

The role of TASL in B cells and autoimmune disease



Julia Johnstone
Lincoln College
University of Oxford

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Abstract

Systemic lupus erythematosus (SLE) is a highly sexually dimorphic chronic autoimmune disease, characterised by auto-antibodies to nuclear antigens. Genome wide association studies (GWAS) have revealed an association between SLE and *CXORF21*, an X-linked gene, which encodes the protein 'TLR-adaptor interacting with SLC15A4 on the lysosome' (TASL). TASL acts as an innate immune adaptor in the IRF5 pathway, and is required for the phosphorylation of IRF5 and the downstream transcription of interferon-stimulated genes (ISGs) in response to TLR7 or TLR9 stimulation. In this thesis, I investigated the role of TASL in B cells, both in response to immunisation and in the B6.MRL^{Lpr} murine model of SLE. I found that TASL is not required for the development or homeostasis of B cells, but is essential for the full activation of B cells by endolysosomal TLR signalling, and the subsequent production of cytokines and expression of ISGs. I also found that TASL plays an important role in the extrafollicular response, and in its absence the production of plasmablasts in response to T-dependent and T-independent antigens is dramatically reduced in females. Additionally, I identified that TASL is essential for the development of age-associated B cells (ABCs), a population that is expanded in aged people and autoimmune disease. TASL deletion also prevented the development of autoimmunity in the B6.MRL^{Lpr} model. Taken together, the results presented here suggest that TASL is important in the extrafollicular response and development of autoimmunity, and may play a role in the sexual dimorphism observed in SLE. This work also supports further investigation of TASL as a therapeutic target for SLE treatment.

Memorandum

This thesis is the result of my own original work and was carried out at the Kennedy Institute of Rheumatology, Oxford. All experimental data presented were collected and analysed by me. No part of this thesis has previously been submitted for any degree at this or any other university, however some of this work has formed the basis of a preprint:

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List of Abbreviations

ANAs	Anti-nuclear antibodies
APC	Antigen-presenting cell
BCR	B cell receptor
BM	Bone marrow
FDC	Follicular dendritic cell
GC	Germinal centre
GCBC	Germinal centre B cell
IFN	Interferon
IL-6	Interleukin 6
ISG	Interferon-stimulated gene
ISRE	Interferon-stimulated response element
IRF	Interferon response factor
LLPCs	Long-lived plasma cells
MHC II	Major histocompatibility complex class II
SHM	Somatic hypermutation
SNP	Single nucleotide polymorphism
SLE	Systemic Lupus Erythematosus
SRBCs	Sheep red blood cells
TASL	TLR adaptor interacting with SLC15A4 on the lysosome
Tfh	T follicular helper cell
Tfr	T follicular regulatory cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
PB	Plasmablast
PC	Plasma cell

pDC		Plasmacytoid dendritic cell
WT		Wild type
XCI		X-chromosome inactivation

1

Introduction

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1.1 B cells and their functions

B cells are lymphocytes that are responsible for the humoral immune response, but also play a critical role in other aspects of immunity. Whilst the most studied

role of B cells is their ability to produce antibodies, B cells play numerous roles in the immune system, including their ability to produce cytokines and act as antigen-presenting cells (APCs).

1.1.1 B cell development

B cells begin their development in the bone marrow. Common lymphoid progenitor cells commit to the B cell lineage, becoming pre-pro B cells. The heavy chain of the B cell receptor (BCR) is then formed, via rearrangement of VDJ segments, producing pro-B cells. Successful BCRs, with VDJ segments all in the correct reading frame, are then able to pair with surrogate light chains to form the pre-BCR, and surface expression of this receptor marks the transition to pre-B cells. In pre-B cells, the V and J segments of the light chain are rearranged to form a functional BCR. Once this has been achieved, pre-pro B cells proliferate, and alternative splicing of the heavy chain leads to expression of both IgD and IgM on the cell surface, forming naive B cells that are released into the periphery. Depending on the stimulus that the B cell receives, it may undergo either the germinal centre (GC) reaction or enter the extrafollicular response.

1.1.2 The GC reaction

The GC reaction occurs in secondary lymphoid tissue, and is critical for the development of high-affinity antibodies, long-lived plasma cells (LLPCs) and memory B cells (Fig. 1.1). GCs begin to form approximately one week after immunisation, although this time period, as well as the amount of time GCs remain for, depends on the antigen or pathogen[1]. During the GC reaction, B cells cycle between the anatomical dark zone (DZ) and light zone (LZ). In the DZ, B cells proliferate rapidly and undergo somatic hypermutation (SHM), a process in which point mutations are introduced into the variable region of the immunoglobulin gene locus by the enzyme activation-induced cytidine deaminase (AID)[2], thereby potentially altering the antigen affinity slightly. B cells then migrate to the LZ, where they test their new receptors on antigen bound to follicular dendritic cells (FDCs), and then compete

for help from T follicular helper (Tfh) cells, in order to receive signals to survive and proliferate, resulting in the selection of B cells with higher affinity BCRs, in a process known as affinity maturation. GC B cells (GCBCs) may then re-enter the DZ, repeating this process, or differentiate into plasma cells or memory B cells. The long-lived plasma cells produced by GCs are typically enriched for the highest affinity BCRs, leading to the theory that higher affinity causes a preference for signals for PC differentiation[3]. However, other evidence has shown that the affinity of pre-PC cells, a population defined by single cell sequencing of LZ GC B cells, is no different to other cells that remain in the GC, suggesting that the process of LLPC selection is affinity-independent[4].

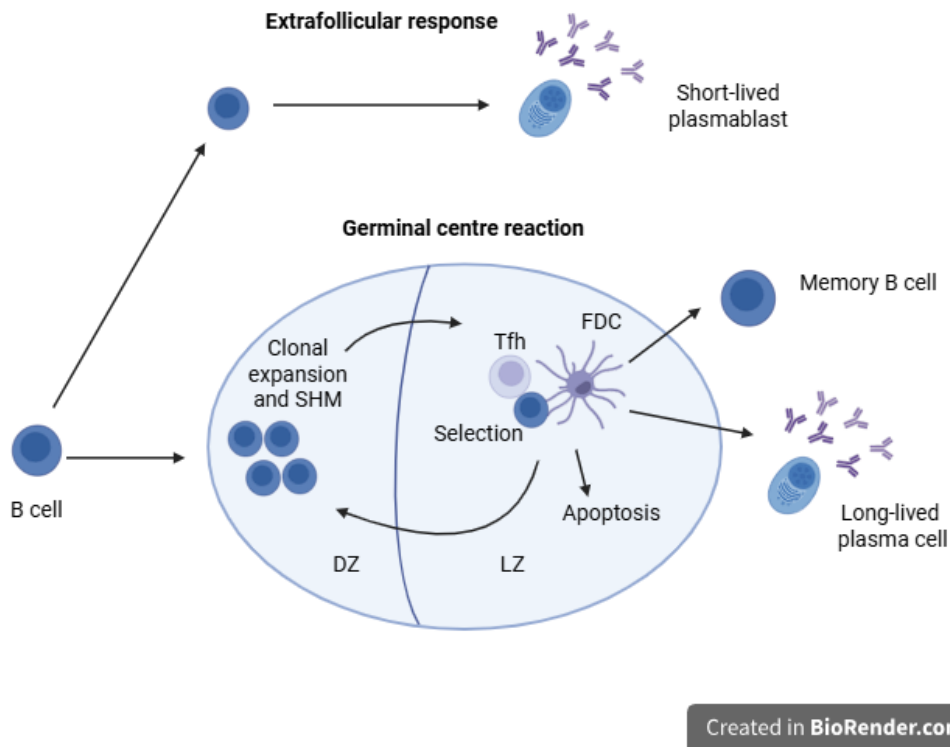


Figure 1.1: The germinal centre reaction Depending on the stimulus, B cells may either enter the germinal centre (GC) reaction or the extrafollicular response. During the GC reaction B cells proliferate and undergo somatic hypermutation (SHM) in the dark zone (DZ), then migrate to the light zone (LZ), where they test their receptors on antigen presented by follicular dendritic cells (FDCs) and compete for help from T follicular helper cells (Tfh) to receive signals to survive and proliferate, resulting in the selection of B cells with higher affinity B cell receptors (BCRs). Surviving GC B cells may then either re-enter the DZ, cycling through this process again, or may differentiate into long-lived plasma cells or memory B cells. Alternatively, B cells may enter the extrafollicular response, which results in the quick production of short-lived plasmablasts. Figure created in <https://BioRender.com>

In addition to Tfh cells, T follicular regulatory (Tfr) cells also play an important role in controlling the GC response. Tfr cells suppress the GC and selection of auto-reactive B cells, and additionally limit GC lifespan and help to terminate the GC reaction[5][6].

As well as point mutations in the variable region, AID can also introduce mutations that produce double-stranded DNA breaks in the switch region of immunoglobulin genes. This leads to class-switch recombination to the antibody isotypes IgG, IgA, and IgE. Class-switch recombination has traditionally been

thought to occur in the GC, however more recently it has been shown that this occurs mainly in pre-GC B cells[7].

Bcl6 is a key transcriptional regulator of the GC response, and is expressed both by GCBCs and Tfh cells. B cells upregulate *Bcl6* after activation, and expression of *Bcl6* is essential for sustained interaction with Tfh cells[8]. Bcl6 is a transcriptional repressor with a number of targets. One of these is Blimp1, which is encoded by *Prdm1* and is a transcription factor essential for plasmablast (PB) development. By inhibiting expression of *Blimp1*, Bcl6 delays the differentiation of PBs, thereby enabling time for SHM and affinity maturation to occur before subsequent plasmablast differentiation[9][10].

Upon activation, B cells also upregulate Epstein-Barr virus induced molecule-2 (EBI2). EBI2 plays a critical role in the positioning of B cells in the follicle. B cells that downregulate EBI2 expression move to a central location in the GC follicle, whereas high EBI2 expression leads to B cells localising to the outer follicle and extrafollicular region[11][12]. The downregulation of EBI2 and subsequent positioning of B cells in the centre of the follicle is essential for the GC reaction to occur[11]. In the absence of EBI2, mice were unable to generate early antibody responses to T-dependent antigens[11], demonstrating that EBI2 is also important in the regulation of the extrafollicular response by directing the localisation of B cells.

1.1.3 The extrafollicular response

The extrafollicular response begins around two days after antigen exposure, at which point B cells begin differentiating into PBs by upregulating Blimp1, a transcriptional repressor that promotes PB differentiation by inhibiting numerous genes required for alternative pathways, including AID and Bcl6[13]. The extrafollicular response rapidly produces short-lived PBs, providing quick protection against invading pathogens. The duration of the extrafollicular response differs dramatically for different pathogens, and can last from weeks to months. For instance, *Salmonella* induces an extrafollicular response that peaks around 7-10 days post-infection, but can last a month or more[14].

Traditionally, SHM and class-switching were thought to only occur in the GC reaction. However, as discussed above, it has now definitively been shown that class-switching occurs before the upregulation of Bcl6 and the entry of B cells into the GC[7]. There is increasing evidence that SHM also occurs extrafollicularly, both in the context of chronic bacterial infection by *Salmonella* and *E. muris*, and in murine models of SLE[15][16][17][18]. In MRL^{Lpr} mice, it was shown that active SHM occurred at the T zone-red pulp border, and not in the GC[17], and estimates showed that the rate of this SHM in autoimmune mice was similar to that in GCs generated by NP-CGG immunisation[19]. There is also evidence of positive selection for increased affinity in this extrafollicular environment[20].

In addition to plasmablasts, the extrafollicular response can also produce age-associated B cells (ABCs) [21]. ABCs were initially described in aged mice, found predominantly in the spleen, but are also increased in autoimmune disease, especially in females[22][23]. Toll-like receptor 7 (TLR7) or TLR9 signalling is critical to direct differentiation to ABCs, along with signalling from the cytokines IFN γ or IL-21[24]. ABC differentiation also requires the transcription factors Zeb2 and T-bet[25][26][27]. ABCs play a protective role in viral infection, and the population increases in the acute phases of infection. For example, ABC-derived antibody-secreting cells are found following influenza infection[28]. A subset of ABCs also persists in the spleen following resolution, contributing to B cell memory responses[29].

1.1.4 Antibody production

Both the GC and extrafollicular responses lead to the production of antibody-secreting plasma cells, and the production of antibodies is one of the key functions of B cells (Fig 1.2). Antibodies have different effector functions depending on their isotype, but the broad categories of effector function are activation of complement, and activation of immune cells by binding to Fc receptors (FcRs), which can induce antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP), thereby providing protection from pathogens.

All antibodies have two identical antigen-binding sites, and are comprised of two heavy and two light chains. There are five different classes of heavy chain, namely α , δ , γ , ϵ and μ , which determines antibody isotype as being IgA, IgD, IgE, IgG or IgM, respectively. There are also subclasses of IgG and IgA. These antibody isotypes are produced at different stages of, and have different functions within, the immune response. At the initiation of the humoral immune response, before SHM has begun, B cells produce IgM, which has low affinity but high avidity due to its pentameric structure. As described above, isotype switching then occurs, leading to the production of other antibody isotypes. IgA is predominantly found in secretions in mucosal epithelial tissues, such as in the gut and respiratory tract, and provides protection against pathogens and irritants in the mucosa[30].

IgE binds with high avidity to mast cells, and can control the secretion of histamine and other inflammatory mediators, and is critical in the immune response to parasites and helminth infection, as well as playing a role in hypersensitivity reactions[31].

IgG is the most prevalent isotype in the blood and extracellular fluid. There are four different subclasses, IgG1, IgG2, IgG3 and IgG4, that differ in their structure and function. Of these, IgG1 is the most abundant[32]. IgG1 and IgG3 are the most effective at initiating the complement cascade, and bind efficiently to most types of Fc γ Rs, whereas IgG2 and IgG4 bind with high affinity to only a few types of Fc γ R. IgG2, which is mostly produced in response to polysaccharide antigens, mainly binds to Fc γ RIIa, which is expressed widely on myeloid cells, and can also trigger the alternative complement cascade. IgG4 is the least prevalent subclass, and is not an effective inducer of immune effector responses. Instead, IgG4 undergoes fragment-antigen binding (Fab)-arm exchange, resulting in functionally monovalent antibodies that can bind antigen, but do not cross-link antigens or form immune complexes, thereby acting as a 'block' on the immune system, preventing excessive activation[33].

In SLE, the main pathogenic isotype associated with clinical manifestations is IgG, although other isotypes of autoantibodies are also often present[34]. Immune

complexes formed from IgG and auto-antigens trigger inflammation, and are deposited in tissues and organs, causing tissue damage[35].

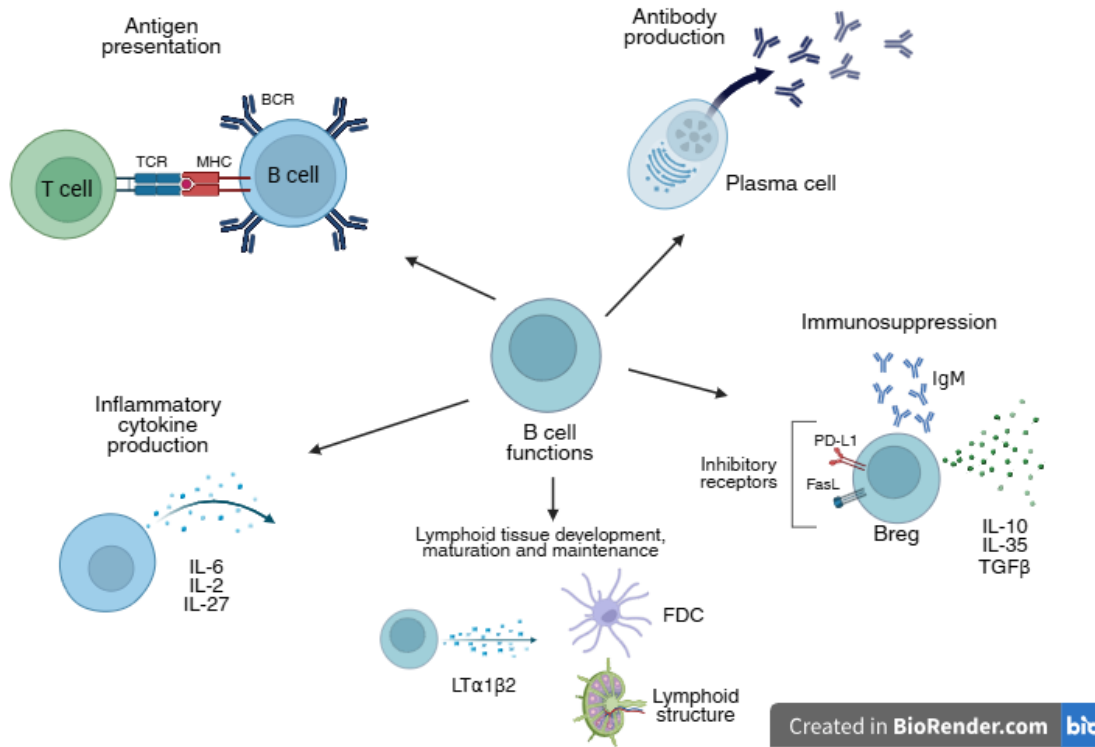


Figure 1.2: B cell functions B cells play a number of important roles in the immune cells. These include antibody production, antigen presentation, the production of inflammatory cytokines which direct T cell, as well as other immune, responses, production of the cytokine lymphotoxin $\alpha 1\beta 2$ (LT $\alpha 1\beta 2$), which induces the development of follicular dendritic cells (FDCs), and contributes to the development of lymphoid structures in the spleen, gut and tertiary lymphoid structures. Additionally, regulatory B cells (Bregs) play a role in immunosuppression, by secreting immunosuppressive cytokines such as IL-10, IL-35 and TGF- β , and by direct cell-to-cell contact via inhibitory receptors. PD-L1 = Programmed Death-Ligand 1. FasL = Fas-Ligand. Figure created in <https://BioRender.com>

1.1.5 B cells as APCs

In addition to producing antibodies, B cells can also act as professional APCs, expressing major histocompatibility complex class II molecules (MHC II) and costimulatory molecules such as CD86, enabling them to present antigen to and prime naive CD4⁺ T cells[36]. Compared to other APCs, such as dendritic cells, B cells are particularly efficient at presenting antigen at low concentrations[37]. Both naive

and GCBCs can uptake and present antigen, but GCBCs typically require a higher affinity BCR to internalise antigen than naive B cells[38]. Different B cell subsets also have differing antigen-presentation capacities, with MZ B cells being more effective APCs than follicular B cells[39]. ABCs are also extremely potent APCs, forming stable interactions with T cells at the T cell/B cell border in the spleen[40].

The binding of an antigen to the BCR triggers the formation of antigen-BCR clusters, which are then endocytosed. As the endosome matures and fuses with the lysosome, the pH increases, the antigen is processed and the resulting peptides are loaded onto MHC II. The peptide-MHC complex can then be transported to the B cell surface and presented to $CD4^+$ T cells[41].

B cells act as APCs in a number of different contexts in the immune system. In the thymus, B cells express self-reactive BCRs, and some thymic B cells additionally express autoimmune regulator (AIRE), leading to the promiscuous expression of normally peripheral tissue-restricted antigens, in order for negative selection of self-reactive T cells or maintenance of Treg populations[42][43].

Antigen presentation is also central to the GC reaction, and the amount of antigen captured and presented by B cells to Tfh cells is directly proportional to its affinity, and is therefore a critical factor in GCBC selection[44][45]. The role of B cells as APCs in the formation and maintenance of memory T cell responses is less clear. Some studies show that low levels of antigen presentation by B cells during the resolution of the initial immune response to a pathogen contribute to the generation and maintenance of quiescent memory $CD4^+$ T cells[46][47]. However, the importance of B cells compared to other professional APCs remains in question, as depletion of B cells did not impact formation or reactivation of antigen-specific $CD4^+$ memory T cells in the bone marrow[48].

1.1.6 Cytokine secretion

Another critical role of B cells is their ability to produce cytokines. Cytokine production by B cells is not only important for the adaptive immune response

and inflammation, but also for lymphoid tissue development, the innate immune response, and negative regulation of immune responses[49].

The secretion of tumour necrosis factor (TNF) and lymphoid toxin $\alpha 1\beta 2$ (LT $\alpha 1\beta 2$) by B cells induces the development of FDCs in the spleen[50], as well as contributing to the development of the T cell and marginal zones in the spleen[51]. LT $\alpha 1\beta 2$ is also required for the maturation of isolated lymphoid follicles in the gut[52], and the development of tertiary lymphoid structures[53], as well as the maintenance and remodelling of secondary lymphoid structures during infection[54].

The cytokines produced by B cells also play a role in determining both the CD8⁺ and CD4⁺ T cell response. For example, following infection with influenza, B cells produce large amounts of IL-6, which leads to Tfh cell development in the lymph nodes[55]. IL-6 production by B cells is also implicated in autoimmune disease, as increased levels of IL-6 are secreted and promote excessive T helper 1 (Th1) and T helper 17 (Th17) cell activation and responses, which can be normalised by B cell depletion[56].

Alternatively, IL-2 secretion by B cells promotes Th2 cell responses, including memory Th2 responses[57]. IL-27 production, which is stimulated by TLR2, TLR4 or TLR9, and CD40 and IL-21 signalling, leads to the development of cytotoxic CD8⁺ T cells, and also plays a role in the development of the primary CD8⁺ T cell response after vaccination[58][59].

As well as producing cytokines that promote inflammation and immune responses, B cells can also regulate immune responses via the cytokines IL-10 and IL-35. An interesting example of this is the induction of IL-10 producing B cells by *Schistosoma mansoni*, which provides protection against allergic hypersensitivity in the airway[60]. IL-10 production is a hallmark feature of regulatory B cells (Bregs), leading to their ability to suppress inflammation and maintain regulatory T cell (Treg) populations, although Bregs also suppress inflammation by other mechanisms, including the secretion of other anti-inflammatory cytokines and direct cell-contact[61].

