANSAL, S., JIANG, C., BANDYOPADHYAY, S., HU, Y., A. FURIE, R. & G. NADLER, S. 2017. *A Systemic Lupus Erythematosus Endophenotype Characterized by Increased CD8 Cytotoxic Signature Associates with Renal Involvement*.

MANOLIO, T. A., COLLINS, F. S., COX, N. J., GOLDSTEIN, D. B., HINDORFF, L. A., HUNTER, D. J., MCCARTHY, M. I., RAMOS, E. M., CARDON, L. R., CHAKRAVARTI, A., CHO, J. H., GUTTMACHER, A. E., KONG, A., KRUGLYAK, L., MARDIS, E., ROTIMI, C. N., SLATKIN, M., VALLE, D., WHITTEMORE, A. S., BOEHNKE, M., CLARK, A. G., EICHLER, E. E., GIBSON, G., HAINES, J. L., MACKAY, T. F., MCCARROLL, S. A. & VISSCHER, P. M. 2009. Finding the missing heritability of complex diseases. *Nature,* 461**,** 747-53.

MARCONDES, A. M., LI, X., TABELLINI, L., BARTENSTEIN, M., KABACKA, J., SALE, G. E., HANSEN, J. A., DINARELLO, C. A. & DEEG, H. J. 2011. Inhibition of IL-32 activation by alpha-1 antitrypsin suppresses alloreactivity and increases survival in an allogeneic murine marrow transplantation model. *Blood,* 118**,** 5031-9.

MARCONDES, A. M., MHYRE, A. J., STIREWALT, D. L., KIM, S. H., DINARELLO, C. A. & DEEG, H. J. 2008. Dysregulation of IL-32 in myelodysplastic syndrome and chronic myelomonocytic leukemia modulates apoptosis and impairs NK function. *Proc Natl Acad Sci U S A,* 105**,** 2865-70.

MARIANI, S. M. 2004. Genes and autoimmune diseases - a complex inheritance. *MedGenMed,* 6**,** 18.

MARSHALL, N. B. & SWAIN, S. L. 2011. Cytotoxic CD4 T cells in antiviral immunity. *J Biomed Biotechnol,* 2011**,** 954602.

MARTIN, V. G., WU, Y. C. B., TOWNSEND, C. L., LU, G. H. C., O'HARE, J. S., MOZEIKA, A., COOLEN, A. C. C., KIPLING, D., FRATERNALI, F. & DUNN-WALTERS, D. K. 2016. Transitional B Cells in Early Human B Cell Development - Time to Revisit the Paradigm? *Frontiers in Immunology,* 7.

MASUDA, K., KUWANO, Y., NISHIDA, K., ROKUTAN, K. & IMOTO, I. 2013. NF90 in posttranscriptional gene regulation and microRNA biogenesis. *Int J Mol Sci,* 14**,** 17111-21.

MATSUSHITA, T. & SATO, S. 2005. [The role of BAFF in autoimmune diseases]. *Nihon Rinsho Meneki Gakkai Kaishi,* 28**,** 333-42.

MCCARTHY, M. I. & HIRSCHHORN, J. N. 2008. Genome-wide association studies: potential next steps on a genetic journey. *Hum Mol Genet,* 17**,** R156-65.

MCCARTY, C. A., MUKESH, B. N., DIMITROV, P. N. & TAYLOR, H. R. 2003. Incidence and progression of cataract in the Melbourne Visual Impairment Project. *Am J Ophthalmol,* 136**,** 10-7.

MCKINNEY, E. F., LEE, J. C., JAYNE, D. R., LYONS, P. A. & SMITH, K. G. 2015. T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection. *Nature,* 523**,** 612-6.

MEAGHER, M. J. & BRAUN, R. E. 2001. Requirement for the murine zinc finger protein ZFR in perigastrulation growth and survival. *Mol Cell Biol,* 21**,** 2880-90.

MEDZHITOV, R. 2008. Origin and physiological roles of inflammation. *Nature,* 454**,** 428-35.

MEHTA, J., GENIN, A., BRUNNER, M., SCALZI, L. V., MISHRA, N., BEUKELMAN, T. & CRON, R. Q. 2010. Prolonged expression of CD154 on CD4 T cells from pediatric lupus patients correlates with increased CD154 transcription, increased nuclear factor of activated T cell activity, and glomerulonephritis. *Arthritis Rheum,* 62**,** 2499-509.

MELAMED, D., BENSCHOP, R. J., CAMBIER, J. C. & NEMAZEE, D. 1998. Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor selection. *Cell,* 92**,** 173-82.

MELCHERS, F. 1995. B-Cell Differentiation in Bone-Marrow. *Clinical Immunology and Immunopathology,* 76**,** S188-S191.

MERRILL, M. K. & GROMEIER, M. 2006. The double-stranded RNA binding protein 76:NF45 heterodimer inhibits translation initiation at the rhinovirus type 2 internal ribosome entry site. *J Virol,* 80**,** 6936-42.

MESTAS, J. & HUGHES, C. C. 2004. Of mice and not men: differences between mouse and human immunology. *J Immunol,* 172**,** 2731-8.

MESTECKY, J., RUSSELL, M. W., JACKSON, S. & BROWN, T. A. 1986. The human IgA system: a reassessment. *Clin Immunol Immunopathol,* 40**,** 105-14.

METZLER, B., BURKHART, C. & WRAITH, D. C. 1999. Phenotypic analysis of CTLA-4 and CD28 expression during transient peptide-induced T cell activation in vivo. *Int Immunol,* 11**,** 667-75.

MIKULKOVA, Z., PRAKSOVA, P., STOURAC, P., BEDNARIK, J., STRAJTOVA, L., PACASOVA, R., BELOBRADKOVA, J., DITE, P. & MICHALEK, J. 2010. Numerical defects in CD8(+)CD28(-) T-suppressor lymphocyte population in patients with type 1 diabetes mellitus and multiple sclerosis. *Cellular Immunology,* 262**,** 75-79.

MIOSGE, L. A., FIELD, M. A., SONTANI, Y., CHO, V., JOHNSON, S., PALKOVA, A., BALAKISHNAN, B., LIANG, R., ZHANG, Y., LYON, S., BEUTLER, B., WHITTLE, B., BERTRAM, E. M., ENDERS, A., GOODNOW, C. C. & ANDREWS, T. D. 2015. Comparison of predicted and actual consequences of missense mutations. *Proc Natl Acad Sci U S A,* 112**,** E5189-98.

MITTRUCKER, H. W., VISEKRUNA, A. & HUBER, M. 2014. Heterogeneity in the differentiation and function of CD8(+) T cells. *Arch Immunol Ther Exp (Warsz),* 62**,** 449-58.

MIZUI, M. & TSOKOS, G. C. 2016. Low-Dose IL-2 in the Treatment of Lupus. *Curr Rheumatol Rep,* 18**,** 68.

MOISINI, I. & DAVIDSON, A. 2009. BAFF: a local and systemic target in autoimmune diseases. *Clinical and Experimental Immunology,* 158**,** 155-163.

MOON, Y. M., YOON, B. Y., HER, Y. M., OH, H. J., LEE, J. S., KIM, K. W., LEE, S. Y., WOO, Y. J., PARK, K. S., PARK, S. H., KIM, H. Y. & CHO, M. L. 2012. IL-32 and IL-17 interact and have the potential to aggravate osteoclastogenesis in rheumatoid arthritis. *Arthritis Res Ther,* 14**,** R246.

MOSCHEN, A. R., MOLNAR, C., GEIGER, S., GRAZIADEI, I., EBENBICHLER, C. F., WEISS, H., KASER, S., KASER, A. & TILG, H. 2010. Anti-inflammatory effects of excessive weight loss: potent suppression of adipose interleukin 6 and tumour necrosis factor alpha expression. *Gut,* 59**,** 1259-64.

MOSER, K. L., KELLY, J. A., LESSARD, C. J. & HARLEY, J. B. 2009. Recent insights into the genetic basis of systemic lupus erythematosus. *Genes and Immunity,* 10**,** 373-379.

MOULTON, V. R. & TSOKOS, G. C. 2015. T cell signaling abnormalities contribute to aberrant immune cell function and autoimmunity. *J Clin Invest,* 125**,** 2220-7.

MUELLER, D. L. & JENKINS, M. K. 1997. Autoimmunity: when self-tolerance breaks down. *Curr Biol,* 7**,** R255-7.

MUNROE, M. E., LU, R., ZHAO, Y. D., FIFE, D. A., ROBERTSON, J. M., GUTHRIDGE, J. M., NIEWOLD, T. B., TSOKOS, G. C., KEITH, M. P., HARLEY, J. B. & JAMES, J. A. 2016. Altered type II interferon precedes autoantibody accrual and elevated type I interferon activity prior to systemic lupus erythematosus classification. *Ann Rheum Dis,* 75**,** 2014-2021.

MURPHY, K. & WEAVER, C. 2016. *Janeway's immunobiology,* New York, NY, Garland Science/Taylor & Francis Group, LLC.

NADDA, N., SETIA, S., VAISH, V. & SANYAL, S. N. 2013. Role of cytokines in experimentally induced lung cancer and chemoprevention by COX-2 selective inhibitor, etoricoxib. *Molecular and Cellular Biochemistry,* 372**,** 101-112.

NAGASAWA, T. 2006. Microenvironmental niches in the bone marrow required for B-cell development. *Nature Reviews Immunology,* 6**,** 107-116.

NAKADAI, T., FUKUDA, A., SHIMADA, M., NISHIMURA, K. & HISATAKE, K. 2015. The RNA binding complexes NF45-NF90 and NF45-NF110 associate dynamically with the c-fos gene and function as transcriptional coactivators. *J Biol Chem,* 290**,** 26832-45.

NAKAYAMA, M., NIKI, Y., KAWASAKI, T., TAKEDA, Y., IKEGAMI, H., TOYAMA, Y. & MIYAMOTO, T. 2013. IL-32-PAR2 axis is an innate immunity sensor providing alternative signaling for LPS-TRIF axis. *Sci Rep,* 3**,** 2960.

NAPIREI, M., KARSUNKY, H., ZEVNIK, B., STEPHAN, H., MANNHERZ, H. G. & MOROY, T. 2000. Features of systemic lupus erythematosus in Dnase1-deficient mice. *Nat Genet,* 25**,** 177-81.