1.2 Introduction to SLE

Systemic lupus erythematosus (SLE) is a chronic autoimmune condition characterised by the presence of autoantibodies against nuclear antigens. It is around nine times more prevalent in females than in males, making it one of the most sexually dimorphic autoimmune diseases[62][63]. Around 400,000 people are diagnosed with SLE every year worldwide[64]. SLE is more prevalent in African-American, Asian and Hispanic populations than Europeans[65]. SLE incidence varies hugely with geography, and this is thought to be due to differences in population demographics, but also due to differences in socioeconomic factors and environmental exposures[66][65]. The global incidence of SLE is also increasing with time[67][68][69][70], although this could be due to an increased number of diagnoses accompanying better recognition of the condition.

Symptoms of SLE can be wide-ranging, as can the severity of the disease, affecting multiple organ systems, including musculoskeletal, renal, mucocutaneous, respiratory and neurological systems[71] (Fig. 1.3).

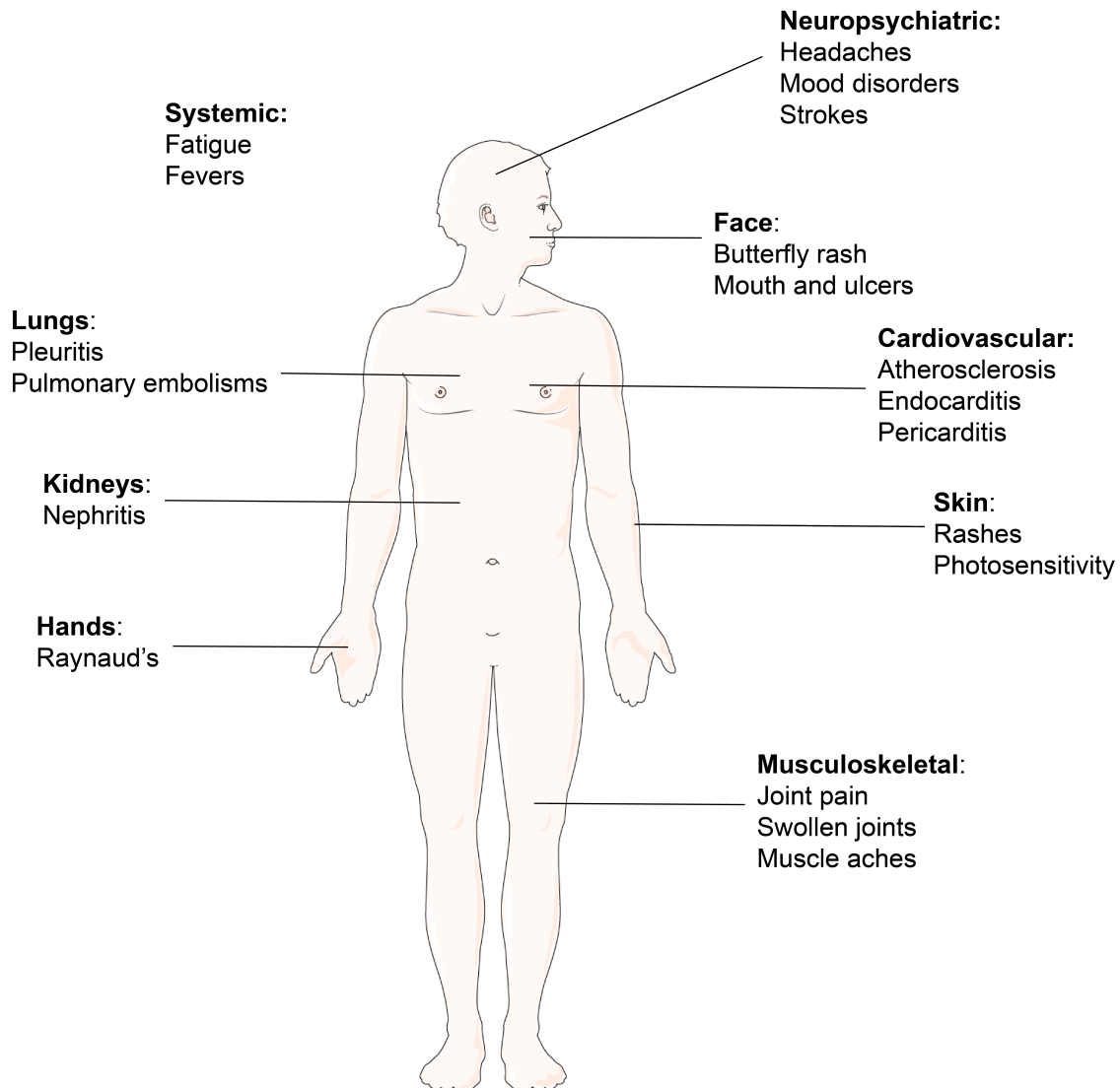


Figure 1.3: SLE symptoms Systemic lupus erythematosus (SLE) is a very heterogenous condition that can present with many different clinical symptoms, affecting multiple different organ systems in the body, including neurological, respiratory, cardiovascular, renal and musculoskeletal systems

Whilst mortality in those with SLE has decreased significantly over the past few decades, it remains around three times higher than in the general population, and the most common causes of death for people with SLE are infection, renal disease and cardiovascular disease[72]. SLE is also one of the leading causes of death in young females[73], showing that there is clear unmet clinical need.

Genetics, epigenetics and environmental factors all contribute to the development of SLE[74], and concordance in monozygotic twins is 24%, and 2% in dizygotic

twins [75]. Whilst there are rare instances of monogenic forms of SLE, this tends to present in childhood, with severe symptoms. Mutations that can cause monogenic SLE are mostly in nucleic acid sensing and degradation pathways, type I interferon signalling, the complement pathway, or defects in apoptosis[76]. For instance, a gain-of-function mutation in the RNA-sensing TLR, TLR7, causes SLE, and this mutation was sufficient to cause SLE in a murine model[77].

The majority of SLE cases are polygenic. Genome-wide association studies (GWAS) have identified over 150 common genetic variants associated with SLE, most of which are located in non-coding regions[78]. Interestingly, genetic associations differ between those of European and African ancestry, with associations in those with European ancestry being more often in innate immune pathways, and in individuals with African ancestry, genetic associations are more often in B cell activation pathways, linked to metabolic dysfunction and cell stress[79]. Environmental factors that can contribute to the development of SLE include smoking cigarettes[80], silica exposure[81], oral contraceptives, and hormone replacement therapy (HRT)[82]. Other environmental factors such as UV light[83] and pollution[84] are also thought to be linked to SLE exacerbation and pathogenesis, but the evidence is not conclusive[85]. There has recently been interest in potential links between the gut microbiome and SLE, and there is some evidence in murine models that antigens from the gut could lead to autoantibody production via molecular mimicry, with additional evidence of impaired gut barrier function in SLE[86][87][88]. Several infections are also associated with the development of SLE, and the strongest association is with Epstein-Barr Virus (EBV)[89][90].

1.3 Molecular mechanisms of SLE

As discussed above, many of the genetic associations of SLE lie in complement pathways, DNA sensing pathways, and type I Interferon (IFN) signalling. The exact mechanisms underlying the pathogenesis of SLE are not fully understood, but the key players involved are slowly starting to be revealed.

1.3.1 Defective cell corpse clearance

Autoantigens in SLE are thought to arise from the defective clearance of apoptotic cells and neutrophil extracellular traps (NETs). Macrophages from SLE patients have decreased phagocytic capacity and decreased expression of surface molecules involved in adhesion and clearance of apoptotic debris[91][92]. Neutrophil function is often also altered in SLE, and higher numbers of apoptotic neutrophils have been identified in the blood of SLE patients[93]. The production of NETs is induced by proinflammatory cytokines, including type I IFNs. In SLE, neutrophils can also produce type I IFN in response to chromatin antigens, which may lead to increased priming of neutrophils to produce NETs[94]. The degradation of NETs is also defective in SLE, and this causes complement activation and inflammation, and may also act as a source of autoantigens[95][96].

The clearance of apoptotic cells also relies on the complement system for opsonisation of debris. Defects in this pathway are strongly associated with development of SLE[97]. Additionally, as SLE progresses patients may develop an acquired deficiency of complement proteins due to their chronic activation and consumption, leading to decreased clearance of immune complexes and therefore tissue damage[98].

The accumulation of autoantigens means they may be presented by FDCs in the GC, or by marginal-zone resident DCs in the extrafollicular response[99], to B cells, leading to a break in tolerance and the positive selection of autoreactive B cells.

1.3.2 Type I IFN and SLE

For decades, it has been known that individuals with SLE have increased levels of circulating type I IFNs[100]. Observational studies have also shown that treatment of malignancy with IFN α leads to the development of autoantibodies and autoimmunity in some patients[101]. Several of the loci associated with SLE from GWAS studies are linked to IFN pathway activation[102]. Many patients with SLE also have increased expression of type I-IFN regulated genes, termed an 'IFN signature'[103].

A high level of expression of type I-IFN regulated genes is also correlated with increased disease activity[104].

There are three types of IFN: type I, II and III. Type I IFN constitutes the largest family, and can be divided into 5 subtypes, namely IFN- α , β , ϵ , κ and ω [105]. Type I IFNs bind to the IFN receptor (IFNAR), and stimulate a number of immunomodulatory effects via the induction of interferon stimulated genes (ISGs).

The source of type I IFN in SLE is a matter of debate. Immune complexes bound to self-antigen can trigger the endolysosomal TLRs TLR7 and TLR9, leading to the translocation of Interferon Response Factors (IRFs) IRF3, IRF5 and IRF7 to the nucleus, where they bind interferon-stimulated response elements (ISREs), triggering the expression of *IFNA* and *IFNB*, along with other ISGs. Whilst most nucleated cells can secrete IFNs, pDCs are a professional producer of type I IFNs, especially in viral infections[106]. pDCs have long been thought to be the main producers of Type I IFN in SLE, and, indeed, mice lacking pDCs, or even having their pDCs transiently depleted, are protected from the development of features of SLE and do not develop autoantibodies[107][108]. Anti-BDCA2 antibodies, which specifically deplete pDCs, have also had some success treating cutaneous SLE in a phase II clinical trial[109].

However, although pDCs have been shown to secrete large amounts of type I IFN in the initial stages of disease, later in disease in mice they seem to lose their ability to produce IFN α [110]. Additionally, pDCs in the circulation of SLE patients were unable to produce IFN α [111]. This has therefore led to a search for other producers of IFN α in individuals with SLE, to explain the IFN signature seen in these patients even once pDCs have become inert. This has revealed that in the circulation, subsets of neutrophils and macrophages are able to produce IFN α [112][113], but also that at sites of inflammation, production of IFN α by non-immune cells plays a significant role. For instance, keratinocytes in the skin produce IFN κ [114][115], and tubular epithelial cells in the kidney have also been shown to express high amounts of IFN α [116]. Further research is needed to clarify which cells are critical in producing IFN in SLE.

1.3.3 Extrafollicular or GC?

Traditionally, GCs have been considered the dominant source of autoantibodies and a key part of the pathogenesis of SLE, as spontaneous GCs are observed in SLE, as are isotype-switched and affinity-matured autoantibodies[117]. However, recent evidence has shown that, at least in some experimental models, GCs are dispensable for the development of autoimmunity, implicating the extrafollicular response instead. Additionally, as previously discussed, it has now been identified that class switching occurs prior to the GC reaction[7], and SHM can occur outside the GC[15][18]. It has now been demonstrated in most murine models of SLE that the extrafollicular response is a key source of autoantibodies, including for MRL^{Lpr} mice and R848-induced models[21].

TLR7 plays a central role in SLE pathogenesis. Copy number variations or polymorphisms in *TLR7* are associated with increased susceptibility to SLE, and a gain-of-function mutation in *TLR7* causes monogenic SLE[118][77]. In most SLE patients there is increased TLR7 activation in B cells[119]. TLR7 signalling is thought to induce both a GC and extrafollicular response, compared with TLR9 which causes a predominantly extrafollicular response, and inhibits the GC reaction, although the mechanism behind this remains unclear[120]. However, although TLR7 signalling induces a GC response, it has recently been shown that even in the absence of these GCs, autoimmunity still develops. For instance, conditional deletion of *Bcl6* in *Aicda*-Cre mice, which prevents the GC reaction from progressing but does not impact the extrafollicular response, did not prevent the development of autoimmunity in an R848-induced SLE model[121]. B cells deficient in *Bcl6* also contributed to the PB and PC compartment in a competitive bone marrow chimera setting, and demonstrated increased differentiation into PCs compared to *Bcl6*-expressing B cells *in vitro*[121].

It has been suggested that GCs may actually be protective against autoimmunity, by limiting the development of self-reactive B cells, and having strict controls in place mediating positive selection of B cells by Tfh cells and high rates of apoptosis[122], whereas the extrafollicular response has fewer checkpoints[21]. However, in the

context of rare or tissue-specific antigens, protective mechanisms against auto-reactivity in GCBCs are less effective, which can result in the development of autoimmunity[123].

The frequency of ABCs, which are produced by the extrafollicular response, also correlate with SLE severity, and contribute to its development, further implicating the extrafollicular response[124][125].

1.3.4 Altered T cell populations in SLE

Whilst the main focus of this project is examining B cell function, it is important to recognise that T cell populations are also dramatically altered in SLE, contributing to the inflammatory microenvironment, as well as directly impacting their interactions with B cells. CD4⁺ helper T cells drive the production of autoantibodies, and have been found to contribute to tissue injury in the kidneys and skin. Th17 cells, a subset of helper T cells, secrete IL-17, a cytokine whose levels are greatly increased in the peripheral blood of SLE patients. Serum levels of IL-17 correlate with disease activity[126], and IL-17 is thought to play a role in the progression of lupus nephritis, as well as promoting development of spontaneous GCs[127][128]. CD8⁺ cytotoxic T cells are also often functionally impaired in SLE patients[129], and absence of perforin in a mouse model of SLE led to increased disease progression, suggesting the cytolytic function of CD8⁺ T cells is important to regulate autoimmunity[130].

CD3⁺CD4⁻CD8⁻ double negative (DN) T cells are a subset that is greatly expanded in individuals with various chronic autoimmune and autoinflammatory conditions[131], and play an important role in SLE. DN T cells were initially identified in Lpr mice[132], and are increased in patients with SLE[133][127], as well as those with Sjogren' syndrome and other autoimmune conditions[134][135]. DN T cells secrete inflammatory cytokines such as IL-12, IL-4, TNF and IL-17A, and have been shown to enhance autoantibody production by B cells *in vitro*[136][133]. There is some evidence that in SLE, auto-antigens activate self-reactive CD8⁺ T cells, causing them to develop into DN T cells, which then contribute to SLE pathogenesis by producing proinflammatory cytokines[137].

1.4 Sexual dimorphism in SLE

The sexual dimorphism seen in SLE is not fully understood, and there are a number of different hypotheses to explain it. One theory revolves around sex-chromosome dosage, as females have two X chromosomes, whilst males only have one. To compensate for this, in females one X chromosome is randomly inactivated by DNA methylation and consequent transcriptional silencing in each somatic cell, a process termed X-chromosome inactivation (XCI)[138]. However, around 15% of genes are known to escape XCI[139][140], and amongst these are several of the large number of immune-related genes encoded on the X chromosome, including *TLR7* [141]. This results in a higher 'dose' of certain genes encoded on the X chromosome in female cells, which could contribute to development of SLE. This is supported by the fact that men with Klinefelter's syndrome, who possess an additional X chromosome (XXY), are around 14 times more likely to have SLE than males with a normal karyotype[142]. Additionally, females with triple X syndrome have a 2.5-fold increased risk of developing SLE[143], whilst those with Turner's syndrome, who have only one X chromosome (XO), have a lower risk[144]. To separate the impact of sex chromosome dosage from gonadal sex, mouse models have been generated where *Sry*, a sex-determining gene on the Y chromosome, is deleted from the Y chromosome, creating XY^- mice[145]. This enables comparisons between XX and XY^- mice, which both have female hormonal backgrounds but different numbers of X chromosomes. Studies in such mouse models of both pristane-induced SLE and in the genetically-determined NZM2328 SLE model have demonstrated that having XX chromosomes, compared to XY, increased susceptibility to autoimmunity[145][146].

Another contributing factor to the sexual dimorphism is the different hormone levels in males and females. The prevalence of SLE in females aged 20-50, when estradiol and progesterone levels are highest, along with evidence that treatments containing estrogen can increase the risk of flares in SLE, implicate estradiol as playing a role in SLE[147][148]. Additionally, hormone therapy has been linked to the development of SLE in some transgender patients[149]. However, despite evidence that females with SLE have slightly elevated amounts of the hormone

estradiol in their blood and decreased levels of testosterone[150][151], a meta-analysis was inconclusive about whether these hormone levels are consistently altered[152]. Studies in murine models support a role for sex hormones in the pathogenesis of SLE. It has been shown that treatment of MRL^{Lpr} mice with 17 β -estradiol increases mortality and nephritis, and knockout of the estrogen receptor, ER α , protects against immune complex deposition in the kidney and decreases mortality, although the mechanism by which this occurs is unclear[153][154].

Estrogen and androgen receptors are expressed on many types of immune cells, and these hormones are known to modulate immune cell behaviour[155]. Estradiol induces the upregulation of IRF5[156]. IRF5 expression is increased in female immune cells, and is linked to increased IFN α production in SLE[157]. Estradiol also has multiple effects on B cells, including on their survival, development and activation[158][159]. Deletion of the estrogen receptor in B cells in the NZB/NZW F1 murine lupus model decreased the level of autoantibodies and decreased B cell activation[160].

A number of other factors are proposed to play a role in the sexual dimorphism of SLE, including differences in miRNAs, which regulate gene expression. Far more miRNAs are encoded on the X chromosome than on the Y chromosome, and many of these miRNAs are involved in the immune response[161]. Alterations in expression of miRNAs has also been identified in SLE patients[162], leading to altered IFN expression as well as other altered inflammatory effects[163][164].

Further research is required to understand the relative contribution of these factors and others, and the interplay between them, that drives the sexual dimorphism observed in SLE.

1.5 TASL

Genome wide association studies (GWAS) identified *CXORF21*, which was later found to encode the protein ‘TLR adaptor interacting with SLC15A4 on the lysosome’ (TASL), as a risk locus for SLE[165][166]. The risk haplotype of *CXORF21* is associated with increased abundance of the protein in cells, and it is also an

IFN-inducible gene, so expression may be increased further in the inflammatory environment of SLE[166]. *TASL* protein abundance in B cells was correlated with severity of SLE, measured by the clinical SLEDAI score, in females younger than 35 years-old[166]. *CXORF21* is located on the X chromosome, and escapes X-inactivation[167], making it a strong candidate for playing a role in the sexual dimorphism of SLE.

TASL was initially identified as the protein encoded by *CXORF21* due to its interaction with the lysosomal histidine transporter, *SLC15A4*[168]. *SLC15A4* also has genetic associations with SLE[169][170], with several SLE-associated single nucleotide polymorphisms (SNPs) in this locus in Asian and Caucasian populations[171][172]. *SLC15A4* is mainly expressed in dendritic cells, macrophages and B cells[173]. It plays a critical role in TLR7 and TLR9 signalling pathways in pDCs, and is required for the production of IFN α [174]. *SLC15A4* is also crucial for B cell responses to either TLR7 or TLR9 stimulation, and in the absence of *SLC15A4*, production of IgG2c, and autoantibody production by B cells in murine SLE, were dramatically decreased[175].

In initial studies of *SLC15A4* in B cells, it appeared that its function as a lysosomal transporter was critical, and in its absence lysosomal pH, and subsequently mTOR signalling, were altered, leading to dysregulation of the IRF7 pathway[175]. In the pDC cell-line, CAL-1, and also in macrophages, *SLC15A4* also regulates the mTORC1 pathway and subsequent metabolic activity[176][177][178]. However, more recent evidence suggests that in the context of autoimmunity, it may be the ability of *SLC15A4* to recruit *TASL* to the lysosome that is more important[179]. Deletion of either *SLC15A4* or *TASL* specifically inhibits IRF5 signalling pathways, without inhibiting NF- κ B or MAPK signalling[168]. When *TASL* was engineered to be tethered to the endolysosomal compartment in the absence of *SLC15A4*, this rescued the phenotype seen in *SLC15A4*-deficient mice, whereas the transporter activity of *SLC15A4* was dispensable[179]. There is conflicting evidence that increased *TASL* expression may be linked to decreased lysosomal pH, with one group finding that female *TASL*-expressing cells, namely monocytes, B cells and pDCs, had

lower lysosomal pH than equivalent male cells, whilst cells that do not express *TASL*, such as T cells, did not show any sex-specific changes in lysosomal pH[180]. However, the majority of evidence implies that the main role of *TASL* is as an innate adaptor of the IRF5 pathway (Fig. 1.4).

Study of the structure of *TASL* has revealed that it contains a conserved pLxIS motif (where p is a hydrophilic residue, and x is any residue), that enables it to interact with IRF5 and act as an adaptor in the IRF5 pathway, much as STING acts as an adaptor in the IRF3 pathway[168]. In the absence of *TASL*, IRF5 cannot be phosphorylated and translocate to the nucleus, and therefore expression of ISGs and production of inflammatory cytokines is decreased[168][179]. *TASL* itself does not have any kinase activity, and so functional MyD88 and the IKK β kinase activity this triggers, along with the pLxIS domain of *TASL*, are all required for the IRF5 signalling pathway[181].

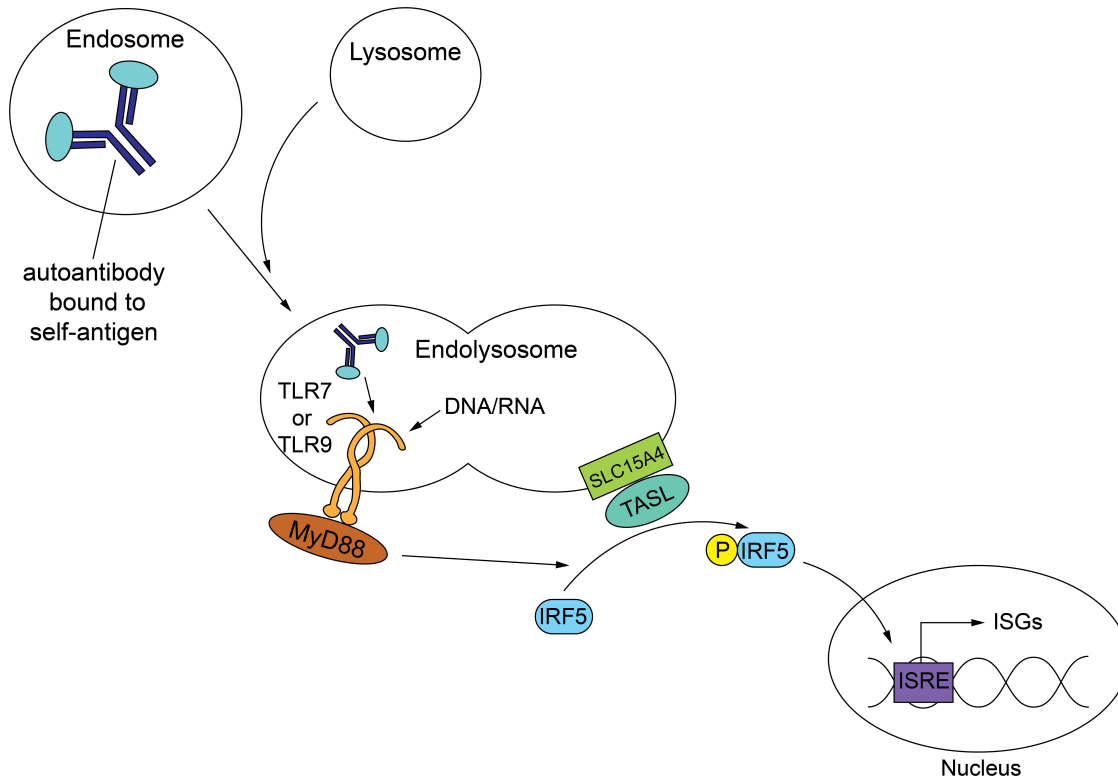


Figure 1.4: TASL acts as an innate adaptor in the IRF5 pathway Immune complexes bound to autoantigens are endocytosed by B cells. The endolysosomal TLRs TLR7 and TLR9, which respond to RNA and single-stranded (ss) DNA, respectively, can be activated by these complexes. This leads to activation of MyD88 and the phosphorylation of IRF5. TASL is essential for the phosphorylation of IRF5 in this signalling pathway. Once IRF5 is phosphorylated it can translocate to the nucleus, where it binds to the ISRE and causes the transcription of interferon-stimulated genes (ISGs).

TASL has also been found to be essential for the development of SLE in induced models of murine SLE with a wild type genetic background. TASL-deficient mice did not develop autoimmunity in response to either pristane or imiquimod, both of which normally cause significant disease[182][183]. A recent paper identified a TASL homologue, TASL2, which seems to play a minor role in IRF5 phosphorylation, and the effects of TASL2-deficiency are much less than that seen with TASL deficiency[183].

Whilst most work on TASL is on its interactions and role in the IRF5 pathway, it has also been suggested that it could potentially act as a biomarker for response to immunotherapy in certain types of tumours[184]. Analysis of TASL expression in 33 different cancer types revealed that TASL is a predictor of poor prognosis in low grade

glioma, an immunologically ‘cold’ tumour, with poor T cell infiltration. However, TASL was a predictor of good prognosis in two immunologically ‘hot’ tumours, lung adenocarcinoma and skin cutaneous melanoma, which have increased infiltration by T cells. TASL was also expressed highly in patients with elevated expression of immune checkpoint-related genes, who often respond better to immunotherapy[185]. This suggests TASL expression may be a predictor of immunotherapy responses[184]. The mechanism behind this association has not yet been fully explored, however, and requires further research.

1.5.1 IRF5 in SLE

TASL acts as an adaptor in the IRF5 pathway, a pathway that is known to be involved in SLE pathogenesis. Several SNPs in the *IRF5* locus are associated with SLE[186][187][188]. IRF5 is a transcription factor highly expressed in monocytes, macrophages, B cells and dendritic cells[189]. In humans IRF5 has 11 distinct isoforms (v1-v11) generated by alternative splicing and specific to different cell types and functions[190]. Certain isoforms, including v2, v9 and v10, are linked to IRF5 overexpression and SLE susceptibility[187]. Importantly, in mice there is one main IRF5 transcript, and only one alternative splice variant that is found at low levels in C57BL/6J mice, which must be considered when interpreting data from mouse models of human diseases linked to IRF5[191].

Activation of TLRs leads to recruitment by the adaptor protein MyD88 of TRAF6 and IRAK1 and 4. IRF5 then interacts with this complex and is phosphorylated and ubiquitinated, enabling it to translocate to the nucleus, where it binds ISRE motifs. This leads to the transcription of pro-inflammatory genes, including the cytokines IL-6, IL-12, TNF- α and type I IFN[192][193].

Mice deficient in IRF5 are protected from the development of SLE in both a pristane-induced model[194] and in genetic models[195][196]. In B cells, IRF5 is known to play a role in plasmablast differentiation in mice by controlling expression of *Prdm1* (which encodes Blimp1)[197], the production of the inflammatory cytokine IL-6[197], and also in SLE to play a role in autoantibody production[198]. Additionally,

IRF5 is critical for the development of ABCs, which are expanded in SLE[199]. Therefore there is much interest in the targeting of this pathway for treatment of autoimmune diseases.

Strategies under investigation for modulating IRF5 activity include reducing the levels of its expression and altering post-translational modifications or otherwise disrupting interactions with proteins required for its signalling[189]. Identifying all the key players in its signalling pathway is therefore critical. TASL acts as an adaptor protein necessary for IRF5 phosphorylation, making it an attractive option to alter IRF5 post-translational modifications. Since TASL is mainly expressed in B cells and pDCs, this could limit off-target effects in other cell types. Investigation of the role of TASL in B cells, and whether this pathway is required for development of autoimmunity, is therefore essential.

1.6 The current state of SLE treatment

The heterogenous and complex multi-organ pathology seen in SLE, along with our incomplete understanding of its pathogenesis, means that whilst there has been much progress made in treatment of this condition, there is still great unmet clinical need. Glucocorticoids, such as prednisolone, remain a mainstay of treatment, but these often have severe side effects, and high doses are associated with irreversible damage in patients with SLE[200]. For this reason, current recommendations are that glucocorticoid usage should be limited where possible if patients are in remission[201].

Hydroxychloriquine (HCQ) treatment is also recommended in all patients who have no contraindications to its use[202]. HCQ reduces organ damage, disease flares and end-stage renal disease, decreases cardiovascular complications and increases survival[203], but there is a risk of retinopathy[204].

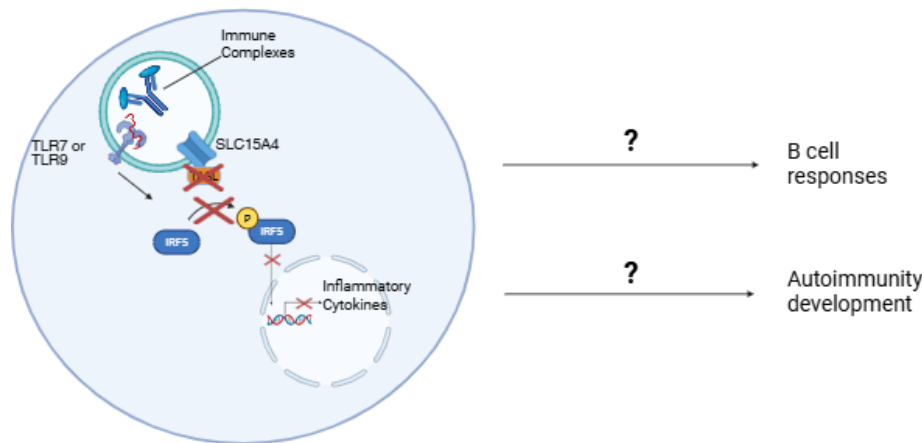
Other, non-corticosteroid immunosuppressants, for instance methotrexate, cyclophosphamide and azathioprine, are also used in the treatment of SLE. However, as well as having side effects, a number of these drugs are also teratogenic[205].

Due to the broad action of these immunosuppressants and their side effects, many trials are ongoing to find more specific treatments for SLE[206]. Two biologics,

belimumab and anifrolumab, are currently approved for treatment of SLE[207][208]. Belimumab targets B-cell activating factor (BAFF), a cytokine that promotes B cell survival, thereby leading to B cell death and a decrease in autoantibody levels[209]. In two phase III trials belimumab was shown to be effective in treating moderate-to-severe SLE[210][211]. The anti-CD20 antibody, rituximab, which targets and depletes B cells, was initially hoped to be a possibility for SLE treatment, but the Phase II/III EXPLORER trial had disappointing results[212]. However, in refractory SLE, rituximab is still used off-label[202], and a review of 188 cases showed that it caused improvement in 91% of these patients[213].

Anifrolumab is the other approved biologic, and targets the type I IFN receptor (IFNAR), thereby blocking type I IFN signalling, and it is especially effective in patients with a strong IFN signature[214][215]. Other biologics, targeting cytokines, such as IL-12, or pDCs are currently also in trials. Litifilimab, a monoclonal antibody targeting BDCA2 on pDCs, has shown some promise in treating cutaneous SLE in a phase II trial[109]. This again emphasises the role of type I IFN in SLE, demonstrating that exploration of this signalling pathway, and how it is altered in disease, is important in the search for new, specific treatments for SLE.

1.7 Project Aims and Hypothesis



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Figure 1.5: Current knowledge about the role of TASL TASL is known to act as an innate adaptor in the IRF5 pathway, and its absence prevents the phosphorylation of IRF5, and the subsequent production of inflammatory cytokines. The precise role it plays in B cells, in humoral immune responses and in the development of autoimmunity is not known. Figure created in <https://BioRender.com>

Based on the literature on the role of TASL in endolysosomal TLR and IRF5 signalling (Fig 1.5), and the importance of this signalling pathway in the development of SLE, the central hypothesis of this project is:

TASL plays an essential role in the activation of B cells by endolysosomal TLRs, B cell function, and the development of autoimmunity.

The main aims of my project are therefore as follows:

1. Characterise whether TASL is required for B cell development, function and activation
2. Investigate the role of TASL in immune responses

3. Investigate the role of TASL in development of SLE in a murine model

2

Materials and Methods

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2.1 Mice

B6.MRL-Fas^{lpr}/J (strain 000482) were purchased from the Jackson Laboratory. C57BL/6J-Tasl^{em3H}/H mice were generated by the Mary Lyon Centre at MRC Harwell, via a CRISPR/Cas9-induced mutation deleting 10 nucleotides in exon ENSMUSE00000697605 of the Tasl gene. B6.Cg-Tg(TcraTcrb)425Cbn/J OT II mice

were kindly donated by the Cornall lab (Centre for Human Genetics, University of Oxford). Females and males of ages 8-16 weeks were used, except where separately stated for individual experiments. Mice were bred and maintained under specific pathogen-free conditions at the Kennedy Institute of Rheumatology, University of Oxford, with regular checks performed to confirm the absence of pathogens. All procedures and experiments were performed in accordance with the UK Scientific Procedures Act (1986) under a project license authorized by the UK Home Office (PPL no. PP1971784).

2.2 B cell isolation and culture

Spleens were crushed through a 70 μ M strainer (Falcon), to obtain a single cell suspension of splenocytes. B cells were then isolated by using the Pan B cell Isolation II Kit (Milteny Biotec) as per manufacturer's protocol, with resulting purity of over 90%. For proliferation analysis, cells were incubated with Cell Trace Violet (Life Technologies) in PBS for 20 mins at room temperature, before adding complete media and washing. Isolated B cells were cultured in RPMI-1640 medium (Gibco) with 10% FCS (Gibco), 50 μ M 2-mercaptoethanol (Gibco), 1mM glutamine (Gibco), 0.01% sodium pyruvate (Gibco), 10mM HEPES buffer (Gibco) and 1% penicillin and streptomycin (Life Technologies Ltd).

For TLR stimulation, either 100nM CpG-B (ODN1826) (Miltenyi Biotec), 5 μ g/ml IMQ (Invitrogen) or 10 μ g/ml LPS (Merck) was used, for times indicated in figures. Agonist concentrations were determined based on the literature for CpG[216][217], IMQ[218], and LPS[217], as well as optimisation studies for CpG and IMQ, in which multiple concentrations and timepoints were tested before selecting the dose that caused sufficient response.

2.3 B cell-OT II T cell coculture

Method adapted from previous protocol[219]. B cells were isolated using the Pan B cell Isolation II Kit (Milteny Biotec), then plated at 2×10^5 /ml in a 96

U-bottomed plate (Scientific Laboratory Supplies Ltd.) and stimulated for 24h with 100nM CpG-B (ODN1826) (Miltényi Biotec) at 37° C. For the last 30 mins of this incubation, 100 μ g/ml Ova 323-339 peptide was added to B cells. The stimulated B cells were then washed and resuspended in complete media. Naive CD4⁺ T cells were isolated from the spleens of B6.Cg-Tg(TcraTcrb)425Cbn/J OT II mice using the MojoSortTM Mouse CD4 T Cell Isolation Kit (BioLegend) as per manufacturer's protocol. Isolated T cells were then incubated with Cell Trace Violet (Life Technologies) for 20 mins at room temperature, before complete media was added and cells washed. 2 $\times$ 10⁵/ml T cells were then added to pre-stimulated B cells and incubated for 72h at 37° C.

2.4 ABC differentiation

B cells were isolated using the Pan B cell Isolation II Kit (Miltényi Biotec). Cells were then plated in complete RPMI at a concentration of 1 $\times$ 10⁶/ml in a 24-well plate (SLS Ltd.) For ABC in vitro culture, a differentiation cocktail of 1 μ g/ml agonistic anti-CD40 (clone FGK45.5, Miltényi Biotec), 1 μ g/ml anti-IgM F(ab')₂ (Jackson), and 50ng/ml IL-21 (PeproTech) was used[25][26][220], either with or without 0.5 μ g/ml IMQ or 100nm CpG as stated. Cells were cultured for 3 days and then analysed.

2.5 Flow cytometry

Spleens were crushed through a 70 μ m strainer (VWR). Red blood cells were lysed with ACK Lysis buffer (Gibco) for 3 mins. Bone marrow (BM) cells were obtained by flushing the femur and tibia with FACS buffer (PBS with 0.5% BSA and 2mM EDTA). Single-cell suspensions were incubated with Fixable Viability Dye eFluor 780 (eBioscience) or Zombie UV Fixable Viability Dye (BioLegend) at 1:1000 in PBS for 30 min on ice. Fc block (BD Biosciences) was then added for 5 mins, followed by surface antibodies in a 50:50 solution of FACS buffer and Brilliant Stain buffer (BD Biosciences) for 30 mins on ice. Cells were fixed in freshly prepared 4% PFA for 10 mins at room temperature. For intracellular staining, cells were

fixed using FoxP3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (eBioscience) for 30 mins at room temperature, then permeabilised in Permeabilization buffer (eBioscience). Cells were then incubated with intracellular antibodies diluted in permeabilization buffer for 45 mins at room temperature. Antibodies used can be found in Appendix 1.

Cells were resuspended in FACS buffer and acquired on either a BD Fortessa X20 or Cytex Aurora flow cytometer.

2.6 Immunoblotting

Cells were lysed in RIPA buffer (Merck) supplemented with phosphatase and protease inhibitors (cOmpleteTM ULTRA Tablets and PhosSTOPTM, Roche) for 40 mins at 4°C, with vortexing every 15 mins. Cells were then centrifuged at 15,000g for 15 mins at 4°C and the supernatant was collected. Protein was quantified with a PierceTM BCA Protein Assay kit (Thermo Fisher Scientific). Samples were denatured in Laemmli buffer (BioRad) containing 10% β -mercapthoethanol for 5 mins at 95°C, then cooled immediately on ice. 20 μ g protein from samples was then loaded and run on a 10% gel (Mini-PROTEAN TGX Precast Gels, BioRad). Protein was then transferred to a PVDF membrane (BioRad). Membranes were blocked in 2.5% skimmed milk in TBS for 1h. Membranes were washed three times with TBS-Tween20 (0.05%), then incubated with primary antibodies anti-TASL (Atlas) and anti-vinculin (Cell Signaling Technology) diluted 1:1000 in TBS-Tween20 containing 2.5% BSA at 4°C overnight. Membranes were then washed several times with TBS-Tween20 before adding HRP-conjugated goat anti-rabbit secondary antibody (Cell Signaling Technology) diluted 1:20000 in TBS-Tween20 containing 2.5% BSA, and incubating at room temperature for 1h. SuperSignalTM West Pico PLUS Chemiluminescent Substrate (ThermoFisher) was then added to membranes, and chemiluminescence was used to detect signal.

2.7 Quantitative PCR

Cells were lysed in RLT plus (Qiagen) containing 1% β -mercaptoethanol. RNA was isolated using an RNeasy Plus Mini kit (Qiagen), according to the manufacturer's protocol. Isolated RNA was then reverse transcribed to cDNA using the High Capacity RNA-to-cDNA kit (Thermo Fisher). qPCR reaction was performed using TaqManTM Fast Advanced Master Mix (Applied Biosystems) and specific TaqMan probes for *Isg15* (Mm01705338_s1), *Il6* (Mm00446190_m1), *Il10* (Mm01288386_m1), *Irf7* (Mm00516793_g1), *Mx1* (Mm00487796_m1), *Prdm1* (Mm00476128_m1) and *Ubc* (Mm00476128_m1). Data was acquired on an Applied Biosystems Viia7 System, and expression was normalised to *Ubc* expression using the $\Delta\Delta$ CT method.

2.8 Immunisation

Immunisations were performed as per previous protocols[221][222] Either 50 μ g NP(30-39)-CGG (Biosearch Tech) or NP-Ficoll was mixed 1:1 with alum (Thermo Fisher Scientific) and mixed vigorously by pipetting for 5 mins, before being injected intraperitoneally in 100 μ l volume. For NP-CGG, day 14 was used as the primary timepoint, and for NP-Ficoll, spleens and serum were collected at day 7.

For sheep red blood cell (SRBC) immunisation, 1ml of sterile SRBCs in Alsever's solution (Thermo Fisher Scientific) was washed twice with 10ml ice-cold PBS, then reconstituted in 2-3ml of PBS. 200 μ l was then injected intraperitoneally.

2.9 Anti-NP ELISA

Anti-NP ELISA adapted from previous protocols[223][217]. Corning 96-well EIA/RIA flat bottom high bind plates (Scientific Laboratory Supplies) were coated with NP₂ or NP₂₀ conjugated to BSA, at 5 μ g/ml in bicarbonate/carbonate coating buffer overnight at 4°C. Plates were washed in PBS, then blocked with 5% skimmed milk in PBS at 37° C for 2h. Plates were washed with PBS, then sera from immunised mice was diluted in 1% skimmed milk and added to plates and incubated at 37°

C for 1h. Plates were then washed with PBS-0.05% tween (PBS-T) five times. Alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1, IgG2c, IgG3 or IgM detection antibodies were added and incubated at 37°C for 1h. After washing, alkaline phosphatase substrate (Sigma) was added to plates and left at to develop for 5 min at room temperature, after which readings were taken every 3 mins on a FLUOstar Omega plate reader at 405nm.

2.10 Ig Isotyping ELISA

Ig Isotyping was performed using a Mouse Ig Isotyping Uncoated ELISA kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Corning 96-well EIA/RIA flat bottom high bind plates (Scientific Laboratory Supplies) were coated with capture antibody at a 1:250 dilution in coating buffer and left at 4°C overnight. Wells were then washed three times with 200 μ l PBS-T, blotting plates on absorbent paper between washes. The plate was then blocked with 250 μ l blocking buffer for 2h at room temperature. The plate was then washed three times in PBS-T. 100 μ l of serum diluted in assay buffer A was then added to wells, and incubated for 1h at room temperature. After four washes in PBS-T, 100 μ l detection antibody was added and incubated for 1h at room temperature. Plates were then washed four times in PBS-T, before adding TMB substrate solution and incubating at room temperature for 20 mins. 100 μ l stop solution was then added to quench the reaction, and the plate was analysed on FLUOstar Omega plate reader at 450nm.

2.11 Autoantibody ELISA

Corning 96-well EIA/RIA flat bottom high bind plates (Scientific Laboratory Supplies) were coated with UltraPure Salmon Sperm DNA Solution (Invitrogen) at 100 μ g/ml, or 1 μ g/ml Sm/RNP (Arotec Diagnostics) in PBS overnight at 4°C as previously described[224],. The plate was then washed three times with PBS. The plate was blocked with 2% BSA in PBS for one hour at 37°C, then washed three times with PBS. Mouse serum was then diluted in PBS containing 1% BSA,

then added to plates and incubated for 2h at 37°C. Plates were then washed four times in PBS-T. Alkaline phosphatase-conjugated goat anti-mouse IgG, IgG2c or IgM detection antibodies (SouthernBiotech) were then added and then plates were incubated at 37°C for 1h. Plates were then washed five times with PBS-T, and after washing alkaline phosphatase substrate (Sigma) was added to plates and left to incubate for 5 mins at room temperature. Readings were then taken every 3 mins on a FLUOstar Omega plate reader at 405nm.

2.12 Anti-nuclear antibody staining

Anti-nuclear antibody staining was performed as per published protocols[225]. Mouse serum at a 1:100 dilution was added to Kallestad Hep-2 slides (BioRad) and slides were incubated for 30 mins at room temperature in the dark. Slides were washed in PBS, then AF488-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) was added and incubated for 30 mins at room temperature in the dark. Slides were then washed several times in PBS and mounted, before being imaged with a ZEISS LSM 980 and analysed in ImageJ.

2.13 Cytometric bead array

Supernatant was collected from cultured B cells, and then a cytometric bead array assay was performed using the Legendplex Mouse Inflammation Panel kit (BioLegend) according to manufacturer's protocol. Briefly, 25 μ l of assay buffer and 25 μ l of the culture supernatant was added to each well in a 96-well v-bottomed plate. The mouse inflammation panel premixed beads were then vortexed thoroughly, and 25 μ l was added to each well. After being sealed with a plate-sealer, the plate was wrapped in aluminium foil to protect it from light, and shaken for 2 hours at room temperature at 800rpm on a plate shaker. The plate was then centrifuged for 5 mins at 1050rpm, and the supernatant removed by inverting and flicking the plate. Wells were then washed with 200 μ l wash buffer, and the plate was shaken at 800rpm before being centrifuged, and the supernatant removed. 25 μ l detection antibodies

were then added to each well, before again sealing the plate, covering with foil and shaking for 1h at room temperature at 800rpm. 25 μ l of Streptavidin-phycoerythrin (SA-PE) was then added to each well, before re-sealing the plate, wrapping in foil and shaking for 30 min at room temperature at 800rpm. The plate was then washed twice. Beads were then resuspended in 150 μ l wash buffer, and samples were then acquired on a BD Fortessa X20 and data was analysed using FlowJo.