NATH, S. K., HAN, S., KIM-HOWARD, X., KELLY, J. A., VISWANATHAN, P., GILKESON, G. S., CHEN, W., ZHU, C., MCEVER, R. P., KIMBERLY, R. P., ALARCON-RIQUELME, M. E., VYSE, T. J.,

LI, Q. Z., WAKELAND, E. K., MERRILL, J. T., JAMES, J. A., KAUFMAN, K. M., GUTHRIDGE, J. M. & HARLEY, J. B. 2008. A nonsynonymous functional variant in integrin-alpha(M) (encoded by ITGAM) is associated with systemic lupus erythematosus. *Nat Genet,* 40**,** 152-4.

NAVARRA, S. V., GUZMAN, R. M., GALLACHER, A. E., HALL, S., LEVY, R. A., JIMENEZ, R. E., LI, E. K., THOMAS, M., KIM, H. Y., LEON, M. G., TANASESCU, C., NASONOV, E., LAN, J. L., PINEDA, L., ZHONG, Z. J., FREIMUTH, W., PETRI, M. A. & GROUP, B.-S. 2011. Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. *Lancet,* 377**,** 721-31.

NEMAZEE, D. A. & BURKI, K. 1989. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature,* 337**,** 562-6.

NETEA, M. G., AZAM, T., FERWERDA, G., GIRARDIN, S. E., WALSH, M., PARK, J. S., ABRAHAM, E., KIM, J. M., YOON, D. Y., DINARELLO, C. A. & KIM, S. H. 2005. IL-32 synergizes with nucleotide oligomerization domain (NOD) 1 and NOD2 ligands for IL-1beta and IL-6 production through a caspase 1-dependent mechanism. *Proc Natl Acad Sci U S A,* 102**,** 16309-14.

NETEA, M. G., LEWIS, E. C., AZAM, T., JOOSTEN, L. A. B., JAEKAL, J., BAE, S. Y., DINARELLO, C. A. & KIM, S. H. 2008. Interleukin-32 induces the differentiation of monocytes into macrophage-like cells. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 3515-3520.

NIEUWENHUIS, P. & OPSTELTEN, D. 1984. Functional anatomy of germinal centers. *Am J Anat,* 170**,** 421-35.

NISHIDA, A., ANDOH, A., INATOMI, O. & FUJIYAMA, Y. 2009. Interleukin-32 Expression in the Pancreas. *Journal of Biological Chemistry,* 284**,** 17868-17876.

NORIS, M. & REMUZZI, G. 2013. Overview of Complement Activation and Regulation. *Seminars in Nephrology,* 33**,** 479-492.

NOVAK, J., TOMANA, M., MATOUSOVIC, K., BROWN, R., HALL, S., NOVAK, L., JULIAN, B. A., WYATT, R. J. & MESTECKY, J. 2005. IgA1-containing immune complexes in IgA nephropathy differentially affect proliferation of mesangial cells. *Kidney Int,* 67**,** 504-13.

NOVICK, D., RUBINSTEIN, M., AZAM, T., RABINKOV, A., DINARELLO, C. A. & KIM, S. H. 2006. Proteinase 3 is an IL-32 binding protein. *Proc Natl Acad Sci U S A,* 103**,** 3316-21.

NOWICK, K. & STUBBS, L. 2010. Lineage-specific transcription factors and the evolution of gene regulatory networks. *Briefings in Functional Genomics,* 9**,** 65-78.

NURIEVA, R. I., CHUNG, Y., HWANG, D., YANG, X. O., KANG, H. S., MA, L., WANG, Y. H., WATOWICH, S. S., JETTEN, A. M., TIAN, Q. & DONG, C. 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity,* 29**,** 138-149.

NURIEVA, R. I. & CHUNG, Y. S. 2010. Understanding the development and function of T follicular helper cells. *Cellular & Molecular Immunology,* 7**,** 190-197.

O'SULLIVAN, L. A., LIONGUE, C., LEWIS, R. S., STEPHENSON, S. E. & WARD, A. C. 2007. Cytokine receptor signaling through the Jak-Stat-Socs pathway in disease. *Mol Immunol,* 44**,** 2497-506.

OHL, K. & TENBROCK, K. 2015. Regulatory T cells in systemic lupus erythematosus. *Eur J Immunol,* 45**,** 344-55.

OKADA, T., MILLER, M. J., PARKER, I., KRUMMEL, M. F., NEIGHBORS, M., HARTLEY, S. B., O'GARRA, A., CAHALAN, M. D. & CYSTER, J. G. 2005. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol,* 3**,** e150.

OLKHANUD, P. B., DAMDINSUREN, B., BODOGAI, M., GRESS, R. E., SEN, R., WEJKSZA, K., MALCHINKHUU, E., WERSTO, R. P. & BIRAGYN, A. 2011. Tumor-Evoked Regulatory B Cells Promote Breast Cancer Metastasis by Converting Resting CD4(+) T Cells to T-Regulatory Cells. *Cancer Research,* 71**,** 3505-3515.

ONUORA, S. 2016. Autoimmunity: IFNgamma signals control germinal centre formation. *Nat Rev Rheumatol,* 12**,** 312.

ORENGO, J. P., BUNDMAN, D. & COOPER, T. A. 2006. A bichromatic fluorescent reporter for cell-based screens of alternative splicing. *Nucleic Acids Research,* 34.

OSLER, W. 1976. Classics: On the visceral complications of erythema exudativum multiforme. William Osler, M.D., F.R.C.P. Lond. *Am J Med Sci,* 271**,** 106-17.

OSMAN, A., BHUYAN, F., HASHIMOTO, M., NASSER, H., MAEKAWA, T. & SUZU, S. 2014. M-CSF Inhibits Anti-HIV-1 Activity of IL-32, but They Enhance M2-like Phenotypes of Macrophages. *Journal of Immunology,* 192**,** 5083-5089.

OTA, K., KAWAGUCHI, M., FUJITA, J., KOKUBU, F., HUANG, S. K., MORISHIMA, Y., MATSUKURA, S., KUROKAWA, M., ISHII, Y., SATOH, H., SAKAMOTO, T. & HIZAWA, N. 2015. Synthetic double-stranded RNA induces interleukin-32 in bronchial epithelial cells. *Exp Lung Res,* 41**,** 335-43.

OWEN, R. D. 1945. Immunogenetic Consequences of Vascular Anastomoses between Bovine Twins. *Science,* 102**,** 400-1.

OZAKI, K., SPOLSKI, R., ETTINGER, R., KIM, H. P., WANG, G., QI, C. F., HWU, P., SHAFFER, D. J., AKILESH, S., ROOPENIAN, D. C., MORSE, H. C., 3RD, LIPSKY, P. E. & LEONARD, W. J. 2004. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol,* 173**,** 5361-71.

PALIOGIANNI, F., RAPTIS, A., AHUJA, S. S., NAJJAR, S. M. & BOUMPAS, D. T. 1993. Negative Transcriptional Regulation of Human Interleukin-2 (Il-2) Gene by Glucocorticoids through Interference with Nuclear Transcription Factors Ap-1 and Nf-At. *Journal of Clinical Investigation,* 91**,** 1481-1489.

PALSTRA, R. J., DE CRIGNIS, E., ROLING, M. D., VAN STAVEREN, T., KAN, T. W., VAN IJCKEN, W., MUELLER, Y. M., KATSIKIS, P. D. & MAHMOUDI, T. 2018. Allele-specific long-distance regulation dictates IL-32 isoform switching and mediates susceptibility to HIV-1. *Sci Adv,* 4**,** e1701729.

PAPA, I., SALIBA, D., PONZONI, M., BUSTAMANTE, S., CANETE, P. F., GONZALEZ-FIGUEROA, P., MCNAMARA, H. A., VALVO, S., GRIMBALDESTON, M., SWEET, R. A., VOHRA, H., COCKBURN, I. A., MEYER-HERMANN, M., DUSTIN, M. L., DOGLIONI, C. & VINUESA, C. G. 2017. TFH-derived dopamine accelerates productive synapses in germinal centres. *Nature,* 547**,** 318-323.

PARK, J., MUNAGALA, I., XU, H., BLANKENSHIP, D., MAFFUCCI, P., CHAUSSABEL, D., BANCHEREAU, J., PASCUAL, V. & CUNNINGHAM-RUNDLES, C. 2013. Interferon signature in the blood in inflammatory common variable immune deficiency. *PLoS One,* 8**,** e74893.

PARK, M. H., SONG, M. J., CHO, M. C., MOON, D. C., YOON, D. Y., HAN, S. B. & HONG, J. T. 2012. Interleukin-32 enhances cytotoxic effect of natural killer cells to cancer cells via activation of death receptor 3. *Immunology,* 135**,** 63-72.

PARK, Y. S., KANG, J. W., LEE, D. H., KIM, M. S., BAK, Y., YANG, Y., LEE, H. G., HONG, J. & YOON, D. Y. 2014a. Interleukin-32alpha downregulates the activity of the B-cell CLL/lymphoma 6 protein by inhibiting protein kinase Cepsilon-dependent SUMO-2 modification. *Oncotarget,* 5**,** 8765-77.

PARK, Y. S., KANG, J. W., LEE, D. H., KIM, M. S., BAK, Y., YANG, Y., LEE, H. G., HONG, J. & YOON, D. Y. 2014b. Interleukin-32alpha modulates promyelocytic leukemia zinc finger gene activity by inhibiting protein kinase Cvarepsilon-dependent sumoylation. *Int J Biochem Cell Biol,* 55**,** 136-43.

PARKER, D. C. 1993. T-Cell - Dependent B-Cell Activation. *Annual Review of Immunology,* 11**,** 331-360.

PAYNE, K. J. & CROOKS, G. M. 2007. Immune-cell lineage commitment: Translation from mice to humans. *Immunity,* 26**,** 674-677.

PEI, Y., ZHU, P., DANG, Y., WU, J., YANG, X., WAN, B., LIU, J. O., YI, Q. & YU, L. 2008. Nuclear export of NF90 to stabilize IL-2 mRNA is mediated by AKT-dependent phosphorylation at Ser647 in response to CD28 costimulation. *J Immunol,* 180**,** 222-9.

PENE, J., GAUCHAT, J. F., LECART, S., DROUET, E., GUGLIELMI, P., BOULAY, V., DELWAIL, A., FOSTER, D., LECRON, J. C. & YSSEL, H. 2004. Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells. *J Immunol,* 172**,** 5154-7.

PENE, J., GUGLIELMI, L., GAUCHAT, J. F., HARRER, N., WOISETSCHLAGER, M., BOULAY, V., FABRE, J. M., DEMOLY, P. & YSSEL, H. 2006. IFN-gamma-mediated inhibition of human IgE synthesis by IL-21 is associated with a polymorphism in the IL-21R gene. *J Immunol,* 177**,** 5006-13.