2.14 Bone marrow chimera generation

Competitive bone marrow chimeras generated as previously described[217]. Recipient B6.SJL.CD45.1 mice received two doses of 5.5Gy irradiation 4 hours apart. Recipient mice were then injected intravenously with 4x10⁶ bone marrow (BM) cells in 200 μ l PBS, made up of 50:50 CD45.2⁺ Tasl^{KO} and CD45.1⁺ WT, or CD45.2⁺ WT and CD45.2⁺ WT BM cells from age- and sex-matched donor mice. Recipient mice were given antibiotics (Baytril, Bayer corporation) in their drinking water for two weeks. Eight weeks after BM reconstitution, recipient mice were immunised with sheep red blood cells (SRBCs), and spleens were harvested and analysed nine days after immunisation.

2.15 Bulk RNA sequencing

Isolated splenic B cells were stimulated for 24h with 100nM CpG. Cells were then lysed in RLT plus containing 1% 2-mercaptoethanol. RNA was isolated using RNeasy Plus Mini kit (Qiagen). Next generation paired-end sequencing was performed by Azenta using the Illumina NovaSeq 6000 platform, with 20M reads taken per sample. *Salmon* was used to count transcripts. After standard processing, data was filtered to genes that had expression higher than 1CPM in at least 50% of samples. *edgeR* was used to perform normalisation and differential expression analysis. For gene set enrichment analysis (GSEA), the *ClusterProfiler* package was used with gene ontology terms related to biological processes (GO BP). The Benjamini-Hochberg method was used for P-value adjustment to control for false discovery rate.

2.16 Statistical analysis

Statistical analyses and tests are described in respective figure legends, and were conducted using GraphPad Prism v10.0. Distribution of data was examined with normality tests so appropriate statistical tests could be performed. Statistical significance for two-sample comparisons was calculated using unpaired Student's two-tailed t test. For multiple comparisons *in vitro* or for *in vivo* immunisations, statistical significance was calculated using two-way ANOVA with Šidák's multiple testing correction to examine pre-planned comparisons. For *in vivo* experiments examining the impact of TASL knockout on the lupus phenotype, statistical significance was calculated using two-way ANOVA with Tukey's multiple-comparisons test so all pairwise comparisons could be investigated. No statistical methods were used to pre-determine sample sizes, but these are comparable to previous studies[182][183]. For *in vivo* experiments, mice were matched by age and sex. For all *in vivo* experiments, and the majority of *in vitro* experiments, excepting one *in vitro* stimulation experiment where the variation was too large and data were better analysed separately, data pooling before analysis was done in order to achieve required sample sizes. Pooled data were checked visually using Q-Q plots, and the Shapiro-Wilk test was performed to check for normal distribution and ensure correct tests were used. Where data were pooled, this is clearly stated in the figure legend.

Data collection and analysis were generally not performed blindly. For immunisation experiments, mice that showed no germinal centres or Alum spots after immunisation were excluded from analysis due to being considered to have failed intraperitoneal immunisation. All error bars display mean $\pm$ S.E.M, and significance was defined as a P value ≤ 0.05 .

3

TASL is essential for full activation of B cells

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3.1 Introduction

Murine B cells express a variety of different TLRs, although levels of expression differ by B cell subset. However, all murine B cells express the endolysosomal TLRs TLR7 and TLR9, and the surface TLR4[226]. This differs from naive human B cells, which express negligible levels of TLR4 [227][228], although in some inflammatory disease states they may do so[229]. TLR signalling is not required for B cell development[230], but is important in the activation and function of B cells. TLR7 and TLR9 respond to RNA and DNA rich in unmethylated cytosine-phosphate-guanosine (CpG) dinucleotides, respectively. *In vitro* stimulation of B cells with TLR7 or TLR9 ligands leads to activation, antigen presentation, and proliferation, as well as inducing production of cytokines, immunoglobulins, and class-switching[231][232]. Signalling through either TLR7 or TLR9 leads to recruitment of MyD88, and this initiates the formation of the Myddosome complex, consisting of MyD88, IRAK1 and IRAK4. This sets in motion molecular pathways that can activate the transcription factors NF- κ B, MAPK and the Interferon Response Factors (IRFs) IRF3, IRF5 and IRF7. TASL is located on the endolysosomal surface, bound to the lysosomal histidine transporter SLC15A4[168][233].

TASL deficiency has been found to specifically impact IRF5 phosphorylation, without affecting either the NF- κ B or MAPK pathways[168]. Until very recently, the role of TASL *in vivo* and in primary cells remained unexamined. In recent studies, loss of TASL has been found to replicate the phenotype of IRF5-deficient B cells[183].

3.1.1 Chapter aims

In this chapter I have aimed to answer the questions:

- Is TASL required for B cell development or maturation?
- Does TASL play a role in the activation and function of B cells after TLR stimulation?

3.2 Results

3.2.1 TASL is not required for B cell development in the bone marrow

Novel TASL^{KO} mice were generated by the Mary Lyon Centre at the Harwell Institute (Appendix A.2). TASL knockout was confirmed by Western Blot (Fig. 3.1A). TASL^{KO} mice were viable, without any overt signs of immune deficiency.

Initially, I investigated whether TASL plays a role in the development of B cells in the bone marrow. I found that there was no defect in any stage of B cell development in the bone marrow in TASL^{KO} mice, with no differences in numbers or proportions of pro, pre-pro, or pre-B cell populations between WT and TASL^{KO} mice (Fig. 3.1B-C), demonstrating that B cells were able to progress through development without any defects. Mature and immature B cell populations were also unaffected by TASL deficiency (Fig. 3.1D-E). This suggests that TASL is not required for B cell development.

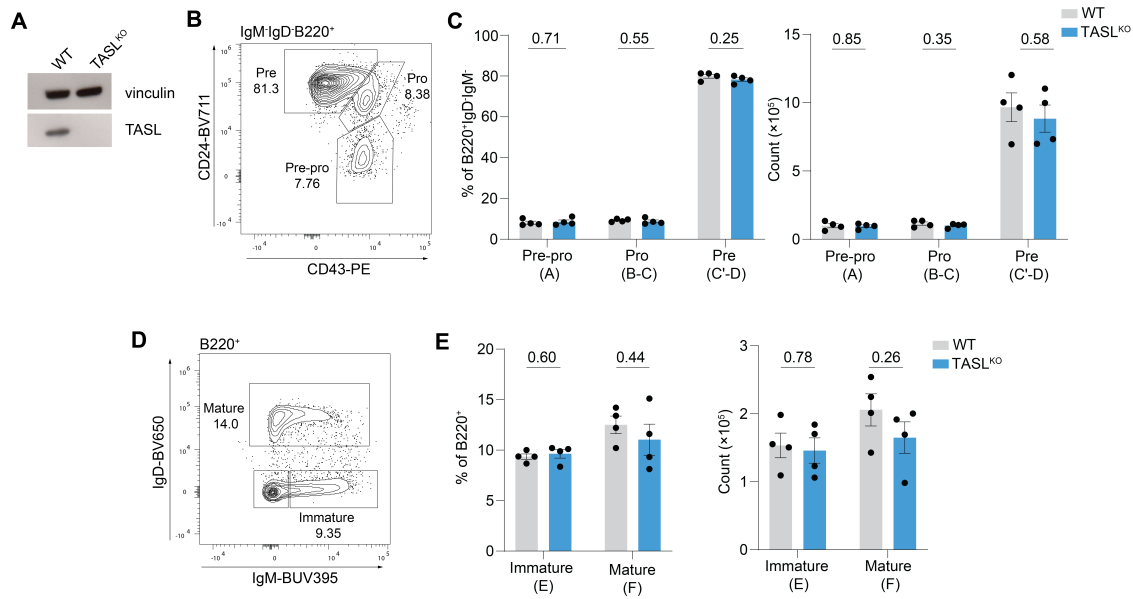


Figure 3.1: TASL is not required for bone marrow B cell development (A) Confirmation of TASL protein knockout by Western blot of splenocytes from WT or $TASL^{KO}$ mice. (B) Representative gating for $IgM^{-}IgD^{-}B220^{+}$ pre-pro-, pro- and pre- B cells in the bone marrow, and (C) quantification of frequency and count of these populations in WT and $TASL^{KO}$ female mice ($n=4$). (D) Representative gating for $B220^{+}IgD^{-}IgM^{+}$ immature B cells and $B220^{+}IgD^{+}$ mature B cells in the bone marrow, and (E) quantification of these populations in WT and $TASL^{KO}$ female mice ($n=4$). Equivalent Hardy Fractions are shown in parentheses. Statistical significance was calculated using two-way ANOVA. Data are representative of 2 independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

3.2.2 TASL is not required for peripheral B cell homeostasis at steady state

To determine the impact of TASL deficiency on the peripheral B cell compartment, I next investigated splenic immune cell populations using flow cytometry. There was no impact on the frequencies or numbers of total B cells (Fig. 3.2A), or any B cell subsets (Fig. 3.2B-F). Whilst a slight increase was seen in transitional B cell populations T1, T2 and T3 (Fig. 3.2F), given that neither total B cell numbers, nor follicular or marginal B cell populations, were altered, it is unlikely this is physiologically important.

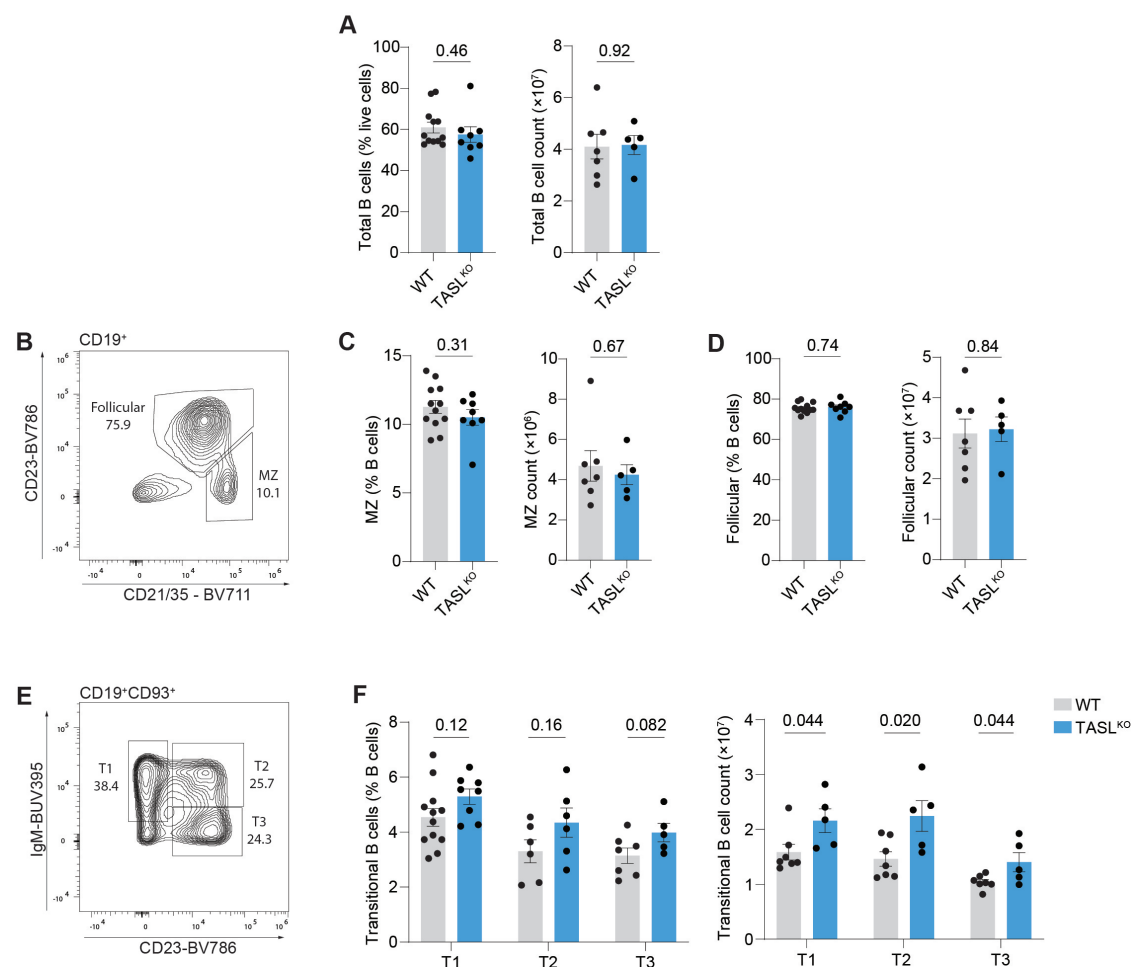


Figure 3.2: Splenic B cell populations at steady-state. (A) Quantification of CD19⁺ B cells in spleens of WT and TASL^{KO} male and female mice. (B) Representative gating for CD19⁺CD23⁻CD21⁺ MZ and CD19⁺CD23⁺CD21^{int} follicular B cells, and quantification of frequency and count of MZ (C) and follicular (D) B cells. (E) Representative gating for CD19⁺CD93⁺ T1 (CD23⁻IgM⁺), T2 (CD23⁺IgM⁺) and T3 (CD23⁺IgM⁻) B cells, and quantification of frequency and count of these populations (F). Statistical significance was calculated using two-tailed t-test (A, C-D) or two-way ANOVA (F). Data are representative of and pooled from 3 independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse (n=5-12).

3.2.3 TASL is not required for maintenance of other splenic immune cell populations at steady state

I next examined whether TASL knockout affected either the total number or proportion of T cells, macrophages or pDCs (Fig. 3.3A). There was no difference in the number or proportion of macrophages or T cells (Fig. 3.3B-C). There was

also no difference in the proportion of pDCs, but there was a slight increase in the number of pDCs in the spleen (Fig. 3.3D-E). This could be due to fewer pDCs being activated in the periphery due to the absence of TASL, which could lead to slightly increased numbers in the spleen.

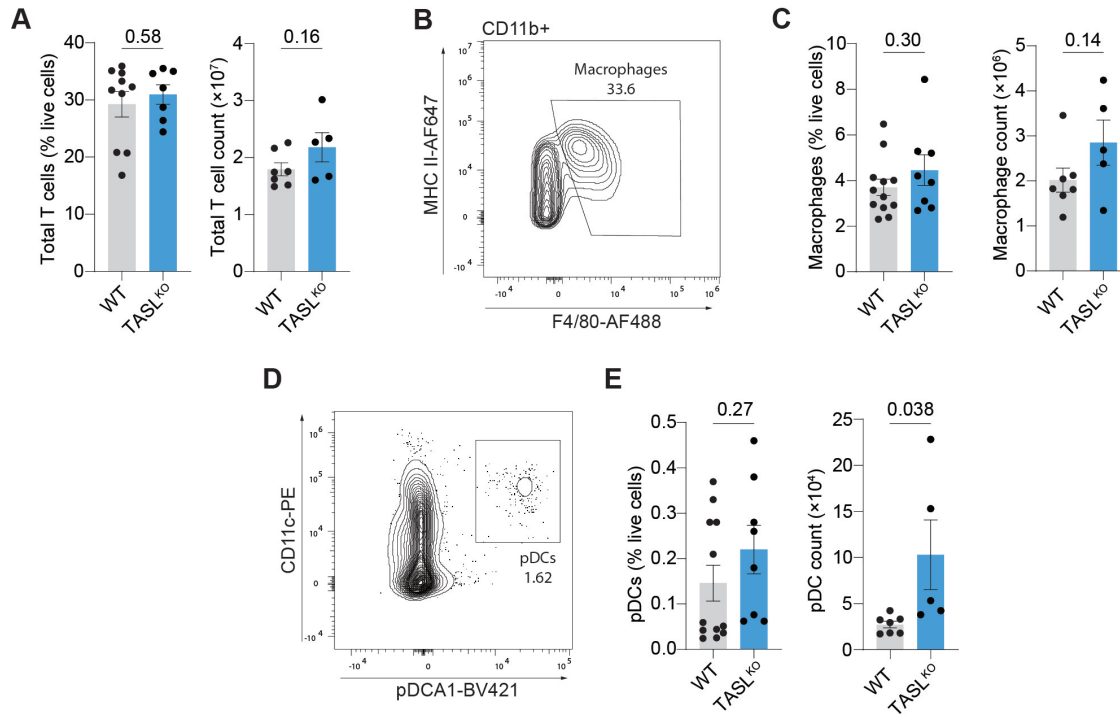


Figure 3.3: Splenic immune cell populations at steady-state. (A) Quantification of total TCRb⁺ T cell frequency and count. (B) Representative gating for CD11b⁺F4/80⁺ macrophages, and quantification of frequency and count (C). (D) Representative gating for CD11c⁺pDCA1⁺ pDCs and quantification (E). Statistical significance was calculated using two-tailed t-test. Data are representative of and pooled from 3 independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse, both male and female mice are shown (n=5-12).

3.2.4 The effect of *Tasl* knockout on B cell viability and proliferation *in vitro*

Having confirmed that TASL is not required for B cell development, I next investigated whether TASL is important for B cell viability and proliferation. I isolated B cells from the spleens of WT and TASL^{KO} mice, and then stimulated these with CpG oligodeoxynucleotides (CpG) or imiquimod (IMQ), TLR9 and TLR7

ligands, respectively (Fig. 3.4A). Alternatively, I stimulated B cells with LPS, a TLR4 ligand that signals via TLR4 and MyD88 at the cell surface, or alternatively by TRIF in the endolysosome, and so is not expected to require TASL. Viability was not affected by TASL deficiency in any of the conditions at 24h, but there was a slight increase in viability in CpG-stimulated B cells after 72h (Fig 3.4B). I labelled B cells with CellTrace Violet (CTV), to examine whether TASL was important in cell proliferation. Following stimulation with IMQ, there was a slight increase in the proliferation index in $TASL^{KO}$ B cells, although overall proliferation was limited, and the majority of cells remained undivided, so this may be an artefact (Fig. 3.4C). I observed a slight decrease in the proliferation index in CpG-stimulated $TASL^{KO}$ B cells (Fig. 3.4C), which fits with which fits with previous studies demonstrating a role for TLR9 stimulated IRF5 signalling in B cell proliferation[234].

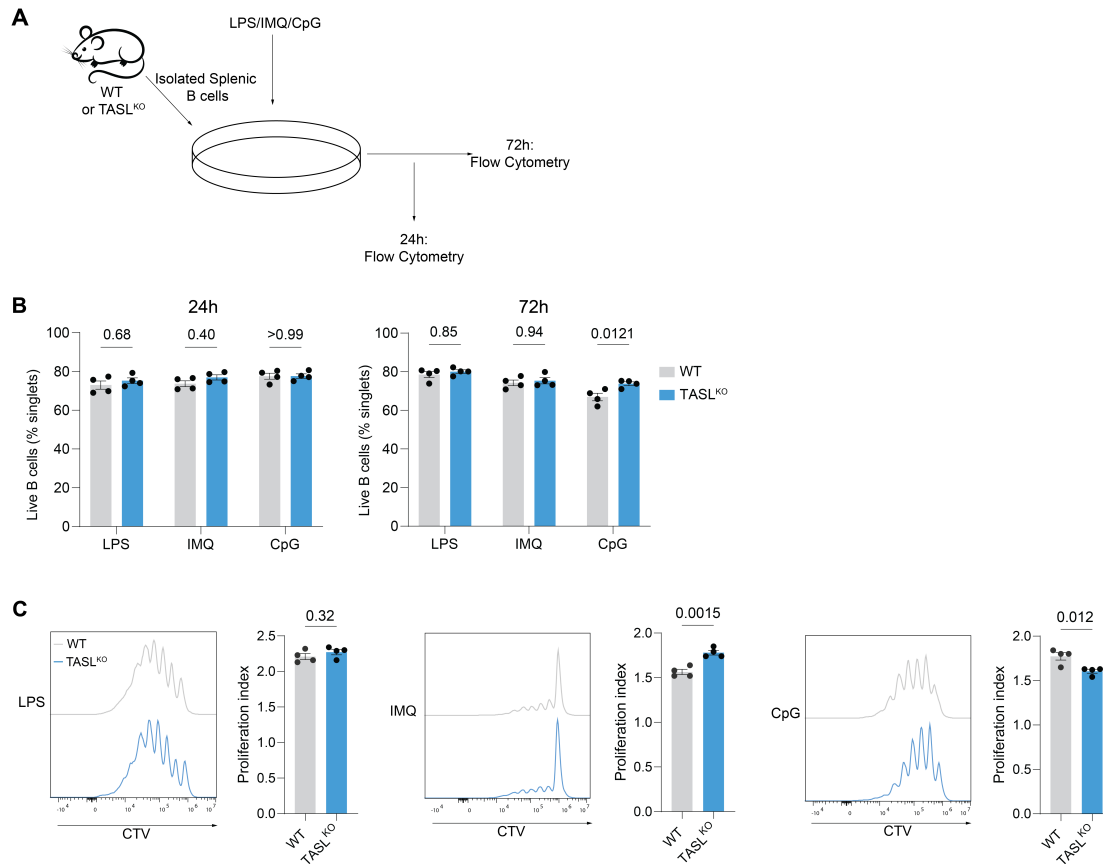


Figure 3.4: The effect of *Tasl* knockout on B cell viability and proliferation *in vitro* (A) Schematic of experimental set-up. B cells were isolated from the spleens of female WT or TASL^{KO} female mice, then stimulated with either 10 μ g/ml LPS, 100nM CpG or 5 μ g/ml IMQ for 24h or 72h. (B) Flow cytometric quantification of live B cells at 24h and 72h (n=4). (C) Representative histograms of Cell Trace Violet (CTV) dilution in live B cells after 72h stimulation with either CpG, IMQ or LPS (n=4). Statistical significance was calculated using a two-way ANOVA with Šidák's multiple testing correction (B) or by an unpaired two-tailed t test (C). Data are representative of at least two independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

3.2.5 TASL is essential for full B cell activation *in vitro*

As *Tasl* is encoded on the X chromosome, and proposed to play a role in the sexual dimorphism of SLE[166], I analysed data from male and female mice separately. Examining surface proteins by flow cytometry 24h after stimulation revealed that levels of CD69, an early activation marker, were decreased on TASL^{KO} B cells following both IMQ- and CpG-stimulation, with levels around a third lower to two-thirds lower in CpG-stimulated TASL^{KO} B cells, and two-thirds lower in both males

and females (Fig. 3.5A-B). This difference was not observed in LPS-stimulated cells, suggesting that the impact of TASL deletion is specific to the endolysosomal TLR pathway (Fig. 3.5A-B). Additionally, after 72h of CpG stimulation, a higher proportion of TASL^{KO} B cells were still IgD⁺, indicating that they were still in a naive state (Fig. 3.5C). Again, this change was seen in both male and female B cells (Fig. 3.5D-E).