PERL, A., FERNANDEZ, D. R., TELARICO, T., DOHERTY, E., FRANCIS, L. & PHILLIPS, P. E. 2009. T-cell and B-cell signaling biomarkers and treatment targets in lupus. *Curr Opin Rheumatol,* 21**,** 454-64.

PEROSA, F., LUCCARELLI, G., PRETE, M., INDIVERI, F. & DAMMACCO, F. 2001. Human CD4 internal antigen anti-idiotypic monoclonal antibody. Immunochemical and sequence analysis. *Clinical and Experimental Medicine,* 1**,** 81-89.

PETRI, M. 2005. Review of classification criteria for systemic lupus erythematosus. *Rheum Dis Clin North Am,* 31**,** 245-54, vi.

PIPKIN, M. E., SACKS, J. A., CRUZ-GUILLOTY, F., LICHTENHELD, M. G., BEVAN, M. J. & RAO, A. 2010. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity,* 32**,** 79-90.

PLANTINGA, T. S., COSTANTINI, I., HEINHUIS, B., HUIJBERS, A., SEMANGO, G., KUSTERS, B., NETEA, M. G., HERMUS, A. R. M. M., SMIT, J. W. A., DINARELLO, C. A., JOOSTEN, L. A. B. & NETEA-MAIER, R. T. 2013. A promoter polymorphism in human interleukin-32 modulates its expression and influences the risk and the outcome of epithelial cellderived thyroid carcinoma. *Carcinogenesis,* 34**,** 1529-1535.

PLATANIAS, L. C. 2005. Introduction: interferon signals: what is classical and what is nonclassical? *J Interferon Cytokine Res,* 25**,** 732.

PLATT, R. J., CHEN, S., ZHOU, Y., YIM, M. J., SWIECH, L., KEMPTON, H. R., DAHLMAN, J. E., PARNAS, O., EISENHAURE, T. M., JOVANOVIC, M., GRAHAM, D. B., JHUNJHUNWALA, S., HEIDENREICH, M., XAVIER, R. J., LANGER, R., ANDERSON, D. G., HACOHEN, N.,

REGEV, A., FENG, G., SHARP, P. A. & ZHANG, F. 2014. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell,* 159**,** 440-55.

PLAYER, A., OGUAMANAM, T., OKANMELU, J., BURRELL, K. & HOLLOMON, M. 2014. Preliminary characterization of IL32 in basal-like/triple negative compared to other types of breast cell lines and tissues. *BMC Res Notes,* 7**,** 501.

PULLABHATLA, V., ROBERTS, A. L., LEWIS, M. J., MAURO, D., MORRIS, D. L., ODHAMS, C. A., TOMBLESON, P., LILJEDAHL, U., VYSE, S., SIMPSON, M. A., SAUER, S., DE RINALDIS, E., SYVANEN, A. C. & VYSE, T. J. 2018. De novo mutations implicate novel genes in systemic lupus erythematosus. *Hum Mol Genet,* 27**,** 421-429.

QI, H., CHEN, X., CHU, C., LU, P., XU, H. & YAN, J. 2014. Follicular T-helper cells: controlled localization and cellular interactions. *Immunol Cell Biol,* 92**,** 28-33.

RABINOVITCH, A., SUAREZ-PINZON, W. L., SHAPIRO, A. M., RAJOTTE, R. V. & POWER, R. 2002. Combination therapy with sirolimus and interleukin-2 prevents spontaneous and recurrent autoimmune diabetes in NOD mice. *Diabetes,* 51**,** 638-45.

RADAEV, S., ZOU, Z., TOLAR, P., NGUYEN, K., NGUYEN, A., KRUEGER, P. D., STUTZMAN, N., PIERCE, S. & SUN, P. D. 2010. Structural and Functional Studies of Ig alpha beta and Its Assembly with the B Cell Antigen Receptor. *Structure,* 18**,** 934-943.

RAHMAN, Z. S. M., RAO, S. P., KALLED, S. L. & MANSER, T. 2003. Normal induction but attenuated progression of germinal center responses in BAFF and BAFF-R signaling deficient mice. *Journal of Experimental Medicine,* 198**,** 1157-1169.

RAMOS, P. S., BROWN, E. E., KIMBERLY, R. P. & LANGEFELD, C. D. 2010. Genetic Factors Predisposing to Systemic Lupus Erythematosus and Lupus Nephritis. *Seminars in Nephrology,* 30**,** 164-176.

RAMOS, P. S., SHEDLOCK, A. M. & LANGEFELD, C. D. 2015. Genetics of autoimmune diseases: insights from population genetics. *J Hum Genet,* 60**,** 657-64.

RAY, A., BASU, S., WILLIAMS, C. B., SALZMAN, N. H. & DITTEL, B. N. 2012. A novel IL-10-independent regulatory role for B cells in suppressing autoimmunity by maintenance of regulatory T cells via GITR ligand. *J Immunol,* 188**,** 3188-98.

RAY, A., WANG, L. & DITTEL, B. N. 2015. IL-10-independent regulatory B-cell subsets and mechanisms of action. *Int Immunol,* 27**,** 531-6.

REES, J. H. 2004. Paraneoplastic syndromes: when to suspect, how to confirm, and how to manage. *J Neurol Neurosurg Psychiatry,* 75 Suppl 2**,** ii43-50.

REESE, T. A., BI, K., KAMBAL, A., FILALI-MOUHIM, A., BEURA, L. K., BURGER, M. C., PULENDRAN, B., SEKALY, R. P., JAMESON, S. C., MASOPUST, D., HAINING, W. N. & VIRGIN, H. W. 2016. Sequential Infection with Common Pathogens Promotes Human-like Immune Gene Expression and Altered Vaccine Response. *Cell Host & Microbe,* 19**,** 713-719.

RIBEIRO-DIAS, F., SAAR GOMES, R., DE LIMA SILVA, L. L., DOS SANTOS, J. C. & JOOSTEN, L. A. 2017. Interleukin 32: a novel player in the control of infectious diseases. *J Leukoc Biol,* 101**,** 39-52.

RICE, G., NEWMAN, W. G., DEAN, J., PATRICK, T., PARMAR, R., FLINTOFF, K., ROBINS, P., HARVEY, S., HOLLIS, T., O'HARA, A., HERRICK, A. L., BOWDEN, A. P., PERRINO, F. W., LINDAHL, T., BARNES, D. E. & CROW, Y. J. 2007. Heterozygous mutations in TREX1 cause familial chilblain lupus and dominant Aicardi-Goutieres syndrome. *Am J Hum Genet,* 80**,** 811-5.

RICE, G. I., DEL TORO DUANY, Y., JENKINSON, E. M., FORTE, G. M., ANDERSON, B. H., ARIAUDO, G., BADER-MEUNIER, B., BAILDAM, E. M., BATTINI, R., BERESFORD, M. W., CASARANO, M., CHOUCHANE, M., CIMAZ, R., COLLINS, A. E., CORDEIRO, N. J., DALE, R. C., DAVIDSON, J. E., DE WAELE, L., DESGUERRE, I., FAIVRE, L., FAZZI, E., ISIDOR, B., LAGAE, L., LATCHMAN, A. R., LEBON, P., LI, C., LIVINGSTON, J. H., LOURENCO, C. M., MANCARDI, M. M., MASUREL-PAULET, A., MCINNES, I. B., MENEZES, M. P., MIGNOT, C., O'SULLIVAN, J., ORCESI, S., PICCO, P. P., RIVA, E., ROBINSON, R. A., RODRIGUEZ, D., SALVATICI, E., SCOTT, C., SZYBOWSKA, M., TOLMIE, J. L., VANDERVER, A., VANHULLE, C., VIEIRA, J. P., WEBB, K., WHITNEY, R. N., WILLIAMS, S. G., WOLFE, L. A., ZUBERI, S. M., HUR, S. & CROW, Y. J. 2014. Gain-of-function mutations in IFIH1 cause a spectrum of human disease phenotypes associated with upregulated type I interferon signaling. *Nat Genet,* 46**,** 503-9.

RICE, J. S., NEWMAN, J., WANG, C., MICHAEL, D. J. & DIAMOND, B. 2005. Receptor editing in peripheral B cell tolerance. *Proc Natl Acad Sci U S A,* 102**,** 1608-13.

RICHARDS, S. M. & CLARK, E. A. 2009. BCR-induced superoxide negatively regulates B-cell proliferation and T-cell-independent type 2 Ab responses. *European Journal of Immunology,* 39**,** 3395-3403.

RICKLIN, D., HAJISHENGALLIS, G., YANG, K. & LAMBRIS, J. D. 2010. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol,* 11**,** 785-97.

RIGO, F., HUA, Y., CHUN, S. J., PRAKASH, T. P., KRAINER, A. R. & BENNETT, C. F. 2012. Synthetic oligonucleotides recruit ILF2/3 to RNA transcripts to modulate splicing. *Nat Chem Biol,* 8**,** 555-61.

RIPPEY, C., WALSH, T., GULSUNER, S., BRODSKY, M., NORD, A. S., GASPERINI, M., PIERCE, S., SPURRELL, C., COE, B. P., KRUMM, N., LEE, M. K., SEBAT, J., MCCLELLAN, J. M. & KING, M. C. 2013a. Formation of chimeric genes by copy-number variation as a mutational mechanism in schizophrenia. *Am J Hum Genet,* 93**,** 697-710.

RIPPEY, C., WALSH, T., GULSUNER, S., BRODSKY, M., NORD, A. S., GASPERINI, M., PIERCE, S., SPURRELL, C., COE, B. P., KRUMM, N., LEE, M. K., SEBAT, J., MCCLELLAN, J. M. & KING, M. C. 2013b. Formation of Chimeric Genes by Copy-Number Variation as a Mutational Mechanism in Schizophrenia. *American Journal of Human Genetics,* 93**,** 697-710.

RIVERA, J. & TESSAROLLO, L. 2008. Genetic background and the dilemma of translating mouse studies to humans. *Immunity,* 28**,** 1-4.

RODRIGUEZ-PINTO, I., ESPINOSA, G. & CERVERA, R. 2016. The problems and pitfalls in systemic lupus erythematosus drug discovery. *Expert Opinion on Drug Discovery,* 11**,** 525-527.

RÖHLICH, K. 1930. Beitrag zur Cytologie der Keimzentren der Lymphknoten. *Z. Mikrosk. Anat. Forsch,* 20**,** 287-297.

ROLLINGS, C. M., SINCLAIR, L. V., BRADY, H. J. M., CANTRELL, D. A. & ROSS, S. H. 2018. Interleukin-2 shapes the cytotoxic T cell proteome and immune environment-sensing programs. *Sci Signal,* 11.

RONNBLOM, L., ELORANTA, M. L. & ALM, G. V. 2006. The type I interferon system in systemic lupus erythematosus. *Arthritis Rheum,* 54**,** 408-20.

RONNBLOM, L. & PASCUAL, V. 2008. The innate immune system in SLE: type I interferons and dendritic cells. *Lupus,* 17**,** 394-9.

ROONEY, J. W., SUN, Y. L., GLIMCHER, L. H. & HOEY, T. 1995. Novel NFAT sites that mediate activation of the interleukin-2 promoter in response to T-cell receptor stimulation. *Mol Cell Biol,* 15**,** 6299-310.

ROSENBERG, S. A. 2014. IL-2: the first effective immunotherapy for human cancer. *J Immunol,* 192**,** 5451-8.

ROSENZWAJG, M., CHURLAUD, G., MALLONE, R., SIX, A., DERIAN, N., CHAARA, W., LORENZON, R., LONG, S. A., BUCKNER, J. H., AFONSO, G., PHAM, H. P., HARTEMANN, A., YU, A., PUGLIESE, A., MALEK, T. R. & KLATZMANN, D. 2015. Low-dose interleukin-2 fosters a dose-dependent regulatory T cell tuned milieu in T1D patients. *J Autoimmun,* 58**,** 48-58.

ROSIGNOLI, G., LIM, C. H., BOWER, M., GOTCH, F. & IMAMI, N. 2009. Programmed death (PD)-1 molecule and its ligand PD-L1 distribution among memory CD4 and CD8 T cell subsets in human immunodeficiency virus-1-infected individuals. *Clin Exp Immunol,* 157**,** 90-7.

ROSSER, E. C. & MAURI, C. 2015. Regulatory B Cells: Origin, Phenotype, and Function. *Immunity,* 42**,** 607-612.

RUBICZ, R., ZHAO, S., GEYBELS, M., WRIGHT, J. L., KOLB, S., KLOTZLE, B., BIBIKOVA, M., TROYER, D., LANCE, R., OSTRANDER, E. A., FENG, Z., FAN, J. B. & STANFORD, J. L. 2016. DNA methylation profiles in African American prostate cancer patients in relation to disease progression. *Genomics*.

SABOURI, Z., SCHOFIELD, P., HORIKAWA, K., SPIERINGS, E., KIPLING, D., RANDALL, K. L., LANGLEY, D., ROOME, B., VAZQUEZ-LOMBARDI, R., ROUET, R., HERMES, J., CHAN, T. D., BRINK, R., DUNN-WALTERS, D. K., CHRIST, D. & GOODNOW, C. C. 2014. Redemption of autoantibodies on anergic B cells by variable-region glycosylation and mutation away from self-reactivity. *Proc Natl Acad Sci U S A,* 111**,** E2567-75.

SAGE, P. T., FRANCISCO, L. M., CARMAN, C. V. & SHARPE, A. H. 2013. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol,* 14**,** 152-61.

SAGE, P. T., PATERSON, A. M., LOVITCH, S. B. & SHARPE, A. H. 2014. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity,* 41**,** 1026-39.

SAGE, P. T., RON-HAREL, N., JUNEJA, V. R., SEN, D. R., MALERI, S., SUNGNAK, W., KUCHROO, V. K., HAINING, W. N., CHEVRIER, N., HAIGIS, M. & SHARPE, A. H. 2016. Suppression by T-FR\ cells leads to durable and selective inhibition of B cell effector function. *Nature Immunology,* 17**,** 1436-1446.

SAGE, P. T., SCHILDBERG, F. A., SOBEL, R. A., KUCHROO, V. K., FREEMAN, G. J. & SHARPE, A. H. 2018. Dendritic Cell PD-L1 Limits Autoimmunity and Follicular T Cell Differentiation and Function. *J Immunol,* 200**,** 2592-2602.

SAGE, P. T. & SHARPE, A. H. 2015a. T follicular regulatory cells in the regulation of B cell responses. *Trends Immunol,* 36**,** 410-8.

SAGE, P. T. & SHARPE, A. H. 2015b. T follicular regulatory cells in the regulation of B cell responses. *Trends in Immunology,* 36**,** 410-418.

SAHOO, A., ALEKSEEV, A., TANAKA, K., OBERTAS, L., LERMAN, B., HAYMAKER, C., CLISE-DWYER, K., MCMURRAY, J. S. & NURIEVA, R. 2015. Batf is important for IL-4 expression in T follicular helper cells. *Nat Commun,* 6**,** 7997.

SAKAGUCHI, S. 2006. Regulatory T cells. *Springer Semin Immunopathol,* 28**,** 1-2.

SAKAGUCHI, S. 2008. Regulatory T cells in the past and for the future. *Eur J Immunol,* 38**,** 901-37.

SAKAGUCHI, S. 2011. Regulatory T cells: history and perspective. *Methods Mol Biol,* 707**,** 3-17.

SAKAGUCHI, S., YAMAGUCHI, T., NOMURA, T. & ONO, M. 2008. Regulatory T cells and immune tolerance. *Cell,* 133**,** 775-87.

SANTAMARIA, P. 2003. Cytokines and chemokines in autoimmune disease: an overview. *Adv Exp Med Biol,* 520**,** 1-7.

SAYED, N., OSPINO, F., HIMMATI, F., LEE, J., CHANDA, P., MOCARSKI, E. S. & COOKE, J. P. 2017. Retinoic Acid Inducible Gene 1 Protein (RIG1)-Like Receptor Pathway Is Required for Efficient Nuclear Reprogramming. *Stem Cells*.

SAYIN, I., RADTKE, A. J., VELLA, L. A., BUGGERT, M., JIN, W. J., WHERRY, E. J., GERMAIN, R. N., BETTS, M. R., HERATI, R. S. & CANADAY, D. H. 2017. Human T follicular regulatory cells (Tfr) able to inhibit antibody production are enriched at the lymph node T-B border and rare in germinal centers. *Journal of Immunology,* 198.

SCHLUNDT, A., NIESSING, D., HEISSMEYER, V. & SATTLER, M. 2016. RNA recognition by Roquin in posttranscriptional gene regulation. *Wiley Interdiscip Rev RNA,* 7**,** 455-69.

SCHMITT, H., SELL, S., KOCH, J., SEEFRIED, M., SONNEWALD, S., DANIEL, C., WINKLER, T. H. & NITSCHKE, L. 2016. Siglec-H protects from virus-triggered severe systemic autoimmunity. *J Exp Med,* 213**,** 1627-44.

SCHORLE, H., HOLTSCHKE, T., HUNIG, T., SCHIMPL, A. & HORAK, I. 1991. Development and Function of T-Cells in Mice Rendered Interleukin-2 Deficient by Gene Targeting. *Nature,* 352**,** 621-624.

SCHUR, P. H. 1988. IgG subclasses. A historical perspective. *Monogr Allergy,* 23**,** 1-11.

SCHWARTZ, M., ZHANG, Y. & ROSENBLATT, J. D. 2016. B cell regulation of the anti-tumor response and role in carcinogenesis. *Journal for Immunotherapy of Cancer,* 4.

SCHWARTZ, R. H. 2003. T cell anergy. *Annu Rev Immunol,* 21**,** 305-34.

SCHWARTZ, R. H. 2012. Historical Overview of Immunological Tolerance. *Cold Spring Harbor Perspectives in Biology,* 4.

SEBZDA, E., MARIATHASAN, S., OHTEKI, T., JONES, R., BACHMANN, M. F. & OHASHI, P. S. 1999. Selection of the T cell repertoire. *Annu Rev Immunol,* 17**,** 829-74.

SEDIGHI, S., AGHAEI, M., MUSAVI, S. & NOMALI, M. 2014. Relationship between Serum Level of Interleukin-2 in Patients with Systemic Lupus Erythematosus and Disease Activity in Comparison with Control Group. *J Clin Diagn Res,* 8**,** MC16-8.

SEO, E. H., KANG, J., KIM, K. H., CHO, M. C., LEE, S., KIM, H. J., KIM, J. H., KIM, E. J., PARK, D. K., KIM, S. H., CHOI, Y. K., KIM, J. M., HONG, J. T. & YOON, D. Y. 2008. Detection of expressed IL-32 in human stomach cancer using ELISA and immunostaining. *Journal of Microbiology and Biotechnology,* 18**,** 1606-1612.

SEOK, J., WARREN, H. S., CUENCA, A. G., MINDRINOS, M. N., BAKER, H. V., XU, W., RICHARDS, D. R., MCDONALD-SMITH, G. P., GAO, H., HENNESSY, L., FINNERTY, C. C., LOPEZ, C. M., HONARI, S., MOORE, E. E., MINEI, J. P., CUSCHIERI, J., BANKEY, P. E., JOHNSON, J. L., SPERRY, J., NATHENS, A. B., BILLIAR, T. R., WEST, M. A., JESCHKE, M. G., KLEIN, M. B., GAMELLI, R. L., GIBRAN, N. S., BROWNSTEIN, B. H., MILLER-GRAZIANO, C., CALVANO, S. E., MASON, P. H., COBB, J. P., RAHME, L. G., LOWRY, S. F., MAIER, R. V., MOLDAWER, L. L., HERNDON, D. N., DAVIS, R. W., XIAO, W., TOMPKINS, R. G., INFLAMMATION &

HOST RESPONSE TO INJURY, L. S. C. R. P. 2013. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A,* 110**,** 3507-12.

SHAMANNA, R. A., HOQUE, M., LEWIS-ANTES, A., AZZAM, E. I., LAGUNOFF, D., PE'ERY, T. & MATHEWS, M. B. 2011. The NF90/NF45 complex participates in DNA break repair via nonhomologous end joining. *Mol Cell Biol,* 31**,** 4832-43.

SHAY, T., JOJIC, V., ZUK, O., ROTHAMEL, K., PUYRAIMOND-ZEMMOUR, D., FENG, T., WAKAMATSU, E., BENOIST, C., KOLLER, D., REGEV, A. & CONSORTIUM, I. 2013. Conservation and divergence in the transcriptional programs of the human and mouse immune systems. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 2946-2951.

SHEN, P. & FILLATREAU, S. 2015. Suppressive functions of B cells in infectious diseases. *International Immunology,* 27**,** 513-519.