Intriguingly, surface levels of the costimulatory molecule CD86, whilst being unchanged in IMQ-stimulated B cells, were increased in CpG-stimulated B cells after 72h in both males and females (Fig. 3.5F-G). I also observed a slight, but significant, increase in MHC-II levels in CpG-stimulated B cells (Fig. 3.5H-I). This suggests that not all aspects of B cell activation are equally impacted by TASL deficiency, and potentially that antigen presentation may be increased.

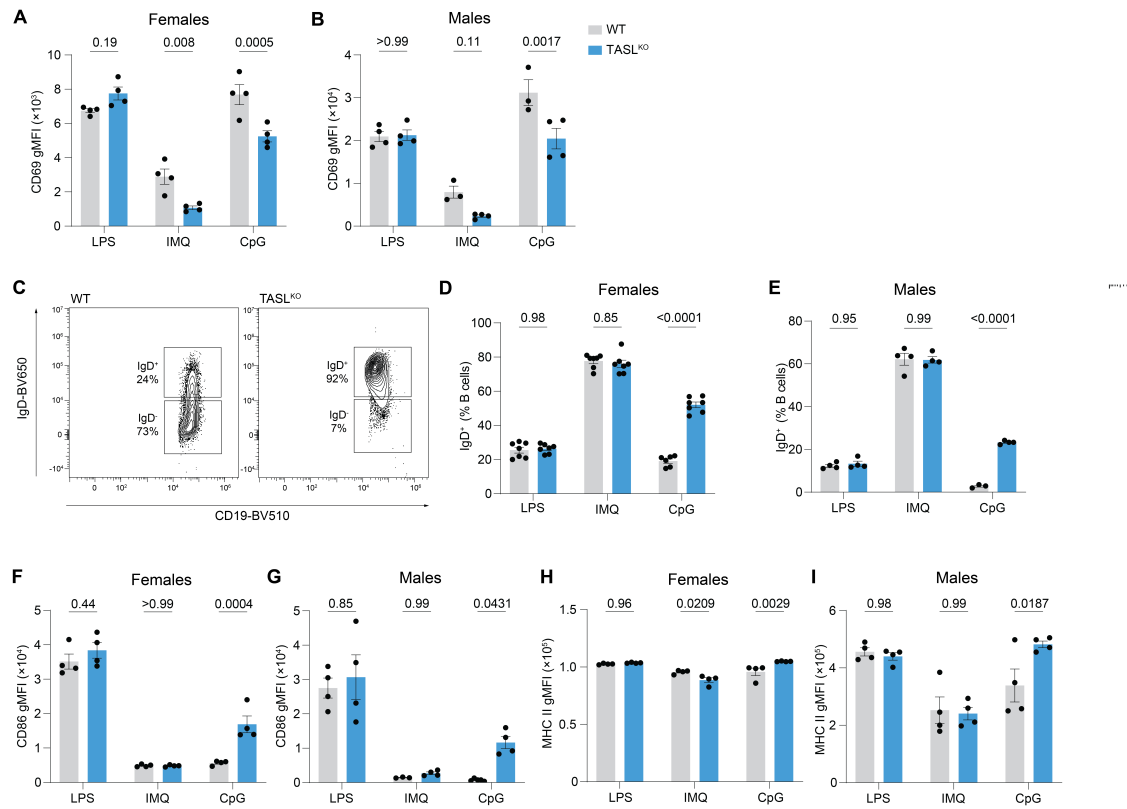


Figure 3.5: TASL is essential for full B cell activation by endolysosomal TLRs

B cells were isolated from the spleens of WT or TASL^{KO} mice, then stimulated with either 10 μ /ml LPS, 100nM CpG or 5 μ g/ml IMQ for 24h or 72h. (A-B) Flow cytometric quantification of CD69 gMFI after 24h stimulation in (A) female (n=4) and (B) male (n=4) B cells. (C) Representative gating of IgD⁺ B cells in WT and TASL^{KO} mice, and (G-H) quantification of IgD⁺ B cells after 72h stimulation of (D) female (n=7) and (E) male (n=4) B cells. (F-G) Flow cytometric quantification of CD86 gMFI after 72h stimulation of (F) female (n=4) and (G) male (n=4) B cells. (H-I) Flow cytometric quantification of MHC II gMFI after 72h stimulation of (H) female (n=4) and (I) male (n=4) B cells. Statistical significance was calculated using a two-way ANOVA with Šidák's multiple testing correction. Data are representative of at least two independent experiments.. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

3.2.6 TASL is required for cytokine production

As TASL plays a role in B cell activation, I next wanted to investigate whether it is required for B cell function. Initially, to determine whether TASL affects the ability of B cells to produce cytokines, I examined the expression of the inflammatory cytokine *Il6* after 24h of stimulation with CpG, IMQ or LPS. (Fig. 3.6A). *Il6* expression was dramatically decreased in TASL^{KO} female and male B cells in response to either CpG or IMQ, but expression was unaltered after LPS stimulation

(Fig. 3.6B-D). I also examined the levels of cytokines present in the supernatant of CpG-stimulated B cells after 72h of stimulation using a cytometric bead array, and found that levels of all cytokines examined, namely IL-6, IL-10, TNF, IFN γ , IL-12p70 and GM-CSF, were decreased to at least half the levels seen in the supernatant of WT B cells (Fig. 3.6E).

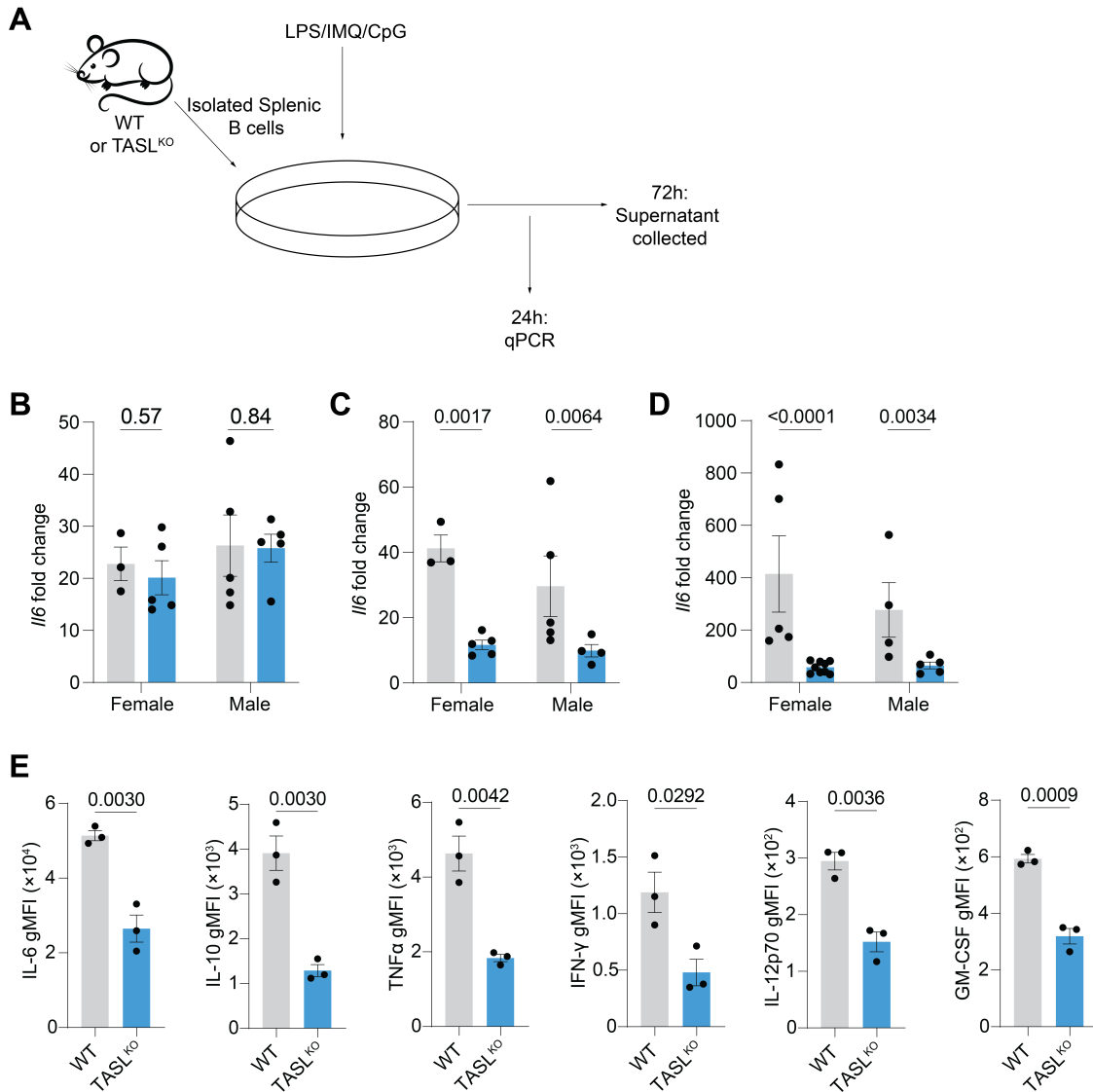


Figure 3.6: TASL is required for cytokine production by B cells. (A) Schematic diagram of experimental set-up. B cells were isolated from the spleens of WT or TASL^{KO} mice, then stimulated with either 10 μ g/ml LPS, 100nM CpG or 5 μ g/ml IMQ for 24h, after which gene expression was analysed, or 72h for collection of culture supernatant. (B-D) Expression of *Il6* and *Il10* in B cells from female WT (n=3-5) and TASL^{KO} (n=5-6) mice or male WT (n=4-5) and TASL^{KO} (n=4-5) mice 24h after stimulation with (B) LPS, (C) IMQ, or (D) CpG. (E) Quantification by cytometric bead array of cytokines in supernatant of CpG-stimulated B cells from female mice after 72h stimulation (n=3, each point represents a single mouse and is the average of 3 technical replicates). Statistical significance was calculated using two-way ANOVA with Šidák's multiple testing correction. Data in (B-D) are representative of two independent experiments, data in (E) is representative of one experiment. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

3.2.7 *Tasl* knockout prevents upregulation of *Prdm1* *in vitro*

I next investigated whether TASL is required for the differentiation of plasmablasts. I isolated splenic B cells and then stimulated them for 24h with either CpG, IMQ or LPS (Fig. 3.7A), to look at the expression of *Prdm1*, which encodes Blimp1, a transcription factor that is essential for plasmablast differentiation. I found that expression of *Prdm1*, was decreased in CpG-stimulated B cells in both males and females (Fig. 3.7B-C). However, there was no difference in *Prdm1* expression between WT and TASL^{KO} B cells when stimulated by either IMQ or LPS.

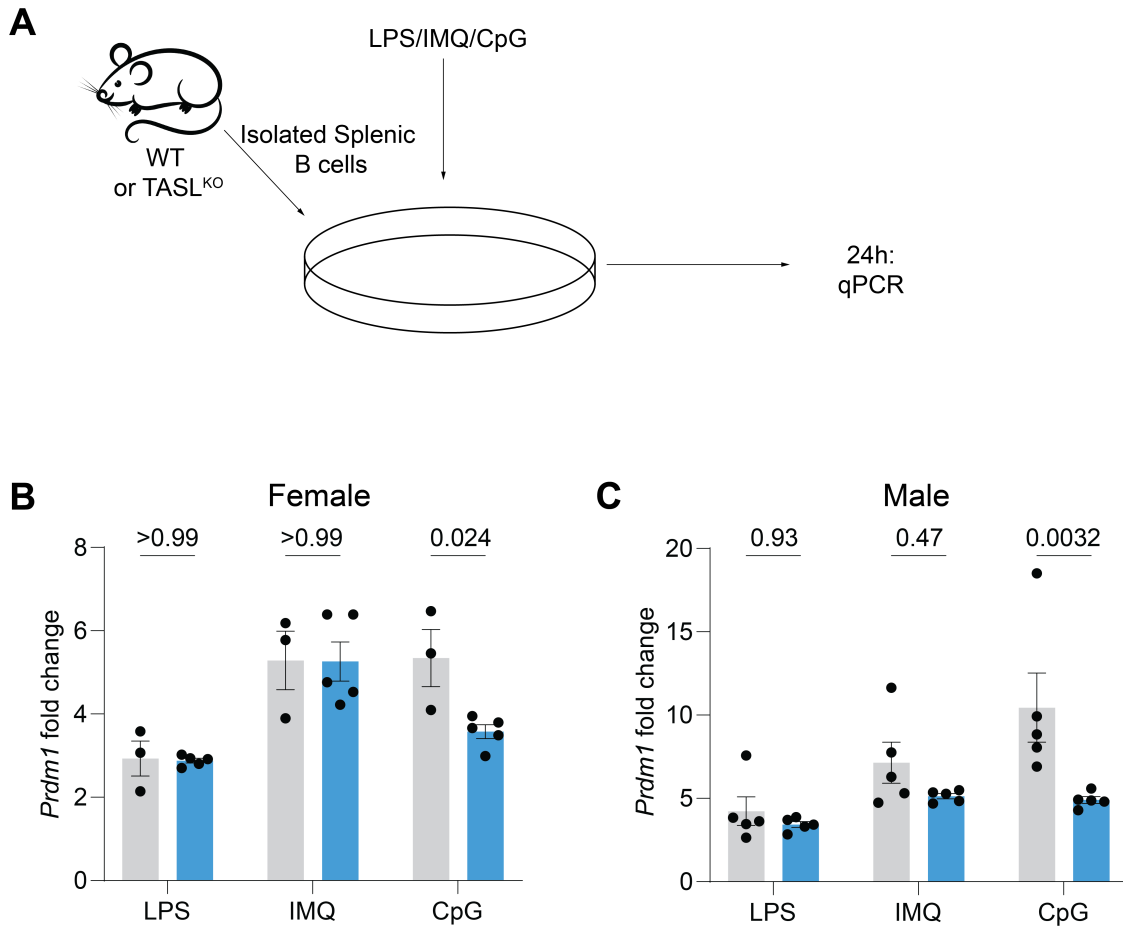


Figure 3.7: TASL knockout prevents upregulation of *Prdm1* *in vitro* (A) Schematic diagram of experimental set-up. B cells were isolated from the spleens of WT or TASL^{KO} mice, then stimulated with either 10 μ g/ml LPS, 100nM CpG or 5 μ g/ml IMQ for 24h. (B-C) Expression of *Blimp1* after 24h of stimulation of isolated B cells from (B) female WT (n=3) or TASL^{KO} mice (n=5) or (C) male WT (n=5) or TASL^{KO} (n=5) mice.

Statistical significance was calculated using a two-way ANOVA with Šidák's multiple testing correction. Data are representative of two independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

3.2.8 TASL is important for plasmablast differentiation *in vitro*

Given the reduction in *Prdm1* expression, I then examined whether the numbers of plasmablasts in culture were altered after 72h of stimulation with CpG, IMQ or LPS (Fig. 3.8A). I observed a decrease in the number of CD19⁺IgD⁻CD138⁺IRF4⁺ plasmablasts at 72h in CpG-stimulated TASL^{KO} B cells (Fig. 3.8B). In female TASL^{KO} cells, only a third as many plasmablasts were present (Fig. 3.8C), and in

males the frequency was reduced by about half (Fig. 3.8D). This demonstrates that TASL is important in the generation of plasmablasts via TLR9 stimulation *in vitro*.

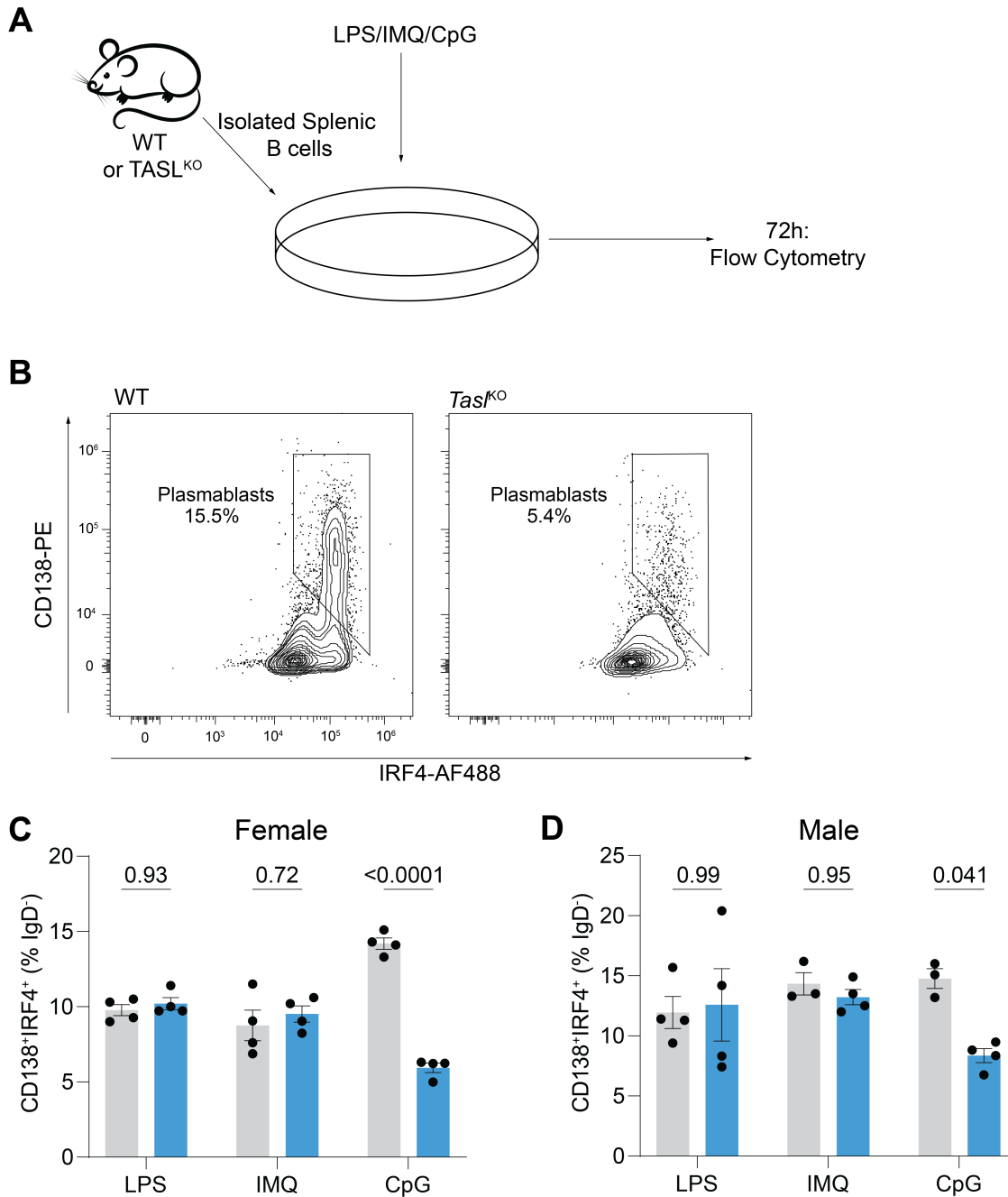


Figure 3.8: TASL is required for *in vitro* plasmablast generation (A) Schematic diagram of experimental set-up. B cells were isolated from the spleens of WT or TASL^{KO} mice, then stimulated with either 10 μ g/ml LPS, 100nM CpG or 5 μ g/ml IMQ for 72h. (B) Representative gating for CD19⁺IgD⁻CD138⁺IRF4⁺ plasmablasts. (C-D) Quantification of (B) in (C) female mice (n=4) and (D) male mice (n=3-4).

Statistical significance was calculated using a two-way ANOVA with Šidák's multiple testing correction. Data are representative of two independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

3.2.9 Impact of *TASL* knockout on B cell gene expression

I next performed RNA sequencing on B cells from female mice stimulated with CpG for 24h. 165 genes were differentially regulated (Fig. 3.9A). Fig. 3.9B shows the top 20 most upregulated and downregulated genes. Amongst the most downregulated genes in *TASL*^{KO} B cells were key interferon-stimulated genes (ISGs), including *Irf7* and *Ifi44*. Indeed, gene set enrichment analysis (GSEA) revealed that the interferon-mediated signalling pathway was the most downregulated pathway, along with responses to cytokines (Fig. 3.9C). Interestingly, the antigen processing and presentation pathway was upregulated (Fig. 3.9C), which supports the increase in CD86 and MHC II that I observed in earlier experiments.

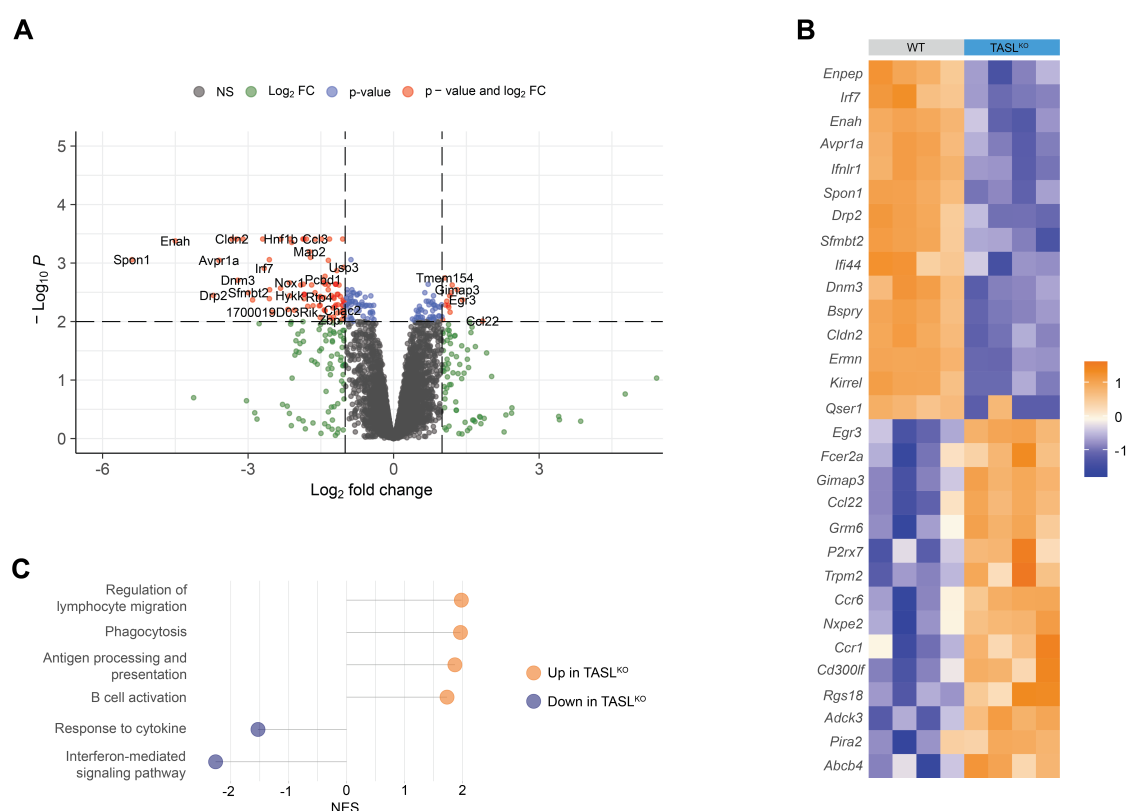


Figure 3.9: Impact of *TASL* deficiency on B cell gene expression (A) Female WT or *TASL*^{KO} B cells (n=4 for each genotype) were stimulated with CpG for 24h then RNA sequencing was performed. (A) Volcano plot of differentially expressed genes. (B) Heatmap of the top 20 upregulated and downregulated genes in *TASL*^{KO} mice from RNA sequencing in (A). (C) Gene set enrichment analysis (GSEA) of RNA sequencing from (A). All pathways shown are statistically significant (adjusted P-value < 0.05). Normalised enrichment score for gene ontology (GO) terms shown.

3.2.10 TASL is critical for ISG upregulation after endolysosomal TLR stimulation

I then confirmed decreased expression of the ISGs *Irf7*, *Isg15* and *Mx1* in CpG-stimulated male and female TASL^{KO} B cells compared to WT B cells using RT-qPCR (Fig. 3.10A). They were also downregulated with IMQ but not LPS stimulation (Fig. 3.10B-C), again showing that TASL only affected the endolysosomal TLR pathway.

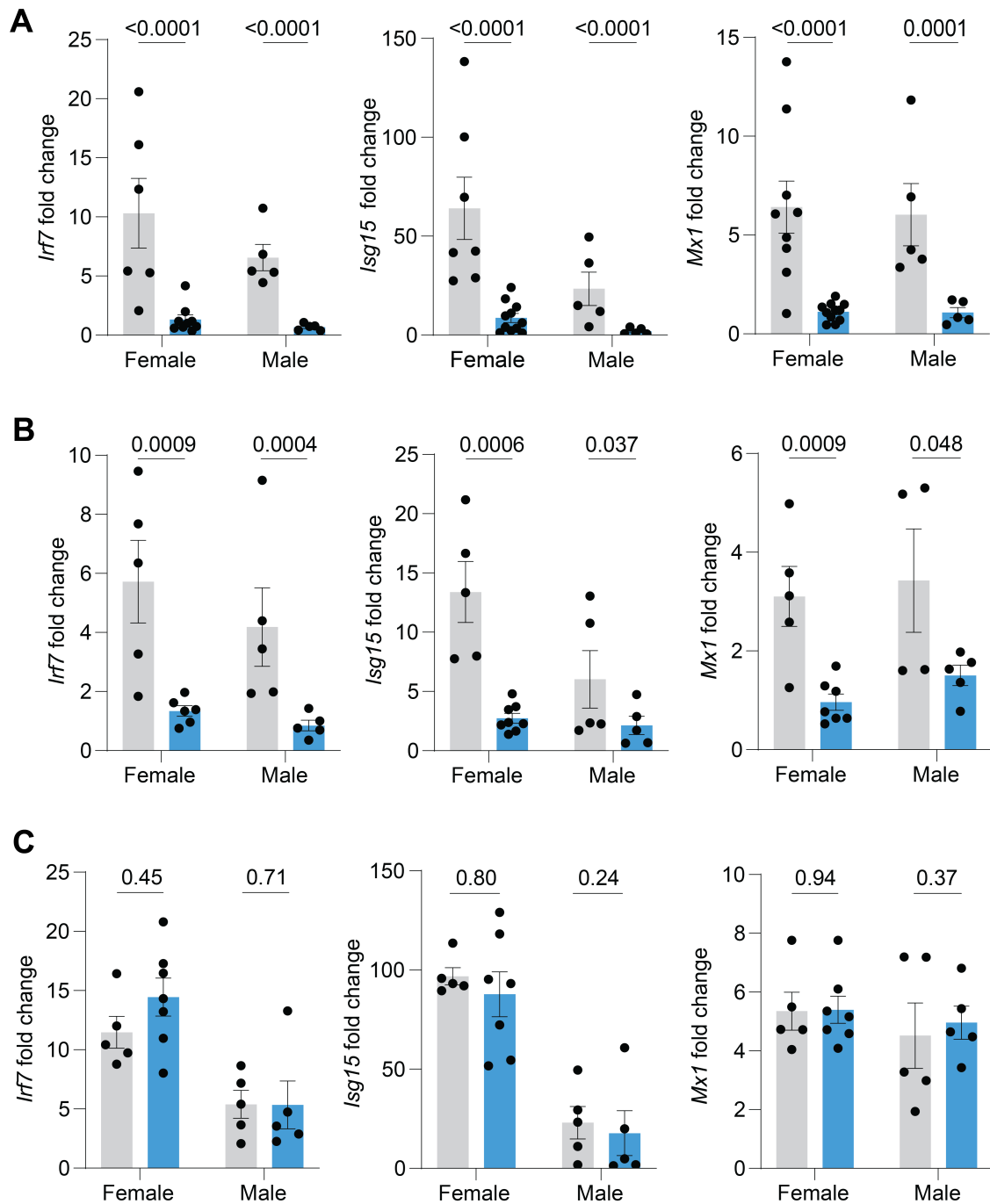


Figure 3.10: TASL is critical for ISG upregulation after endolysosomal TLR stimulation Expression of *Irf7*, *Isg15* and *Mx1* in female WT (n=5-9) and TASL^{KO} (n=6-11) B cells and male WT (n=4-5) and TASL^{KO} (n=5) B cells stimulated for 24h with either (A) 100nM CpG, (B) 5µg/ml IMQ or (C) 10µg/ml LPS.

Statistical significance was calculated using a two-way ANOVA with Šidák's multiple testing correction. Data are representative of and pooled from 2 independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

3.2.11 TASL deficiency impairs antigen presentation

Antigen processing and presentation was one of the most upregulated pathways in TASL^{KO} B cells compared to WT B cells, with key differentially expressed genes in this pathway being shown in Fig. 3.11A. I therefore co-cultured CpG-stimulated TASL^{KO} or WT B cells pulsed with OVA 323-339 peptide at a 1:1 ratio with CD4⁺ T cells isolated from OT II mice (Fig. 3.11B). OT II mice express a transgenic TCR that is specific for the OVA 323-339 peptide presented by MHC II, and which pairs with the CD4 co-receptor, making this a suitable model for examining antigen presentation by B cells to CD4⁺ T cells[235].

CD4⁺ T cell proliferation was not different (Fig. 3.11C). However, CD4⁺ T cells cultured with TASL^{KO} B cells had increased levels the activation marker CD44 (Fig. 3.11D). Additionally, a higher proportion of T cells cultured with TASL^{KO} B cells were IFN γ positive than those cultured with WT B cells (Fig. 3.11E). Levels of BCL6 were also increased in T cells cultured with TASL^{KO} B cells (Fig 3.11F). Together, this suggests that TASL^{KO} B cells do have increased levels of antigen presentation, at least in this *in vitro* setting.

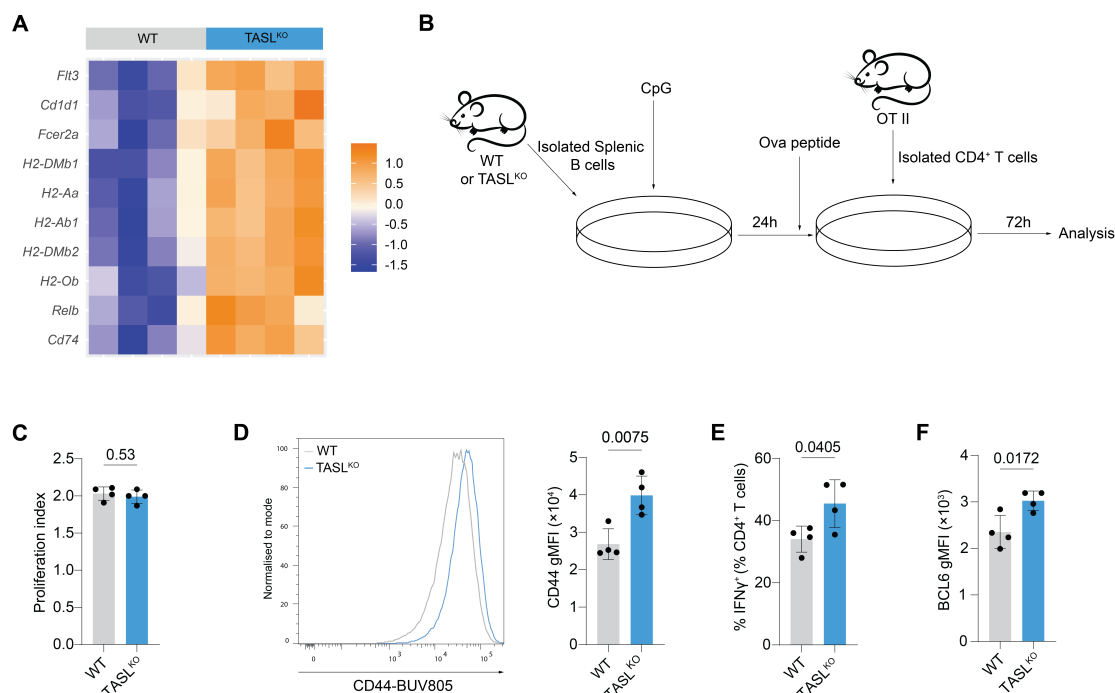


Figure 3.11: TASL deficiency impacts antigen presentation (A) Heatmap displaying genes in the GO-BP antigen processing and presentation pathway that are differentially expressed in *TASL*^{KO} B cells. (B) Schematic diagram of experimental set-up. B cells were isolated from the spleens of WT (n=4) or *TASL*^{KO} (n=4) male mice, then stimulated with 100nM CpG for 24h, and in the last 30 mins OVA-peptide was added. Isolated CD4⁺ T cells from OT II mice were then added in a 1:1 ratio. After 72h co-culture, cells were analysed. (C) Flow cytometric quantification of proliferation index for T cells after 72h co-culture. (D) Representative histogram of CD44 gMFI, and its quantification. (E) Quantification of IFNγ⁺ CD4⁺ T cells. (F) Quantification of BCL6 gMFI in CD4⁺ T cells.

Statistical significance was calculated using a two-tailed unpaired t test. Data are representative of two independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

3.3 Discussion

In summary, I investigated the phenotype of a novel *TASL*^{KO} mouse and found that *TASL* is essential for the full activation of B cells via the endolysosomal TLRs, TLR7 and TLR9, whilst TLR4 stimulation was unaffected. This was expected,

due to the location of TASL in the endolysosome[168][179]. TASL-deficient B cells had decreased expression of ISGs, as well as lower levels of the surface activation marker CD69, and a higher proportion of stimulated B cells retained a naive IgD⁺ phenotype in response to either TLR7 or TLR9 stimulation.

Additionally, TASL^{KO} B cells secreted lower amounts of cytokines *in vitro* compared with WT B cells. IRF5 is crucial for the secretion of several inflammatory cytokines, including IL-6 and IFN γ following endolysosomal TLR stimulation[236], so a significant reduction in cytokine production was expected, and matches results seen previously[183][182]. Using a cytometric bead array, I observed decreased production of all cytokines analysed, including classically anti-inflammatory cytokines such as IL-10, which are regulated by pathways other than endolysosomal TLRs and the IRF5 pathway. This is likely due to decreased paracrine stimulation of B cells in culture - for example, IFN γ can promote IL-10 production in B cells[237], so a decrease in IFN γ stimulation could subsequently impact IL-10 production. Additionally, an *in vitro* setting is not representative of the physiological extracellular environment of B cells, and concentrations of cytokines in culture will be much higher, so analysis of cytokine expression of B cells *in vivo* would be required to confirm this universal decrease in cytokine production.

TASL does not play a role in regulating immune cell populations at steady-state, nor in B cell maturation in the bone marrow. B cell viability was unaltered by TASL deficiency. There was a slight decrease in proliferation in response to TLR9 stimulation, which could be expected as IRF5 deficiency also decreases proliferation in response to TLR9 stimulation[234].

RNA sequencing of CpG-stimulated B cells confirmed that the main pathway downregulated in TASL^{KO} B cells is interferon-mediated signalling pathway, matching results seen by Drobek et al (2025)[183]. Two key ISGs found to be downregulated in the absence of TASL were *Isg15* and *Mx1*. Both these ISGs are upregulated in SLE[238], and *Isg15* expression correlates with disease activity[239]. Patients with active SLE also have ISG15-secreting plasmablasts, proposed to

be a subset of plasma cells causing inflammation in autoimmunity independently of immunoglobulin secretion[240].

Intriguingly, CpG-stimulated $TASL^{KO}$ B cells had increased levels of CD86, along with slightly increased MHC II levels, in contrast to decreased CD86 and MHC II seen in IMQ-stimulated cells. As CD86 is a co-stimulatory molecule, this suggests that $TASL^{KO}$ B cells may have increased antigen presentation capacity. TLR9 stimulation is a potent inducer of an antigen presenting state in B cells [232], and it is interesting that loss of TASL increases antigen presentation, whilst impairing other B cell functions. CD86 levels have also been shown to be increased in IRF5 deficient B cells stimulated with CpG, although the explanation for this remains unclear[198]. RNA-sequencing of CpG-stimulated $TASL^{KO}$ B cells showed an increase in expression of genes in the antigen processing and presentation pathway. Co-culture experiments also confirmed that antigen presentation seems to be increased, with OT-II T cells co-cultured with OVA-peptide pulsed $TASL^{KO}$ B cells increasing levels of the activation marker CD44, $IFN\gamma$ and BCL6, a transcription factor essential for T follicular helper (Tfh) cell development.

RNA sequencing also revealed that *Irf7* is one of the most downregulated genes in $TASL^{KO}$ B cells. *Irf7* is constitutively expressed in B cells[241]. The reduction in *Irf7* expression could be due to a secondary downstream effect of reduced IFN paracrine signalling, as suggested by Drobek et al (2025)[183]. However, as IRF7 plays an important and independent role in the development of autoimmunity and development of plasma cells, as demonstrated in recent work by Fike et al (2025)[242], future work should investigate the interaction of these two pathways and whether TASL has effect on the IRF7 pathway independent of it's role in the IRF5 pathway. Using a mouse model deficient in both IRF5 and TASL could reveal insight in this area.

TASL deficiency additionally impaired the ability of B cells to differentiate into plasmablasts. This was expected, as IRF5 is known to play a role in plasmablast differentiation, and be required for the upregulation of *Blimp1*, a key transcription factor required for plasmablast differentiation[197][234]. Whether TASL impairs the

production of plasmablasts *in vivo* in response to immunisation will be addressed in the next chapter.

As *Tasl* is encoded on the X chromosome, and hypothesised to play a part in the sexual dimorphism seen in SLE[166], I analysed B cells isolated from the spleens of male and females separately, to determine whether sex impacted the effect of TASL deficiency. Sex did not seem to be a significant factor, with male and female TASL^{KO} B cells showing a similar decrease in activation, and increase in antigen presentation. However, an *in vitro* stimulation experimental set-up is not ideal to explore a subject as complicated as sexual dimorphism. Hormone levels, epigenetic modification, and tissue microenvironments are all factors that are not recapitulated in an *in vitro* setting, yet may play important roles in sexual dimorphism. This question will therefore continue to be explored *in vivo* in the following chapters.

4

Investigating the role of TASL in response to immunisation

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4.1 Background

The activating stimulus determines whether a B cell enters the extrafollicular response, or whether it enters the GC reaction. The GC reaction occurs in secondary lymphoid tissue, such as the spleen and lymph nodes. In the GC reaction, B cells cycle through the anatomical dark zone, where they undergo rapid proliferation and somatic hypermutation, a process in which the enzyme activation-induced cytosine deaminase (AID) introduces point mutations into the variable region of immunoglobulin genes, thereby altering B cell specificity. In the light zone, B cells must compete for T cell help in order to survive and proliferate, leading to the selection of higher affinity BCRs in a process called affinity maturation. The GC reaction leads to the production of high-affinity plasma cells, or memory B cells. This differs from the extrafollicular response, which rapidly produces short-lived plasmablasts.

The absence of TASL has been shown to blunt responses to immunisation with CpG and IMQ[183] in murine models, but the role of TASL in broader T-independent and T-dependent humoral responses remains unknown.

4.1.1 Chapter aims

In this chapter I have aimed to answer the questions:

- Does TASL play a role in the humoral response to immunisation?
- Is the role of TASL primarily in the germinal centre or extrafollicular response?

4.2 Results

4.2.1 Effect of *Tasl* knockout on baseline immunoglobulin levels

To establish whether TASL deficiency impacts immunoglobulin levels at steady state, I collected serum from unimmunised mice and performed Ig isotyping. At baseline, there was a significantly decreased level of IgG2b in female mice, and

a trend to decreased Ig levels in other antibody isotypes, as well as reduced free kappa light chains (Fig. 4.1A). In male mice, no difference was observed in any isotype between WT and TASL^{KO} mice (Fig. 4.1B).

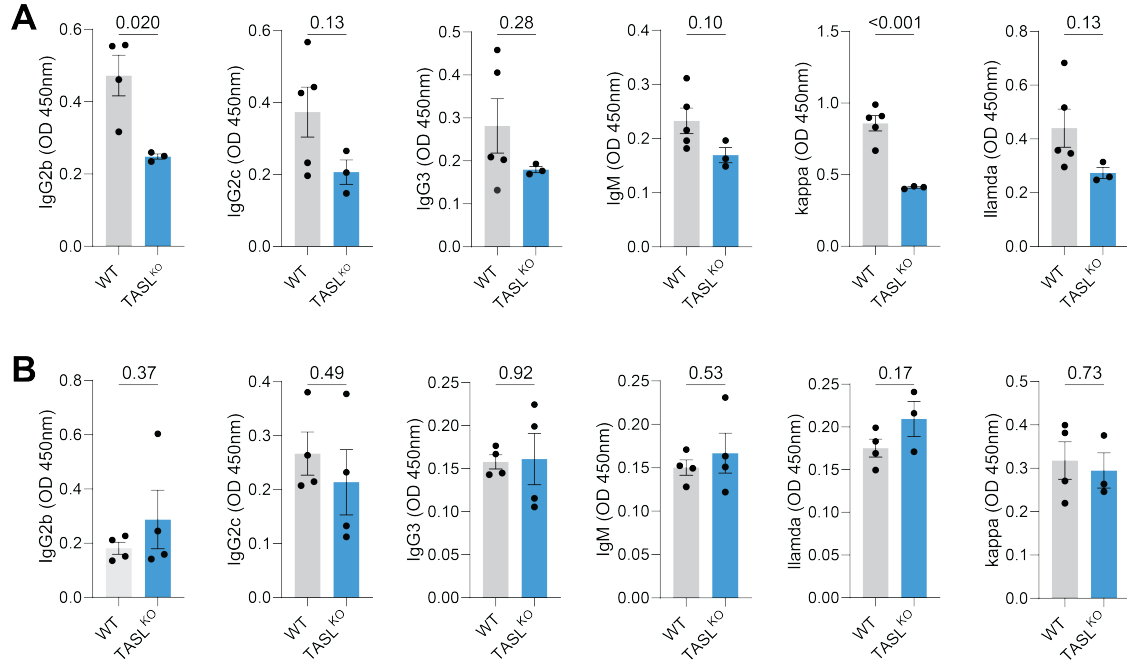


Figure 4.1: Baseline immunoglobulin levels in unimmunised mice. (A) Ig levels in WT (n=4-5) and TASL^{KO} (n=3) female mice (B) Ig levels in WT (n=4) and TASL^{KO} (n=4) male mice. Data representative of and pooled from 2 independent experiments. Statistical significance was calculated using two-tailed t-test. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

4.2.2 Impact of TASL deficiency on formation of GCs in response to immunisation with a T-dependent antigen

In order to investigate the role of TASL in response to an immunogen, I injected WT and TASL^{KO} mice with the model antigen 4-hydroxy-3-nitrophenylacetyl-chicken gamma globulin (NP-CGG), which is a T-dependent protein antigen that induces a robust GC response (Fig. 4.2A). The GC reaction peaks about 14 days after NP-CGG immunisation, so at this point I collected the spleens of the mice and examined them by flow cytometry. There was a reduction in the frequency and numbers of CD19⁺IgD⁻CD38⁻GL-7⁺ germinal centre B cells (GCBCs) in both male and female mice (Fig. 4.2B-C).