SHEN, P., ROCH, T., LAMPROPOULOU, V., O'CONNOR, R. A., STERVBO, U., HILGENBERG, E., RIES, S., DANG, V. D., JAIMES, Y., DARIDON, C., LI, R., JOUNEAU, L., BOUDINOT, P., WILANTRI, S., SAKWA, I., MIYAZAKI, Y., LEECH, M. D., MCPHERSON, R. C., WIRTZ, S., NEURATH, M., HOEHLIG, K., MEINL, E., GRUTZKAU, A., GRUN, J. R., HORN, K., KUHL, A. A., DORNER, T., BAR-OR, A., KAUFMANN, S. H. E., ANDERTON, S. M. & FILLATREAU, S. 2014. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature,* 507**,** 366-+.

SHEVACH, E. M. 2012. Application of IL-2 therapy to target T regulatory cell function. *Trends in Immunology,* 33**,** 626-632.

SHI, J., HOU, S., FANG, Q., LIU, X., LIU, X. & QI, H. 2018. PD-1 Controls Follicular T Helper Cell Positioning and Function. *Immunity,* 49**,** 264-274 e4.

SHI, L., GODFREY, W. R., LIN, J., ZHAO, G. & KAO, P. N. 2007a. NF90 regulates inducible IL-2 gene expression in T cells. *J Exp Med,* 204**,** 971-7.

SHI, L., QIU, D., ZHAO, G., CORTHESY, B., LEES-MILLER, S., REEVES, W. H. & KAO, P. N. 2007b. Dynamic binding of Ku80, Ku70 and NF90 to the IL-2 promoter in vivo in activated T-cells. *Nucleic Acids Res,* 35**,** 2302-10.

SHI, L., ZHAO, G., QIU, D., GODFREY, W. R., VOGEL, H., RANDO, T. A., HU, H. & KAO, P. N. 2005. NF90 regulates cell cycle exit and terminal myogenic differentiation by direct binding to the 3'-untranslated region of MyoD and p21WAF1/CIP1 mRNAs. *J Biol Chem,* 280**,** 18981-9.

SHIH, T. C., HSIEH, S. Y., HSIEH, Y. Y., CHEN, T. C., YEH, C. Y., LIN, C. J., LIN, D. Y. & CHIU, C. T. 2008. Aberrant activation of nuclear factor of activated T cell 2 in lamina propria mononuclear cells in ulcerative colitis. *World J Gastroenterol,* 14**,** 1759-67.

SHIM, J., LIM, H., J, R. Y. & KARIN, M. 2002a. Nuclear export of NF90 is required for interleukin-2 mRNA stabilization. *Mol Cell,* 10**,** 1331-44.

SHIM, J., LIM, H., YATES, J. R. & KARIN, M. 2002b. Nuclear export of NF90 is required for interleukin-2 mRNA stabilization. *Molecular Cell,* 10**,** 1331-1344.

SHIMOMURA, Y., MIZOGUCHI, E., SUGIMOTO, K., KIBE, R., BENNO, Y., MIZOGUCHI, A. & BHAN, A. K. 2008. Regulatory role of B-1B cells in chronic colitis. *International Immunology,* 20**,** 729-737.

SHIOYA, M., NISHIDA, A., YAGI, Y., OGAWA, A., TSUJIKAWA, T., KIM-MITSUYAMA, S., TAKAYANAGI, A., SHIMIZU, N., FUJIYAMA, Y. & ANDOH, A. 2007. Epithelial overexpression of interleukin-32alpha in inflammatory bowel disease. *Clin Exp Immunol,* 149**,** 480-6.

SHODA, H., FUJIO, K., YAMAGUCHI, Y., OKAMOTO, A., SAWADA, T., KOCHI, Y. & YAMAMOTO, K. 2006. Interactions between IL-32 and tumor necrosis factor alpha contribute to the exacerbation of immune-inflammatory diseases. *Arthritis Res Ther,* 8**,** R166.

SHPARAGO, N., ZELAZOWSKI, P., JIN, L., MCINTYRE, T. M., STUBER, E., PECANHA, L. M., KEHRY, M. R., MOND, J. J., MAX, E. E. & SNAPPER, C. M. 1996. IL-10 selectively regulates murine Ig isotype switching. *Int Immunol,* 8**,** 781-90.

SHULGA-MORSKAYA, S., DOBLES, M., WALSH, M. E., NG, L. G., MACKAY, F., RAO, S. P., KALLED, S. L. & SCOTT, M. L. 2004. B cell-activating factor belonging to the TNF family acts through separate receptors to support B cell survival and T cell-independent antibody formation. *Journal of Immunology,* 173**,** 2331-2341.

SIEWE, B., STAPLETON, J. T., MARTINSON, J., KESHAVARZIAN, A., KAZMI, N., DEMARAIS, P. M., FRENCH, A. L. & LANDAY, A. 2013. Regulatory B cell frequency correlates with markers of HIV disease progression and attenuates anti-HIV CD8(+) T cell function in vitro. *Journal of Leukocyte Biology,* 93**,** 811-818.

SIMPSON, N., GATENBY, P. A., WILSON, A., MALIK, S., FULCHER, D. A., TANGYE, S. G., MANKU, H., VYSE, T. J., RONCADOR, G., HUTTLEY, G. A., GOODNOW, C. C., VINUESA, C. G. & COOK, M. C. 2010. Expansion of Circulating T Cells Resembling Follicular Helper T Cells Is a Fixed Phenotype That Identifies a Subset of Severe Systemic Lupus Erythematosus. *Arthritis and Rheumatism,* 62**,** 234-244.

SINGLETON, A. B., HARDY, J., TRAYNOR, B. J. & HOULDEN, H. 2010. Towards a complete resolution of the genetic architecture of disease. *Trends in Genetics,* 26**,** 438-442.

SLOOT, Y. J. E., SMIT, J. W., JOOSTEN, L. A. B. & NETEA-MAIER, R. T. 2018. Insights into the role of IL-32 in cancer. *Semin Immunol,* 38**,** 24-32.

SMITH, A. J., TOLEDO, C. M., WIETGREFE, S. W., DUAN, L., SCHACKER, T. W., REILLY, C. S. & HAASE, A. T. 2011. The immunosuppressive role of IL-32 in lymphatic tissue during HIV-1 infection. *J Immunol,* 186**,** 6576-84.

SMITH, C. D. & CYR, M. 1988. The history of lupus erythematosus. From Hippocrates to Osler. *Rheum Dis Clin North Am,* 14**,** 1-14.

SMITH, K. A. 1988. Interleukin-2: inception, impact, and implications. *Science,* 240**,** 1169-76.

SOLOMOU, E. E., JUANG, Y. T., GOURLEY, M. F., KAMMER, G. M. & TSOKOS, G. C. 2001. Molecular basis of deficient IL-2 production in T cells from patients with systemic lupus erythematosus. *Journal of Immunology,* 166**,** 4216-4222.

SORRENTINO, C. & DI CARLO, E. 2009a. Expression of IL-32 in Human Lung Cancer Is Related to the Histotype and Metastatic Phenotype. *American Journal of Respiratory and Critical Care Medicine,* 180**,** 769-779.

SORRENTINO, C. & DI CARLO, E. 2009b. Expression of IL-32 in human lung cancer is related to the histotype and metastatic phenotype. *Am J Respir Crit Care Med,* 180**,** 769-79.

SPECTOR, D. L. & LAMOND, A. I. 2011. Nuclear speckles. *Cold Spring Harb Perspect Biol,* 3.

SPOLSKI, R., KASHYAP, M., ROBINSON, C., YU, Z. & LEONARD, W. J. 2008. IL-21 signaling is critical for the development of type I diabetes in the NOD mouse. *Proc Natl Acad Sci U S A,* 105**,** 14028-33.

SPRENT, J. 1993. The thymus and T-cell tolerance. *Ann N Y Acad Sci,* 681**,** 5-15.

SPRENT, J. & WEBB, S. R. 1987. Function and specificity of T cell subsets in the mouse. *Adv Immunol,* 41**,** 39-133.

STOHL, W., XU, D., KIM, K. S., KOSS, M. N., JORGENSEN, T. N., DEOCHARAN, B., METZGER, T. E., BIXLER, S. A., HONG, Y. S., AMBROSE, C. M., MACKAY, F., MOREL, L., PUTTERMAN,

C., KOTZIN, B. L. & KALLED, S. L. 2005. BAFF overexpression and accelerated glomerular disease in mice with an incomplete genetic predisposition to systemic lupus erythematosus. *Arthritis Rheum,* 52**,** 2080-91.

STONE, E. L., PEPPER, M., KATAYAMA, C. D., KERDILES, Y. M., LAI, C. Y., EMSLIE, E., LIN, Y. C., YANG, E., GOLDRATH, A. W., LI, M. O., CANTRELL, D. A. & HEDRICK, S. M. 2015. ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. *Immunity,* 42**,** 239-251.

STRIOGA, M., PASUKONIENE, V. & CHARACIEJUS, D. 2011. CD8(+) CD28(-) and CD8(+) CD57(+) T cells and their role in health and disease. *Immunology,* 134**,** 17-32.

SU, T. T. & RAWLINGS, D. J. 2002. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. *Journal of Immunology,* 168**,** 2101-2110.

SUSTER, S. & ROSAI, J. 1992. Cystic thymomas. A clinicopathologic study of ten cases. *Cancer,* 69**,** 92-7.

TAKAO, K. & MIYAKAWA, T. 2015. Genomic responses in mouse models greatly mimic human inflammatory diseases (vol 112, pg 1167, 2014). *Proceedings of the National Academy of Sciences of the United States of America,* 112**,** E1163-E1167.

TAKEUCHI, O. & AKIRA, S. 2010. Pattern recognition receptors and inflammation. *Cell,* 140**,** 805-20.

TANG, F. & ZHENG, P. 2018. Tumor cells versus host immune cells: whose PD-L1 contributes to PD-1/PD-L1 blockade mediated cancer immunotherapy? *Cell and Bioscience,* 8.

TANG, Q. 2015. Therapeutic window of interleukin-2 for autoimmune diseases. *Diabetes,* 64**,** 1912-3.

TANG, Q., ADAMS, J. Y., PENARANDA, C., MELLI, K., PIAGGIO, E., SGOUROUDIS, E., PICCIRILLO, C. A., SALOMON, B. L. & BLUESTONE, J. A. 2008. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity,* 28**,** 687-697.

TAO, L. L. & REESE, T. A. 2017. Making Mouse Models That Reflect Human Immune Responses. *Trends in Immunology,* 38**,** 181-193.

TARLINTON, D., RADBRUCH, A., HIEPE, F. & DORNER, T. 2008. Plasma cell differentiation and survival. *Curr Opin Immunol,* 20**,** 162-9.

TARLINTON, D. M. & SMITH, K. G. 2000. Dissecting affinity maturation: a model explaining selection of antibody-forming cells and memory B cells in the germinal centre. *Immunol Today,* 21**,** 436-41.

TELL, G., DAMANTE, G., CALDWELL, D. & KELLEY, M. R. 2005. The intracellular localization of APE1/Ref-1: More than a passive phenomenon? *Antioxidants & Redox Signaling,* 7**,** 367-384.

TER BORG, E. J., HORST, G., HUMMEL, E. J., LIMBURG, P. C. & KALLENBERG, C. G. 1990. Measurement of increases in anti-double-stranded DNA antibody levels as a predictor of disease exacerbation in systemic lupus erythematosus. A long-term, prospective study. *Arthritis Rheum,* 33**,** 634-43.

THOMPSON, I. M., ANKERST, D. P., CHI, C., LUCIA, M. S., GOODMAN, P. J., CROWLEY, J. J., PARNES, H. L. & COLTMAN, C. A., JR. 2005. Operating characteristics of prostate-specific antigen in men with an initial PSA level of 3.0 ng/ml or lower. *JAMA,* 294**,** 66-70.

TIEGS, S. L., RUSSELL, D. M. & NEMAZEE, D. 1993. Receptor editing in self-reactive bone marrow B cells. *J Exp Med,* 177**,** 1009-20.

TOELLNER, K. M. 2014. Cognate interactions: extrafollicular IL-4 drives germinal-center reactions, a new role for an old cytokine. *Eur J Immunol,* 44**,** 1917-20.

TOMINAGA-YAMANAKA, K., ABDELMOHSEN, K., MARTINDALE, J. L., YANG, X., TAUB, D. D. & GOROSPE, M. 2012. NF90 coordinately represses the senescence-associated secretory phenotype. *Aging (Albany NY),* 4**,** 695-708.

TSOKOS, G. C. 2011. Systemic lupus erythematosus. *N Engl J Med,* 365**,** 2110-21.

TSUBATA, T., WU, J. & HONJO, T. 1993. B-cell apoptosis induced by antigen receptor crosslinking is blocked by a T-cell signal through CD40. *Nature,* 364**,** 645-8.

UGARTE-GIL, M. F., PONS-ESTEL, G. J., MOLINEROS, J., WOJDYLA, D., MCGWIN, G., JR., NATH, S. K., PONS-ESTEL, B. A., ALARCON-RIQUELME, M. & ALARCON, G. S. 2016. Disease features and outcomes in United States lupus patients of Hispanic origin and their Mestizo counterparts in Latin America: a commentary. *Rheumatology (Oxford),* 55**,** 436-40.

UHLEN, M., FAGERBERG, L., HALLSTROM, B. M., LINDSKOG, C., OKSVOLD, P., MARDINOGLU, A., SIVERTSSON, A., KAMPF, C., SJOSTEDT, E., ASPLUND, A., OLSSON, I., EDLUND, K., LUNDBERG, E., NAVANI, S., SZIGYARTO, C. A., ODEBERG, J., DJUREINOVIC, D., TAKANEN, J. O., HOBER, S., ALM, T., EDQVIST, P. H., BERLING, H., TEGEL, H., MULDER, J., ROCKBERG, J., NILSSON, P., SCHWENK, J. M., HAMSTEN, M., VON FEILITZEN, K., FORSBERG, M., PERSSON, L., JOHANSSON, F., ZWAHLEN, M., VON HEIJNE, G., NIELSEN, J. & PONTEN, F. 2015. Proteomics. Tissue-based map of the human proteome. *Science,* 347**,** 1260419.

VALENCIA, X., YARBORO, C., ILLEI, G. & LIPSKY, P. E. 2007. Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. *J Immunol,* 178**,** 2579-88.

VALENTINE, M. A. & LICCIARDI, K. A. 1992. Rescue from anti-IgM-induced programmed cell death by the B cell surface proteins CD20 and CD40. *Eur J Immunol,* 22**,** 3141-8.

VAN DER SIJDE, M. R., NG, A. & FU, J. Y. 2014. Systems genetics: From GWAS to disease pathways. *Biochimica Et Biophysica Acta-Molecular Basis of Disease,* 1842**,** 1903-1909.

VAN DER VLIET, H. J. J. & NIEUWENHUIS, E. E. 2007. IPEX as a result of Mutations in FOXP3. *Clinical & Developmental Immunology*.

VAN EWIJK, W., BREKELMANS, P. J., JACOBS, R. & WISSE, E. 1988. Lymphoid microenvironments in the thymus and lymph node. *Scanning Microsc,* 2**,** 2129-40.

VANCE, C., ROGELJ, B., HORTOBAGYI, T., DE VOS, K. J., NISHIMURA, A. L., SREEDHARAN, J., HU, X., SMITH, B., RUDDY, D., WRIGHT, P., GANESALINGAM, J., WILLIAMS, K. L., TRIPATHI, V., AL-SARAJ, S., AL-CHALABI, A., LEIGH, P. N., BLAIR, I. P., NICHOLSON, G., DE BELLEROCHE, J., GALLO, J. M., MILLER, C. C. & SHAW, C. E. 2009. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science,* 323**,** 1208-11.

VEERMAN, K. M., WILLIAMS, M. J., UCHIMURA, K., SINGER, M. S., MERZABAN, J. S., NAUS, S., CARLOW, D. A., OWEN, P., RIVERA-NIEVES, J., ROSEN, S. D. & ZILTENER, H. J. 2007. Interaction of the selectin ligand PSGL-1 with chemokines CCL21 and CCL19 facilitates efficient homing of T cells to secondary lymphoid organs. *Nat Immunol,* 8**,** 532-9.

VELU, V., TITANJI, K., ZHU, B., HUSAIN, S., PLADEVEGA, A., LAI, L., VANDERFORD, T. H., CHENNAREDDI, L., SILVESTRI, G., FREEMAN, G. J., AHMED, R. & AMARA, R. R. 2009. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature,* 458**,** 206-10.

VENIGALLA, R. K., TRETTER, T., KRIENKE, S., MAX, R., ECKSTEIN, V., BLANK, N., FIEHN, C., HO, A. D. & LORENZ, H. M. 2008. Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25high,CD127 -/low regulatory T cells in patients with active systemic lupus erythematosus. *Arthritis Rheum,* 58**,** 2120-30.

VICTORA, G. D. & NUSSENZWEIG, M. C. 2012. Germinal centers. *Annu Rev Immunol,* 30**,** 429-57.

VICTORA, G. D., SCHWICKERT, T. A., FOOKSMAN, D. R., KAMPHORST, A. O., MEYER-HERMANN, M., DUSTIN, M. L. & NUSSENZWEIG, M. C. 2010. Germinal Center Dynamics Revealed by Multiphoton Microscopy with a Photoactivatable Fluorescent Reporter. *Cell,* 143**,** 592-605.

VILAR, M. J. & SATO, E. I. 2002. Estimating the incidence of systemic lupus erythematosus in a tropical region (Natal, Brazil). *Lupus,* 11**,** 528-32.

VINCENT, F. B., MORAND, E. F., SCHNEIDER, P. & MACKAY, F. 2014. The BAFF/APRIL system in SLE pathogenesis. *Nat Rev Rheumatol,* 10**,** 365-73.

VINUESA, C. G., COOK, M. C., ANGELUCCI, C., ATHANASOPOULOS, V., RUI, L., HILL, K. M., YU, D., DOMASCHENZ, H., WHITTLE, B., LAMBE, T., ROBERTS, I. S., COPLEY, R. R., BELL, J. I., CORNALL, R. J. & GOODNOW, C. C. 2005a. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature,* 435**,** 452-8.

VINUESA, C. G., LINTERMAN, M. A., YU, D. & MACLENNAN, I. C. 2016. Follicular Helper T Cells. *Annu Rev Immunol,* 34**,** 335-68.

VINUESA, C. G., SANZ, I. & COOK, M. C. 2009. Dysregulation of germinal centres in autoimmune disease. *Nat Rev Immunol,* 9**,** 845-57.

VINUESA, C. G., TANGYE, S. G., MOSER, B. & MACKAY, C. R. 2005b. Follicular B helper T cells in antibody responses and autoimmunity. *Nature Reviews Immunology,* 5**,** 853-865.

VINUESA, C. G., TANGYE, S. G., MOSER, B. & MACKAY, C. R. 2005c. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol,* 5**,** 853-65.

VITETTA, E. S., MELCHER, U., MCWILLIAMS, M., LAMM, M. E., PHILLIPS-QUAGLIATA, J. M. & UHR, J. W. 1975. Cell surface immunoglobulin. XI. The appearance of an IgD-like molecule on murine lymphoid cells during ontogeny. *J Exp Med,* 141**,** 206-15.

VON BOEHMER, H. & FEHLING, H. J. 1997. Structure and function of the pre-T cell receptor. *Annu Rev Immunol,* 15**,** 433-52.

VON BULOW, G. U., VAN DEURSEN, J. M. & BRAM, R. J. 2001. Regulation of the T-independent humoral response by TACI. *Immunity,* 14**,** 573-582.

VON HERRATH, M. G. & NEPOM, G. T. 2005. Lost in translation: barriers to implementing clinical immunotherapeutics for autoimmunity. *Journal of Experimental Medicine,* 202**,** 1159-1162.

VON SPEE-MAYER, C., SIEGERT, E., ABDIRAMA, D., ROSE, A., KLAUS, A., ALEXANDER, T., ENGHARD, P., SAWITZKI, B., HIEPE, F., RADBRUCH, A., BURMESTER, G. R., RIEMEKASTEN, G. & HUMRICH, J. Y. 2016. Low-dose interleukin-2 selectively corrects regulatory T cell defects in patients with systemic lupus erythematosus. *Ann Rheum Dis,* 75**,** 1407-15.

VORA, K. A., WANG, L. C., RAO, S. P., LIU, Z. Y., MAJEAU, G. R., CUTLER, A. H., HOCHMAN, P. S., SCOTT, M. L. & KALLED, S. L. 2003. Cutting edge: Germinal centers formed in the absence of B cell-activating factor belonging to the TNF family exhibit impaired maturation and function. *Journal of Immunology,* 171**,** 547-551.