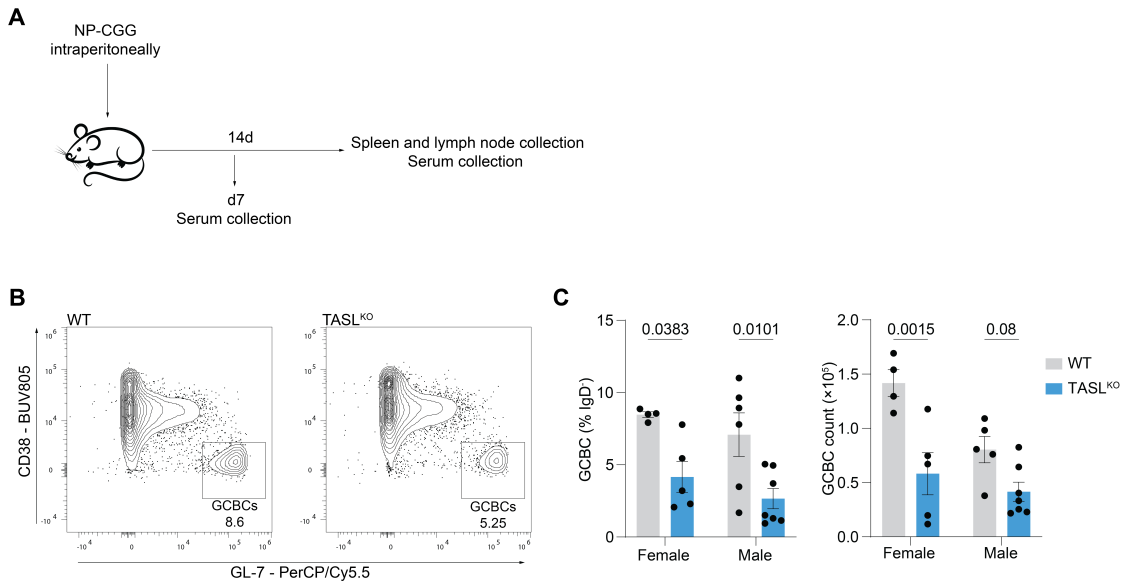


Figure 4.2: Impact of TASL deficiency on formation of GCs in response to immunisation with a T-dependent antigen.

(A) Schematic of immunisation schedule. Mice were immunised intraperitoneally with NP-CGG, then spleens and serum were collected 14 days post-immunisation. Serum was also collected at day 7. (B) Representative gating of CD19⁺IgD⁻CD38⁻GL-7⁺ germinal centre B cells (GCBCs) 14 days after NP-CGG immunisation in the spleens of female WT (n=4) and TASL^{KO} (n=5) mice and male WT (n=6) and TASL^{KO} (n=7) mice. (C) Quantification of (B).

Statistical significance was calculated using two-way ANOVA with Šidák's multiple testing correction. Data are representative of and pooled from 3 independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

4.2.3 TASL is required for plasmablast generation in response to a T-dependent antigen in female mice

I next examined the production of plasmablasts in NP-CGG-immunised mice. I observed a decrease in the frequency and count of CD19⁺IgD⁻CD138⁺IRF4⁺ plasmablasts, only in female mice (Fig 4.3A-B).

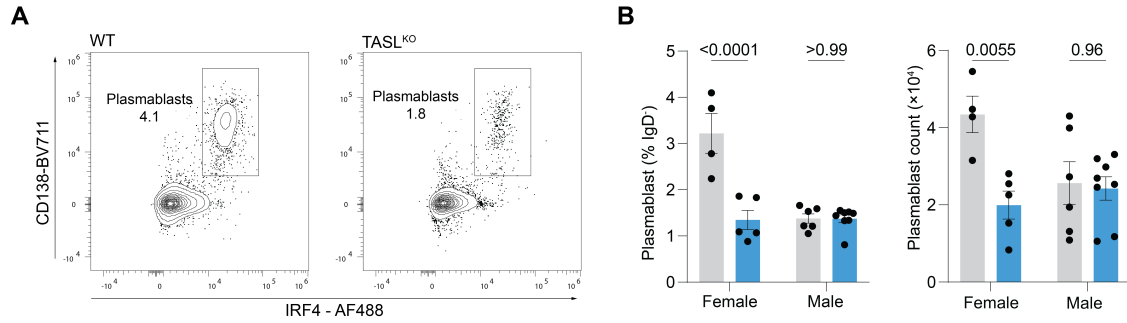


Figure 4.3: TASL is required for PB generation in response to T-dependent antigen in female mice

(A) Representative gating of CD19⁺IgD⁻CD138⁺IRF4⁺ plasmablasts in the spleens of female WT (n=4) and TASL^{KO} (n=5) mice and male WT (n=6) and TASL^{KO} (n=8) mice. (B) Quantification of (A).

Statistical significance was calculated using two-way ANOVA with Šidák's multiple testing correction. Data are representative of and pooled from 3 independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

4.2.4 TASL is dispensable for affinity maturation

The long-lasting nature of the GCs induced by NP-CGG enables affinity maturation to be examined. Low affinity antibodies are only able to bind NP-BSA with a higher conjugation rate (NP₂₀), whereas antibodies with higher affinity will also bind NP-BSA with a low conjugation rate (NP₂). Therefore, the level of affinity maturation can be measured by detecting the difference in the relative binding affinity of IgG to NP₂ and NP₂₀.

I therefore measured the levels of anti-NP₂₀ IgG1 and anti-NP₂ IgG1 in the serum of mice 14 days after immunisation with NP-CGG, and found no difference in the levels in either male or female mice (Fig. 4.4A). There was also no difference in the ratio of anti-NP₂:anti-NP₂₀ IgG1, showing that there is no defect in affinity maturation (Fig. 4.4B). This suggests that even though the numbers of GCBCs are slightly lower in TASL^{KO} mice compared to WT mice, GC function is not significantly impaired.

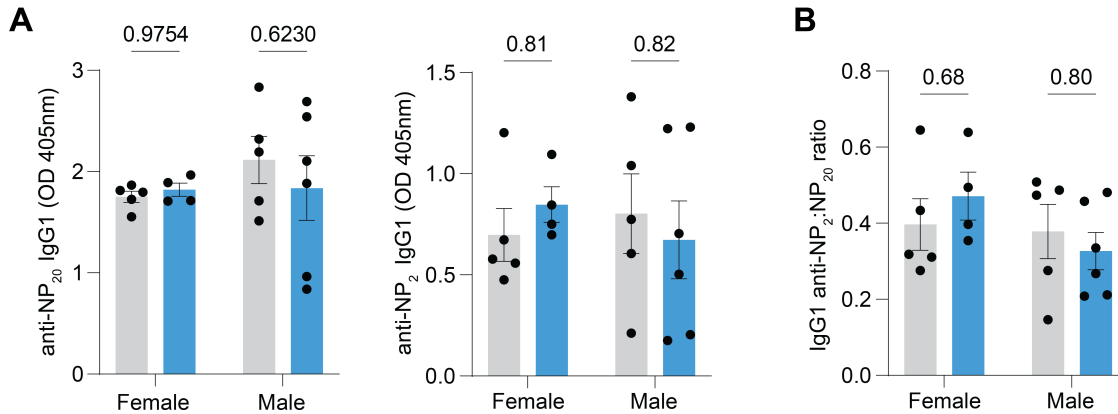


Figure 4.4: TASL is dispensable for affinity maturation. (A) ELISA quantification of IgG1 anti-NP binding to NP_{>20}-BSA and binding to NP₁₋₄-BSA in sera of female WT (n=5) and $TASL^{KO}$ (n=4) and male WT (n=5-6) and $TASL^{KO}$ (n=6) mice 14d after immunisation with NP-CGG. (B) Ratio of high-affinity IgG1 NP-specific antibodies to low affinity IgG1 NP-specific antibodies in (A).

Statistical significance was calculated using two-way ANOVA with Šidák's multiple testing correction. Data are representative of and pooled from 3 independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

4.2.5 TASL is essential for IgG2c production

I next measured the levels of other NP-specific antibody isotypes in the serum 14 days after immunisation with NP-CGG, and found that only NP-specific IgG2c levels were decreased (Fig. 4.5A), whilst all other isotypes measured were unaffected (Fig. 4.5B). As class-switching to IgG2c can happen extrafollicularly, I also looked at the levels in the serum 7 days post-NP-CGG immunisation, before the peak of the GC reaction, and found that, although NP-specific IgG2c levels were relatively low in both WT and $TASL^{KO}$ serum, there was still a significant decrease in the IgG2c levels in the serum of $TASL^{KO}$ mice at this time point (Fig. 4.5C). This suggests the decrease seen in IgG2c could at least in part be due to a defect in the extrafollicular reaction.

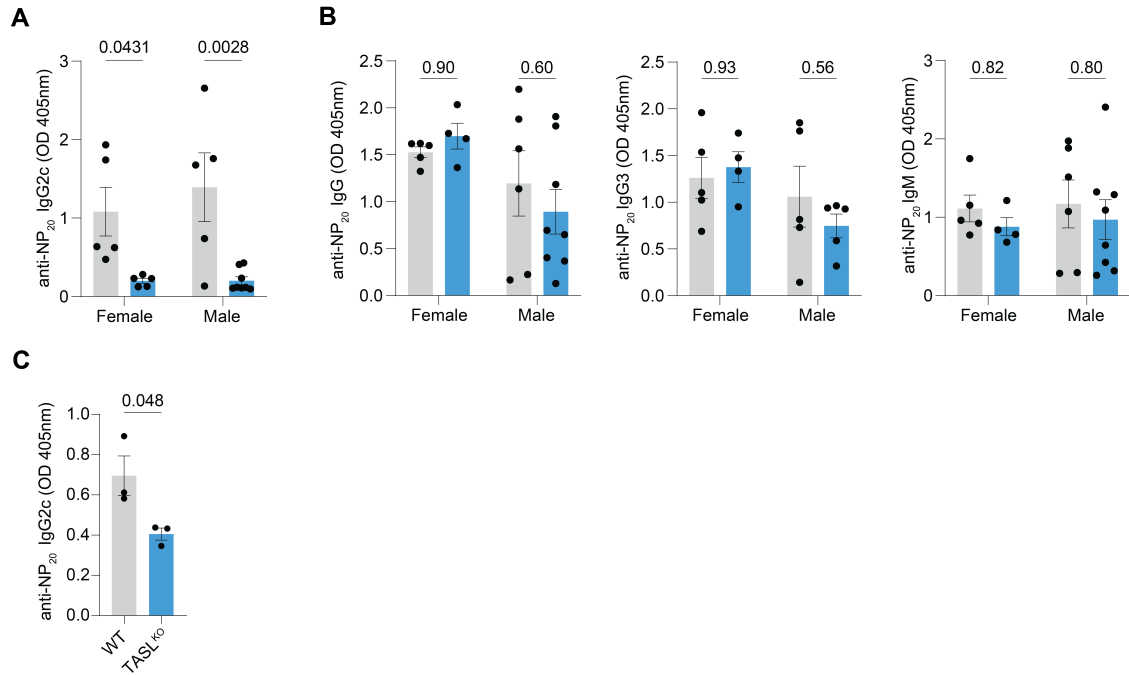


Figure 4.5: TASL is essential for IgG2c production (A) ELISA quantification of NP_{>20}-BSA binding IgG2c in sera of female WT (n=5) and *TASL*^{KO} (n=5) and male WT (n=5) and *TASL*^{KO} (n=8) mice, 14d after immunisation with NP-CGG. Data representative of 3 independent experiments. (B) ELISA quantification of NP_{>20}-BSA binding IgG, IgG3 and IgM in sera of female WT (n=5) and *TASL*^{KO} (n=4) and male WT (n=6) and *TASL*^{KO} (n=5-8) mice, 14d after immunisation with NP-CGG. (C) ELISA quantification of NP_{>20}-BSA binding IgG2c in serum of female WT and *TASL*^{KO} mice (n=3) 7d after NP-CGG immunisation.

Statistical significance was calculated using two-way ANOVA with Šidák's multiple testing correction (A-B) and two-tailed t-test (C). Data are representative of and pooled from 3 independent experiments, except for (C) which is representative of a single experiment. Data are presented as mean ± S.E.M. Each point represents a single mouse.

4.2.6 *Tasl* deletion has no effect on formation of chronic GCs in Peyer's patches

As GCBC numbers were decreased after immunisation, but affinity maturation was unaffected, I wanted to explore further whether TASL has a role in the GC reaction. Peyer's patches in the intestine differ from other secondary lymphoid tissues in that they are constantly exposed to antigens and stimuli from the gut[243]. Due to this chronic antigen exposure, they are a site in which the GC reaction is continually ongoing. I therefore isolated Peyer's patches from the intestine of WT and *TASL*^{KO} mice to identify if there was any defect in the ongoing GC reaction

here. There was no significant difference in the numbers of GCBCs in the Peyer's patches (Fig. 4.6A). Additionally, I looked at the proportions of GCBCs in the dark zone (DZ), where B cells rapidly proliferate and undergo somatic hypermutation, and the light zone (LZ), where B cells compete for T cell help. I did not identify any differences in the proportions of GCBCs in either zone, demonstrating that there is no defect in GC B cell partitioning (Fig. 4.6B). I also examined whether there was any difference in the number of Tfh cells in the Peyer's patches, and again, saw no difference between WT and *TASL*^{KO} mice (Fig. 4.6C). Together, this shows that *TASL* does not affect the formation of these chronic GCs in the gut, and suggests that the role of *TASL* in the germinal centre reaction is minor, if anything.

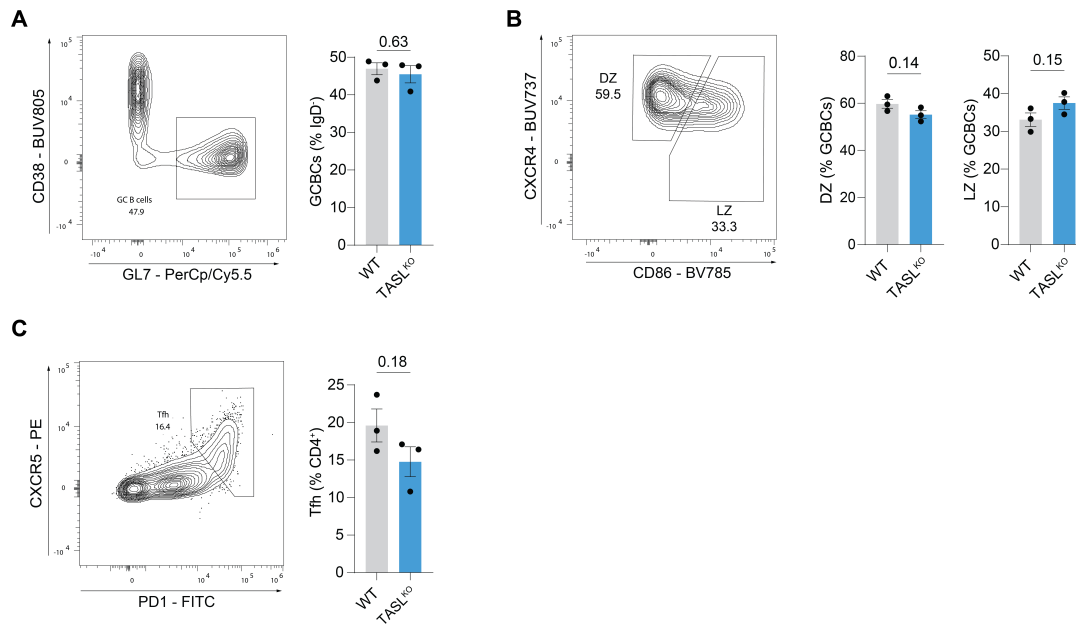


Figure 4.6: Impact of *TASL* on GCs in Peyer's patches (A) Representative gating and quantification of CD19⁺IgD⁻CD38⁻GL-7⁺ germinal centre B cells (GCBCs) in Peyer's patches in female WT and *TASL*^{KO} mice (n=3). (B) Representative gating and quantification of CXCR4^{hi}CD86^{lo} Dark Zone (DZ) and CXCR4^{lo}CD86^{hi}Light Zone (LZ) cells within the GC in female WT and *TASL*^{KO} mice (n=3). (C) Representative gating and quantification of CXCR5⁺PD-1^{hi} T follicular helper (Tfh) cells. Statistical significance was calculated using two-tailed t-test. Data are representative of a single experiment. Data are presented as mean ± S.E.M. Each point represents a single mouse.

4.2.7 TASL is not required for the GC reaction

To further investigate whether TASL plays a role in the GC reaction, I set up competitive bone marrow (BM) chimeras (4.7A). Irradiated CD45.1⁺ recipient mice were injected with a 50:50 ratio of either CD45.2⁺ WT or TASL^{KO} BM cells, and CD45.1⁺ WT BM cells. After 8 weeks of BM reconstitution, I then immunised the mice with sheep red blood cells (SRBCs), which trigger a T-dependent immune response and the formation of GCs. This experimental design enables competition between the WT and TASL^{KO} B cells in an *in vivo* setting, so any defect in the ability of TASL^{KO} cells, or indeed even a slightly reduced capacity to enter or undergo the GC reaction, can be identified. I initially confirmed that the reconstitution had been successful and equal in the bone marrow (Fig. 4.7B). The peak of the GC reaction after SRBC immunisation is 9 days. I therefore analysed the spleens of the chimeras at this timepoint and found no difference in the proportions of CD45.2⁺ WT or TASL^{KO} B cells relative to CD45.1⁺ WT B cells (Fig. 4.7C). Additionally, I looked at the ratio of CD45.2⁺ plasmablasts compared to CD45.1⁺ plasmablasts, and again this ratio was not altered in WT vs TASL^{KO} cells (Fig. 4.7D). Together, this suggests that TASL^{KO} B cells are no less able than WT B cells to undergo the GC reaction in response to SRBCs.

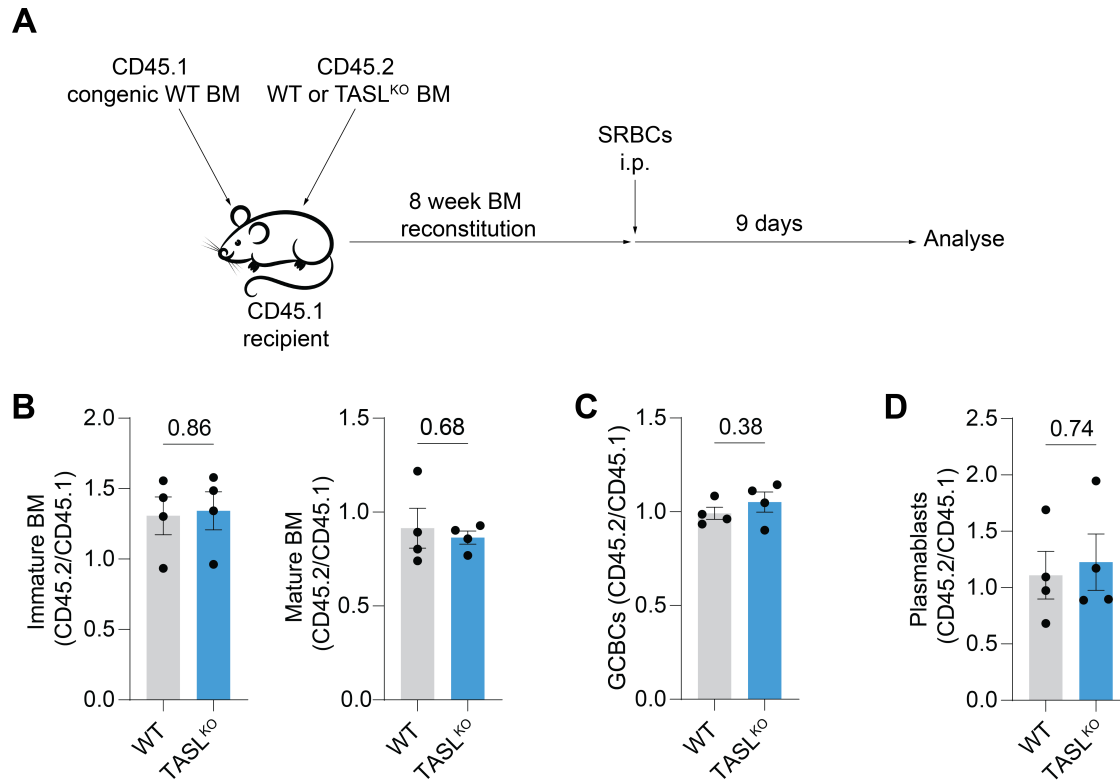


Figure 4.7: Role of TASL in the GC reaction is limited

(A) Schematic of BM chimera set-up and immunisation schedule. Recipient mice received a 50:50 ratio of either CD45.2 WT or CD45.2 TASL^{KO} bone marrow (BM), with $n=4$ mice of each genotype. After 8 weeks, recipient mice were intraperitoneally immunised with sheep red blood cells (SRBCs). Spleens and BM were analysed 9 days post-immunisation. (B) Ratio of CD45.2⁺ WT or TASL^{KO} BM cells normalised to CD45.1⁺ counterpart WT BM cells in recipient mice for both IgD⁺ mature BM cells and IgD⁻IgM⁺ immature BM cells. (C) Ratio of WT or TASL^{KO} CD45.2⁺ splenic GC B cells normalised to CD45.1⁺ counterparts in recipient mice. (D) Ratio of WT or TASL^{KO} CD45.2⁺ plasmablasts normalised to CD45.1⁺ counterparts in recipient mice.

Statistical significance was calculated using two-tailed t-test. Data are representative of a single experiment. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

4.2.8 TASL is important in the extrafollicular response

Having identified that TASL does not play a significant role in the GC reaction, I next wanted to identify if it is important for the extrafollicular reaction. I therefore immunised mice with 4-hydroxy-3-nitrophenyl-Ficoll (NP-Ficoll), a T-independent antigen that triggers a predominantly extrafollicular response, but not the GC reaction[222][244]. I then examined serum and spleens 7 days post-immunisation (Fig. 4.8A). The number of plasmablasts in the spleen was almost halved in female

TASL^{KO} mice, but there was no effect in male mice (Fig. 4.8B-C).