WAKKACH, A., COTTREZ, F. & GROUX, H. 2000. Can interleukin-10 be used as a true immunoregulatory cytokine? *Eur Cytokine Netw,* 11**,** 153-60.

WALKER, L. S. K. & ABBAS, A. K. 2002. The enemy within: Keeping self-reactive T cells at bay in the periphery. *Nature Reviews Immunology,* 2**,** 11-19.

WALPORT, M. J. 2002a. Complement and systemic lupus erythematosus. *Arthritis Research & Therapy,* 4**,** S279-S293.

WALPORT, M. J. 2002b. Complement and systemic lupus erythematosus. *Arthritis Res,* 4 Suppl 3**,** S279-93.

WAN, C. H., BORGESON, B., PHANSE, S., TU, F., DREW, K., CLARK, G., XIONG, X. J., KAGAN, O., KWAN, J., BEZGINOV, A., CHESSMAN, K., PAL, S., CROMAR, G., PAPOULAS, O., NI, Z. Y., BOUTZ, D. R., STOILOVA, S., HAVUGIMANA, P. C., GUO, X. H., MALTY, R. H., SAROV, M., GREENBLATT, J., BABU, M., DERRY, W. B., TILLIER, E. R., WALLINGFORD, J. B., PARKINSON, J., MARCOTTE, E. M. & EMILI, A. 2015. Panorama of ancient metazoan macromolecular complexes. *Nature,* 525**,** 339-+.

WANG, C. M., CHANG, S. W., WU, Y. J., LIN, J. C., HO, H. H., CHOU, T. C., YANG, B., WU, J. & CHEN, J. Y. 2014a. Genetic variations in Toll-like receptors (TLRs 3/7/8) are associated with systemic lupus erythematosus in a Taiwanese population. *Sci Rep,* 4**,** 3792.

WANG, E. C. Y., LAWSON, T. M., VEDHARA, K., MOSS, P. A. H., LEHNER, P. J. & BORYSIEWICZ, L. K. 1997. CD8(high)+ (CD57+) T cells in patients with rheumatoid arthritis. *Arthritis and Rheumatism,* 40**,** 237-248.

WANG, J., VOCK, V. M., LI, S., OLIVAS, O. R. & WILKINSON, M. F. 2002. A quality control pathway that down-regulates aberrant T-cell receptor (TCR) transcripts by a mechanism requiring UPF2 and translation. *J Biol Chem,* 277**,** 18489-93.

WANG, J., WANG, Q., HAN, T., LI, Y. K., ZHU, S. L., AO, F., FENG, J., JING, M. Z., WANG, L., YE, L. B. & ZHU, Y. 2015. Soluble interleukin-6 receptor is elevated during influenza A virus infection and mediates the IL-6 and IL-32 inflammatory cytokine burst. *Cell Mol Immunol,* 12**,** 633-44.

WANG, L., ZHAO, P., MA, L., SHAN, Y., JIANG, Z., WANG, J. & JIANG, Y. 2014b. Increased interleukin 21 and follicular helper T-like cells and reduced interleukin 10+ B cells in patients with new-onset systemic lupus erythematosus. *J Rheumatol,* 41**,** 1781-92.

WANG, Q., LI, L., WANG, X., LIU, H. & YAO, X. 2014c. Understanding the recognition mechanisms of Zalpha domain of human editing enzyme ADAR1 (hZalpha(ADAR1)) and various Z-DNAs from molecular dynamics simulation. *J Mol Model,* 20**,** 2500.

WANG, Y., YANG, Y., ZHU, Y., LI, L., CHEN, F. & ZHANG, L. 2017. Polymorphisms and expression of IL-32: impact on genetic susceptibility and clinical outcome of lung cancer. *Biomarkers,* 22**,** 165-170.

WANG, Y., ZHOU, B., ZHAO, Y., YU, X., LIU, Y. & ZHANG, L. 2016. Association of Plasma IL-32 Levels and Gene Polymorphisms with Systemic Lupus Erythematosus in Chinese Han Population. *Dis Markers,* 2016**,** 2460206.

WANG, Y. T., WANG, H. B., YAO, H., LI, C. S., FANG, J. Y. & XU, J. 2018. Regulation of PD-L1: Emerging Routes for Targeting Tumor Immune Evasion. *Frontiers in Pharmacology,* 9.

WEJKSZA, K., LEE-CHANG, C., BODOGAI, M., BONZO, J., GONZALEZ, F. J., LEHRMANN, E., BECKER, K. & BIRAGYN, A. 2013. Cancer-Produced Metabolites of 5-Lipoxygenase Induce Tumor-Evoked Regulatory B Cells via Peroxisome Proliferator-Activated Receptor alpha. *Journal of Immunology,* 190**,** 2575-2584.

WEST, E. E., JIN, H. T., RASHEED, A. U., PENALOZA-MACMASTER, P., HA, S. J., TAN, W. G., YOUNGBLOOD, B., FREEMAN, G. J., SMITH, K. A. & AHMED, R. 2013. PD-L1 blockade synergizes with IL-2 therapy in reinvigorating exhausted T cells. *J Clin Invest,* 123**,** 2604-15.

WHERRY, E. J. & KURACHI, M. 2015. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol,* 15**,** 486-99.

WING, J. B., ISE, W., KUROSAKI, T. & SAKAGUCHI, S. 2014. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity,* 41**,** 1013-25.

WOLKOWICZ, U. M. & COOK, A. G. 2012. NF45 dimerizes with NF90, Zfr and SPNR via a conserved domain that has a nucleotidyltransferase fold. *Nucleic Acids Res,* 40**,** 9356-68.

WOLLENBERG, I., AGUA-DOCE, A., HERNANDEZ, A., ALMEIDA, C., OLIVEIRA, V. G., FARO, J. & GRACA, L. 2011a. Regulation of the Germinal Center Reaction by Foxp3(+) Follicular Regulatory T Cells. *Journal of Immunology,* 187**,** 4553-4560.

WOLLENBERG, I., AGUA-DOCE, A., HERNANDEZ, A., ALMEIDA, C., OLIVEIRA, V. G., FARO, J. & GRACA, L. 2011b. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol,* 187**,** 4553-60.

WRAMMERT, J. & AHMED, R. 2008. Maintenance of serological memory. *Biol Chem,* 389**,** 537-9.

WU, B. & HUR, S. 2015. How RIG-I like receptors activate MAVS. *Curr Opin Virol,* 12**,** 91-8.

WU, R. C., LIU, S. J., CHACON, J. A., WU, S., LI, Y. F., SUKHUMALCHANDRA, P., MURRAY, J. L., MOLLDREM, J. J., HWU, P., PIRCHER, H., LIZEE, G. & RADVANYI, L. G. 2012. Detection and Characterization of a Novel Subset of CD8(+)CD57(+) T Cells in Metastatic Melanoma with an Incompletely Differentiated Phenotype. *Clinical Cancer Research,* 18**,** 2465-2477.

XING, Y. & HOGQUIST, K. A. 2012. T-Cell Tolerance: Central and Peripheral. *Cold Spring Harbor Perspectives in Biology,* 4.

XU, H., LIU, J., CUI, X., ZUO, Y., ZHANG, Z., LI, Y., TAO, R., LI, Y. & PANG, J. 2015. Increased frequency of circulating follicular helper T cells in lupus patients is associated with autoantibody production in a CD40L-dependent manner. *Cell Immunol,* 295**,** 46-51.

XU, H. P., LI, X. Y., LIU, D., LI, J. F., ZHANG, X., CHEN, X., HOU, S. Y., PENG, L. X., XU, C. G., LIU, W. L., ZHANG, L. F. & QI, H. 2013. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature,* 496**,** 523-+.

XU, S. L. & LAM, K. P. 2001. B-cell maturation protein, which binds the tumor necrosis factor family members BAFF and APRIL, is dispensable for humoral immune responses. *Molecular and Cellular Biology,* 21**,** 4067-4074.

XU, Z., DONG, A., FENG, Z. & LI, J. 2017. Interleukin-32 promotes lipid accumulation through inhibition of cholesterol efflux. *Exp Ther Med,* 14**,** 947-952.

YANG, S., DENG, P., ZHU, Z., ZHU, J., WANG, G., ZHANG, L., CHEN, A. F., WANG, T., SARKAR, S. N., BILLIAR, T. R. & WANG, Q. 2014a. Adenosine deaminase acting on RNA 1 limits RIG-

I RNA detection and suppresses IFN production responding to viral and endogenous RNAs. *J Immunol,* 193**,** 3436-45.

YANG, X., YANG, J., CHU, Y. W., XUE, Y., XUAN, D. D., ZHENG, S. C. & ZOU, H. J. 2014b. T Follicular Helper Cells and Regulatory B Cells Dynamics in Systemic Lupus Erythematosus. *Plos One,* 9.

YANG, Y., MUZNY, D. M., REID, J. G., BAINBRIDGE, M. N., WILLIS, A., WARD, P. A., BRAXTON, A., BEUTEN, J., XIA, F., NIU, Z., HARDISON, M., PERSON, R., BEKHEIRNIA, M. R., LEDUC, M. S., KIRBY, A., PHAM, P., SCULL, J., WANG, M., DING, Y., PLON, S. E., LUPSKI, J. R., BEAUDET, A. L., GIBBS, R. A. & ENG, C. M. 2013. Clinical whole-exome sequencing for the diagnosis of mendelian disorders. *N Engl J Med,* 369**,** 1502-11.

YANG, Y., MUZNY, D. M., XIA, F., NIU, Z., PERSON, R., DING, Y., WARD, P., BRAXTON, A., WANG, M., BUHAY, C., VEERARAGHAVAN, N., HAWES, A., CHIANG, T., LEDUC, M., BEUTEN, J., ZHANG, J., HE, W., SCULL, J., WILLIS, A., LANDSVERK, M., CRAIGEN, W. J., BEKHEIRNIA, M. R., STRAY-PEDERSEN, A., LIU, P., WEN, S., ALCARAZ, W., CUI, H., WALKIEWICZ, M., REID, J., BAINBRIDGE, M., PATEL, A., BOERWINKLE, E., BEAUDET, A. L., LUPSKI, J. R., PLON, S. E., GIBBS, R. A. & ENG, C. M. 2014c. Molecular findings among patients referred for clinical whole-exome sequencing. *JAMA,* 312**,** 1870-9.

YANG, Y., WANG, Z. H., ZHOU, Y. M., WANG, X. X., XIANG, J. B. & CHEN, Z. Y. 2015. Dysregulation of over-expressed IL-32 in colorectal cancer induces metastasis. *World Journal of Surgical Oncology,* 13.

YAP, D. Y. & LAI, K. N. 2013. The role of cytokines in the pathogenesis of systemic lupus erythematosus - from bench to bedside. *Nephrology (Carlton),* 18**,** 243-55.

YARILINA, A., XU, K., CHEN, J. & IVASHKIV, L. B. 2011. TNF activates calcium-nuclear factor of activated T cells (NFAT)c1 signaling pathways in human macrophages. *Proc Natl Acad Sci U S A,* 108**,** 1573-8.

YATES, J., WHITTINGTON, A., MITCHELL, P., LECHLER, R. I., LIGHTSTONE, L. & LOMBARDI, G. 2008. Natural regulatory T cells: number and function are normal in the majority of patients with lupus nephritis. *Clin Exp Immunol,* 153**,** 44-55.

YE, C., BRAND, D. & ZHENG, S. G. 2018. Targeting IL-2: an unexpected effect in treating immunological diseases. *Signal Transduct Target Ther,* 3**,** 2.

YELLIN, M. J., SINNING, J., COVEY, L. R., SHERMAN, W., LEE, J. J., GLICKMAN-NIR, E., SIPPEL, K. C., ROGERS, J., CLEARY, A. M., PARKER, M. & ET AL. 1994. T lymphocyte T cell-B cell-activating molecule/CD40-L molecules induce normal B cells or chronic lymphocytic leukemia B cells to express CD80 (B7/BB-1) and enhance their costimulatory activity. *J Immunol,* 153**,** 666-74.

YOUSEF, S., MARVIN, J., STEINBACH, M., LANGEMO, A., KOVACSOVICS, T., BINDER, M., KROGER, N., LUETKENS, T. & ATANACKOVIC, D. 2015. Immunomodulatory molecule PD-L1 is expressed on malignant plasma cells and myeloma-propagating pre-plasma cells in the bone marrow of multiple myeloma patients. *Blood Cancer Journal,* 5.

YOUSIF, N. G., AL-AMRAN, F. G., HADI, N., LEE, J. & ADRIENNE, J. 2013. Expression of IL-32 modulates NF-kappa B and p38 MAP kinase pathways in human esophageal cancer. *Cytokine,* 61**,** 223-227.

YU, C., GERSHWIN, M. E. & CHANG, C. 2014. Diagnostic criteria for systemic lupus erythematosus: a critical review. *J Autoimmun,* 48-49**,** 10-3.

YU, D., TAN, A. H., HU, X., ATHANASOPOULOS, V., SIMPSON, N., SILVA, D. G., HUTLOFF, A., GILES, K. M., LEEDMAN, P. J., LAM, K. P., GOODNOW, C. C. & VINUESA, C. G. 2007.

Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature,* 450**,** 299-303.

YU, Q., SHARMA, A., OH, S. Y., MOON, H. G., HOSSAIN, M. Z., SALAY, T. M., LEEDS, K. E., DU, H., WU, B., WATERMAN, M. L., ZHU, Z. & SEN, J. M. 2009. T cell factor 1 initiates the T helper type 2 fate by inducing the transcription factor GATA-3 and repressing interferon-gamma. *Nat Immunol,* 10**,** 992-9.

YUSUF, I., KAGEYAMA, R., MONTICELLI, L., JOHNSTON, R. J., DITORO, D., HANSEN, K., BARNETT, B. & CROTTY, S. 2010. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol,* 185**,** 190-202.

ZACCA, E. R., ONOFRIO, L. I., ACOSTA, C. D. V., FERRERO, P. V., ALONSO, S. M., RAMELLO, M. C., MUSSANO, E., ONETTI, L., CADILE, I. I., STANCICH, M. I., BONFANTI, M. C. T., MONTES, C. L., RODRIGUEZ, E. V. A. & GRUPPI, A. 2018. PD-L1(+) Regulatory B Cells Are Significantly Decreased in Rheumatoid Arthritis Patients and Increase After Successful Treatment. *Frontiers in Immunology,* 9.

ZENG, Q. L., LI, S. L., ZHOU, Y. B., OU, W. J., CAI, X. D., ZHANG, L. J., HUANG, W. L., HUANG, L. X. & WANG, Q. Q. 2014. Interleukin-32 contributes to invasion and metastasis of primary lung adenocarcinoma via NF-kappaB induced matrix metalloproteinases 2 and 9 expression. *Cytokine,* 65**,** 24-32.

ZEPP, J. A., NOLD-PETRY, C. A., DINARELLO, C. A. & NOLD, M. F. 2011. Protection from RNA and DNA viruses by IL-32. *J Immunol,* 186**,** 4110-8.

ZHANG, M., XIA, L., YANG, Y., LIU, S., JI, P., WANG, S., CHEN, Y., LIU, Z., ZHANG, Y., LU, S. & WANG, Y. 2018. PD-1 blockade augments humoral immunity through ICOS-mediated CD4(+) T cell instruction. *Int Immunopharmacol,* 66**,** 127-138.

ZHANG, W., PANG, Q., YU, S. F., YUAN, Z., WANG, P. & XIAO, Z. 2016. PD-L1 is Prognostic Factor of Human Esophageal Squamous Cell Carcinoma and Its Association With EGFR in Radiation Therapy. *International Journal of Radiation Oncology Biology Physics,* 96**,** S29-S29.

ZHANG, X., LINDWALL, E., GAUTHIER, C., LYMAN, J., SPENCER, N., ALARAKHIA, A., FRASER, A., ING, S., CHEN, M., WEBB-DETIEGE, T., ZAKEM, J., DAVIS, W., CHOI, Y. S. & QUINET, R. 2015. Circulating CXCR5+CD4+helper T cells in systemic lupus erythematosus patients share phenotypic properties with germinal center follicular helper T cells and promote antibody production. *Lupus,* 24**,** 909-17.

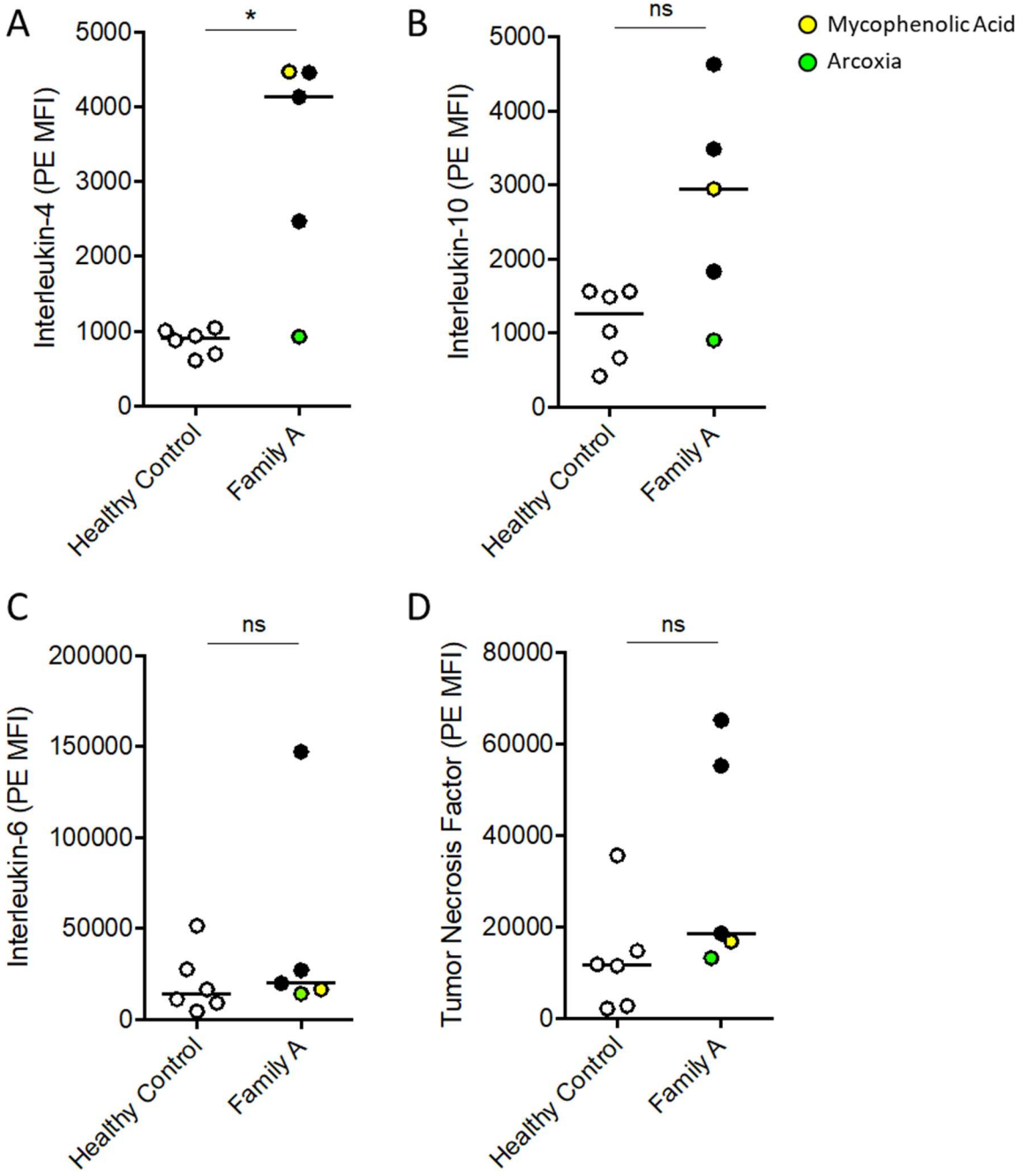
ZHAO, G., SHI, L., QIU, D., HU, H. & KAO, P. N. 2005. NF45/ILF2 tissue expression, promoter analysis, and interleukin-2 transactivating function. *Exp Cell Res,* 305**,** 312-23.

ZHAO, X., CHEN, M. & TAN, J. 2016. Knockdown of ZFR suppresses cell proliferation and invasion of human pancreatic cancer. *Biol Res,* 49**,** 26.

ZHU, P., JIANG, W., CAO, L., YU, W., PEI, Y., YANG, X., WAN, B., LIU, J. O., YI, Q. & YU, L. 2010. IL-2 mRNA stabilization upon PMA stimulation is dependent on NF90-Ser647 phosphorylation by protein kinase CbetaI. *J Immunol,* 185**,** 5140-9.

Appendices

Appendix A.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Mycophenolic Acid
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Healthy Control
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Healthy Control
Family A