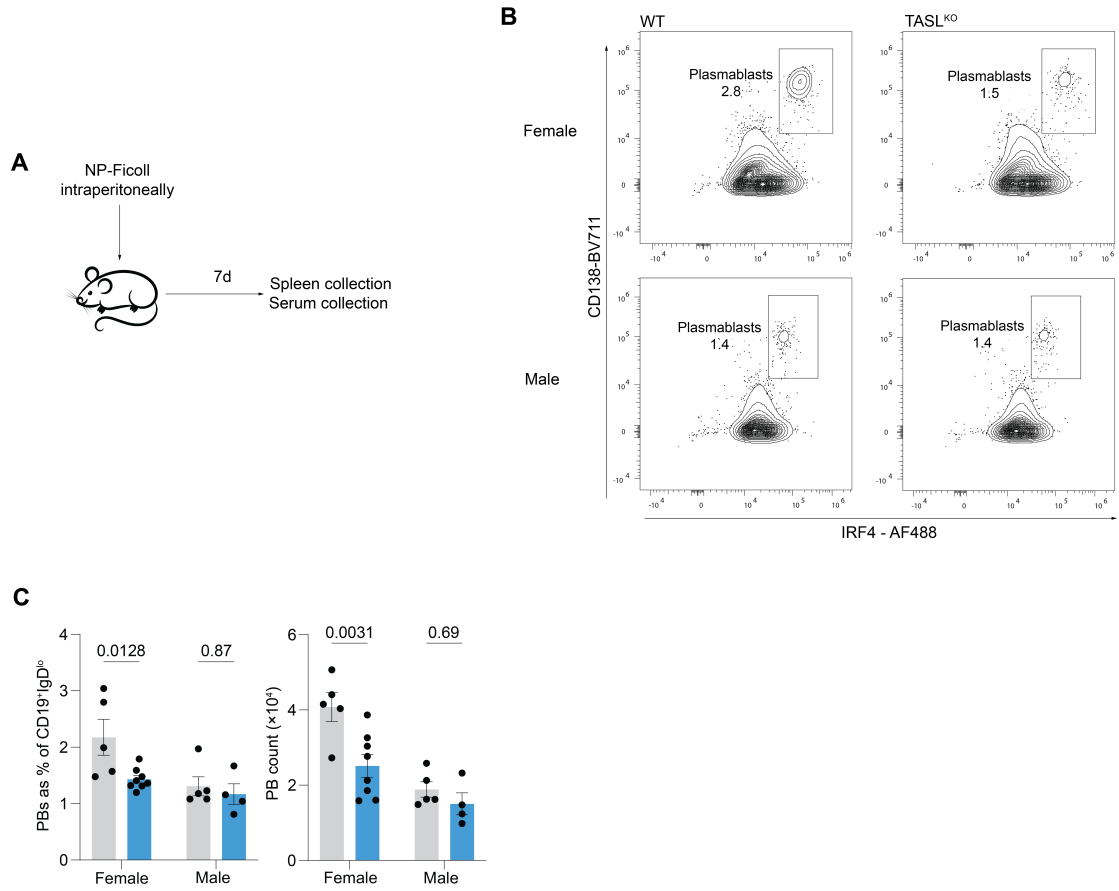


Figure 4.8: TASL is important in the extrafollicular response (A) Schematic diagram of immunisation schedule. Mice were immunised intraperitoneally with NP-Ficoll, then spleens and serum were collected 7 days post-immunisation. (B) Representative gating of CD19⁺IgD⁺IRF4⁺CD138⁺ plasmablasts in spleens of female and male WT and TASL^{KO} mice (C) Quantification of (B).

Statistical significance was calculated using a two-way ANOVA with Šidák's multiple testing correction. Data are representative of and pooled from two independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

4.2.9 The role of TASL in antibody production in response to a T-independent antigen

To measure the antigen-specific response to NP-Ficoll, I determined the levels of NP-specific IgM in the serum of the mice. I found that this was roughly halved in female mice, and also significantly decreased in male mice (Fig. 4.9A) IgG is not the predominant isotype at this timepoint, but I still found that levels of NP-specific IgG were decreased significantly (Fig. 4.9B). This, together with the decreased

number of plasmablasts after NP-Ficoll immunisation, suggests that TASL has an impact in the extrafollicular response.

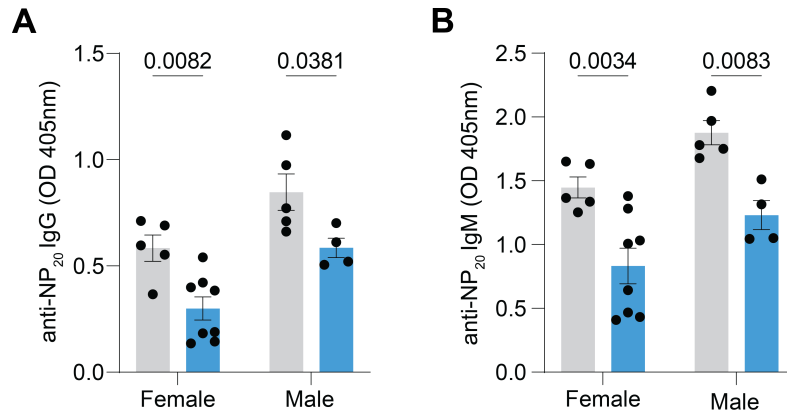


Figure 4.9: The role of TASL in antibody production in response to a T-independent antigen ELISA quantification of NP-specific (A) IgG and (B) IgM in sera of female WT (n=5) and TASL^{KO} (n=8) mice and male WT (n=5) and TASL^{KO} (n=4) mice 7 days after immunisation with NP-Ficoll.

Statistical significance was calculated using a two-way ANOVA with Šidák's multiple testing correction. Data are representative of and pooled from two independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

4.2.10 ABCs are dramatically reduced in aged TASL^{KO} mice

ABCs are a B cell subset primarily produced in the extrafollicular response[27]. They accumulate with age and in autoimmunity, and are more frequent in female mice and humans[23][245][246]. I therefore examined whether there were any alterations in this population in aged TASL^{KO} mice. I found that indeed the frequency and count of ABCs is much increased in 8-month old female mice compared to male mice, but in both sexes the absence of TASL almost completely prevented the development of this cell subset (Fig. 4.10A-B).

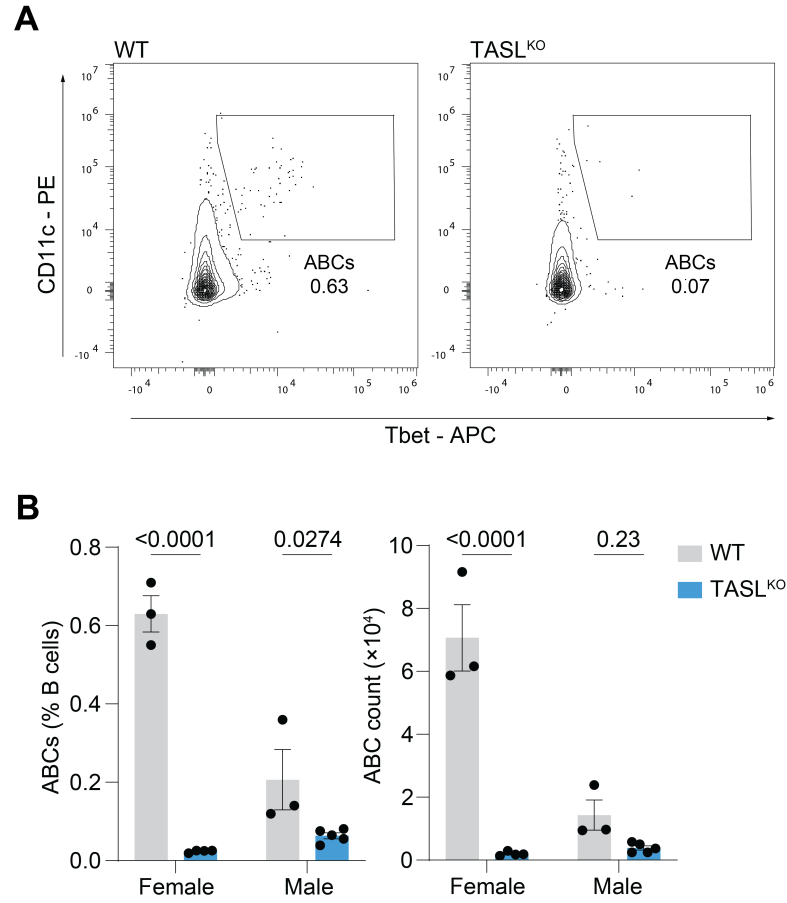


Figure 4.10: ABCs are dramatically reduced in aged TASL^{KO} mice (A) Representative gating of CD11b⁺CD11c⁺Tbet⁺ age-associated B cells (ABCs) from female WT and TASL^{KO} mice. (B) Quantification of ABCs in spleens of aged (8 months) female WT (n=3) and TASL^{KO} (n=4) mice and male WT (n=3) and TASL^{KO} (n=5) mice.

Statistical significance was calculated using a two-way ANOVA with Šidák's multiple testing correction. Data are representative of and pooled from two independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

4.2.11 TASL is required for the development of ABCs

To determine whether the reduction in ABCs was B cell-intrinsic, I isolated B cells from the spleens of WT and TASL^{KO} mice and cultured them for 3 days with anti-CD40, anti-IgM F(ab')₂, and IL-21, and either IMQ or CpG, to induce ABC differentiation[220][25]. I found that B cells from TASL^{KO} mice were unable to differentiate into ABCs with either IMQ or CpG stimulation (Fig4.11A-C). IL-21 is important for the development of ABCs, but is not as effective at generating them

alone in culture without TLR stimulation. However, I still found that with IL-21 alone, there was no difference between the frequency of ABCs between WT and $TASL^{KO}$ B cells (Fig. 4.11D), whereas with IMQ stimulation alone there was still a difference (Fig. 4.11E), suggesting that it is the role of TASL in the TLR pathway that is responsible for the reduction in ABCs seen in $TASL^{KO}$ mice.

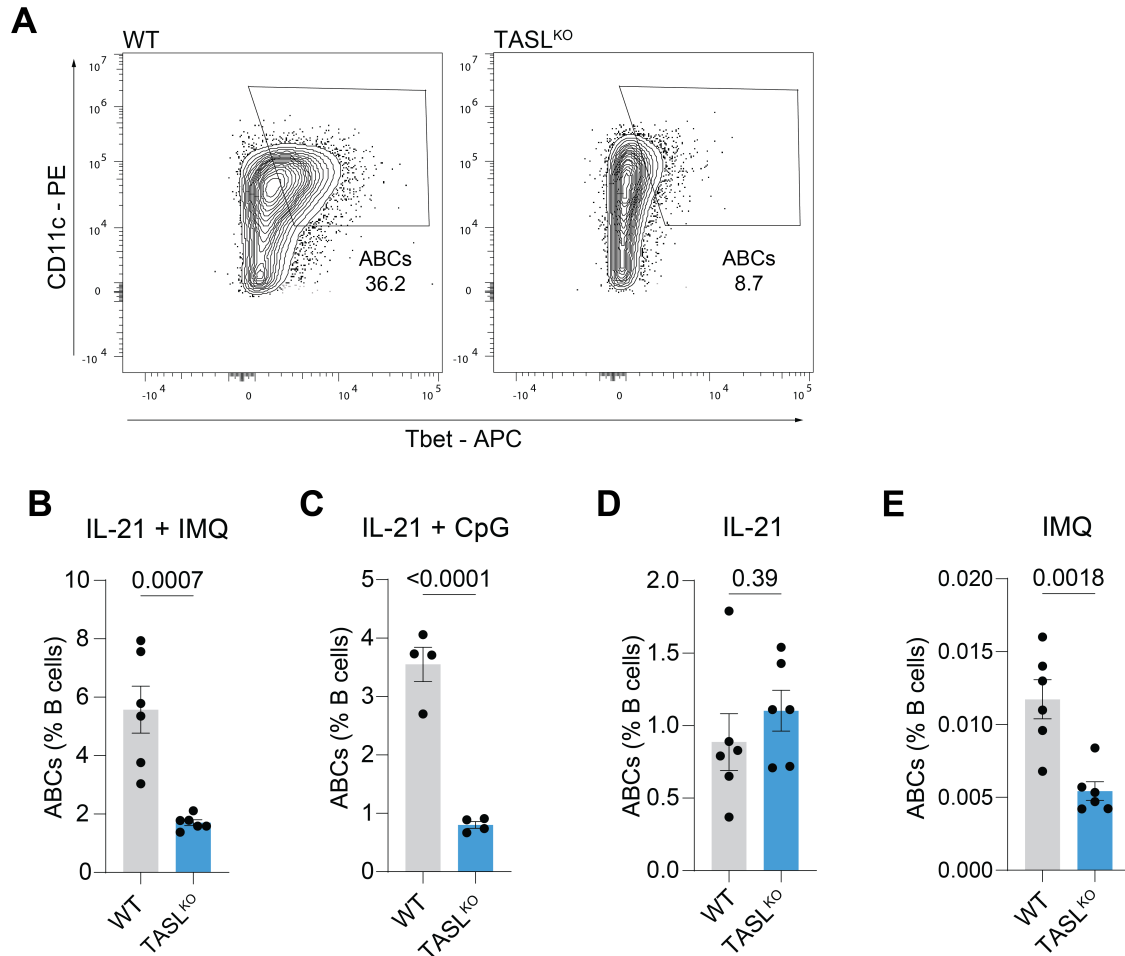


Figure 4.11: TASL is required for the development of ABCs

(A) Representative gating of $CD11b^{+}CD11c^{+}Tbet^{+}$ ABCs from culture of WT and $TASL^{KO}$ B cells with IL-21 and IMQ. (B-E) Quantification of ABC frequency after 3 days of WT (n=4-6) and $TASL^{KO}$ (n=4-6) with either (B) IL-21 + IMQ, (C) IL-21 + CpG, (D) IL-21, and (E) IMQ.

Statistical significance was calculated using an unpaired two-tailed t-test (D, E). Data are representative of and pooled from two independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

4.2.12 *Tasl* deletion decreases IgG2c production in ABC *in vitro* culture

ABCs are potent producers of IgG2c, and T-bet plays an important role in class-switching to the IgG2c isotype[247]. I therefore measured the levels of IgG2c in the supernatant of B cells cultured in the ABC differentiation cocktail by ELISA. I found that the levels of IgG2c were significantly decreased in the cultures of the TASL^{KO} B cells (Fig. 4.12A-B).

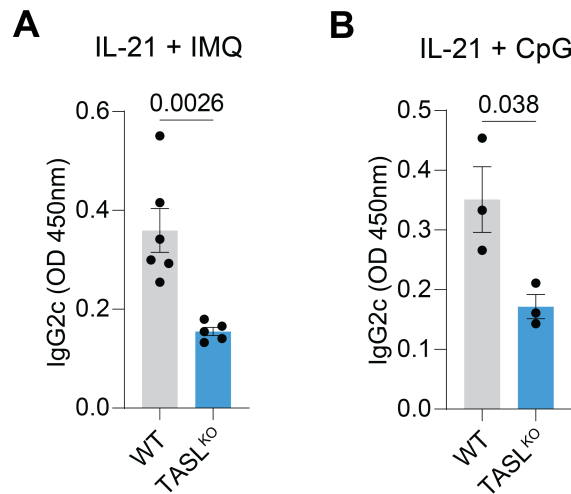


Figure 4.12: *Tasl* deletion decreases IgG2c production in ABC *in vitro* culture ELISA quantification of IgG2c in the supernatant of WT or TASL^{KO} B cells cultured with either (A) IL-21 + IMQ (n=5-6) or (B) IL-21 + CpG (n=3) for 3 days. Statistical significance was calculated using an unpaired two-tailed t-test. Data are representative of and pooled from two independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

4.3 Discussion

In this chapter, I demonstrated that TASL is dispensable for the GC reaction, but appears to play a role in the extrafollicular response, and is essential for the development of ABCs.

Whilst I found that the number and frequency of GCBCs, was diminished in response to the T-dependent antigen NP-CGG, antibody affinity maturation was not impaired, suggesting that the GC reaction is still functional. Another piece of evidence supporting the conclusion that TASL is not essential for the GC reaction is

that there was no alteration in the frequency or numbers of GCBCs seen in Peyer's patches in the intestine, where the GC reaction occurs continually due to constant exposure to antigens in the gut[243]. Additionally, in a competitive bone marrow chimera, *TASL*^{KO} B cells were found in an equal ratio to their WT counterparts in response to immunisation with SRBCs, another T-dependent antigen. This shows that *TASL*^{KO} B cells were not less able to form GCs than WT B cells.

In *Irf5* knockout mice, GC frequency is decreased in response to T-dependent immunisation[248]. IRF5 was found to play a critical role in the generation of Tfh cells, and the ability of Tfh cells to support GC formation, whilst a B-cell intrinsic role was not clear. As the expression of *TASL* is more limited than that of IRF5, and *TASL* mainly functions in B cells and pDCs[182], this could explain why *TASL* deficiency did not have the same impact as the lack of IRF5.

Another key finding in this chapter is that the frequency and number of plasmablasts following immunisation with both T-dependent and T-independent antigens were significantly lower in female mice, but were not impacted in male mice. There is evidence of sexual dimorphism in the role of IRF5 in B cells; for instance the percentage of IRF5⁺ B cells in PBMCs taken from healthy humans is higher in females, and this is linked to increased cytokine production in response to TLR9 stimulation[249], however no mechanism has been described. *Irf5* expression is also increased in female B cells in mice[156]. B cell-intrinsic expression of *IRF5* is also essential for plasmablast differentiation[234], but this has not previously been shown to be different in males and females. As *TASL* is X-linked, and expressed more in female cells[166], it may thus potentially play a larger role in female B cells.

A limitation of the work in this chapter is that the mice are complete knockouts for *Tasl*, therefore other cell types could be playing a role in the effects seen. Notably, pDCs also express high levels of *Tasl* and *TASL* is known to play the same crucial adaptor role in the IRF5 pathway in these cells. *IRF5* expression is also increased in human female pDCs, and increased levels of IRF5 also caused increased IFN α production in response to TLR7 stimulation[157]. Decreased production of cytokines and alterations to the extracellular environment could therefore be

causing the decreased levels of plasmablasts seen in TASL^{KO} females, rather than the impact being fully B cell intrinsic. However, in Chapter 1 I demonstrated that isolated TASL^{KO} B cells were also less able to differentiate into plasmablasts *in vitro*, suggesting at least some part of the effect is B-cell intrinsic.

Another key finding in this chapter is the specific reduction in the IgG2c isotype following *Tasl* deletion, with both slightly decreased levels at baseline, and an almost complete lack of NP-specific IgG2c production after NP-CGG immunisation. IgG2c is an allelic variant of IgG2a, found in the C57BL/6 mouse strain[250]. IRF5 plays a critical role in class-switching to IgG2a/c, both in response to T-dependent and T-independent antigens, as well as in autoimmunity[251][198]. TLR9 and MyD88 signalling is also required for class-switching to IgG2a/c, and there is evidence that IgG2a is a key pathogenic isotype in SLE[252]. IgG2a/c and IgG2b are the only isotypes capable of binding to the activating IgG Fc receptor, FcγRIV, expressed on myeloid cells[253]. This makes IgG2c potent in protective responses against viral and fungal infections[254][255], but also contributes to pathogenesis in autoimmunity. For example, pathogenic IgG2c can recruit FcγRIV-expressing macrophages, which play a role in kidney damage in murine lupus[252].

Class switching to IgG2c can occur quickly in extrafollicular responses to intracellular pathogens[14] and viruses such as influenza[256], and can be induced without T cell-help in response to a TLR7 ligand[257]. This would match with my observation that NP-specific IgG2c was decreased from the early timepoint of 7 days post-NP-CGG immunisation, before the GC response has peaked, as well as at day 14.

I identified a clear role for TASL in the extrafollicular response, seeing a significant decrease in the levels of NP-specific IgG and IgM after immunisation with NP-Ficoll, as well as decreased numbers of plasmablasts in females. In male mice, the frequency and number of plasmablasts was the same in WT and TASL^{KO} mice, but levels of NP-specific IgG and IgM were still decreased, suggesting that there is TASL does play a role in antibody production by male plasmablasts in the extrafollicular response.

Additionally, I found that TASL is essential for the development of ABCs, an extrafollicular-derived subset of B cells. As I found in *in vitro* culture, TLR7 or 9 stimulation are required for the development of ABCs[22]. IRF5 is also critical for ABC development[199], so the role of TASL in the development of this subset makes sense, as it links TLR7 and TLR9 signalling with the IRF5 pathway.

I found that levels of IgG2c were decreased in the culture supernatant of differentiated ABCs. T-bet, a transcription factor that is required for ABC differentiation, is also important for class-switching to IgG2c, especially in response to T-independent stimuli[258][247]. ABCs can act as a memory B cell subtype, demonstrated by a study in which adoptive transfer of ABCs into naive mice, from mice given a primary immune challenge with virus-like particles (VLPs) led to the rapid production of anti-VLP IgG2c and IgG on rechallenge[259]. The generation of ABCs in response to TLR7 stimulation has also been shown to be critical for viral clearance[258], and ABCs are expanded after many viral infections, and after some vaccinations, such as influenza vaccination[260][28]. TASL deficiency prevented mice from controlling chronic LCMV infection[183], and investigating whether TASL prevents effective immune responses, viral clearance and the response to immunisations is therefore a key question that should be explored.

ABCs are expanded in autoimmune disease, and correlate with disease severity in SLE[220][261], and their presence has even been proposed as a prognostic marker in SLE patients[262]. ABCs have also been shown to contribute to sexual dimorphism in an SLE-like syndrome in mice, and TLR7 duplication in male mice was shown to decrease this sexual dimorphism [245]. In this model, IRF5 was also shown to be critical for the development of the ABC population[199]. The role of TASL as an adaptor between TLR7 and IRF5 could therefore provide a putative explanation for its links with disease severity in young females[166].

5

Investigating the role of TASL in the development of SLE in a murine model

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5.1 Background

Due to its role in endolysosomal TLR signalling and the IRF5 pathway, which is strongly linked to lupus, and its genetic association with SLE[166], TASL is hypothesised to play a role in lupus. Indeed, this has recently been demonstrated in two studies of pristane-induced and imiquimod-induced murine models of lupus[183][182]. In the pristane-induced model, mice are intraperitoneally administered pristane (2, 6, 10, and 14 tetramethylpentadecane, TMPD), leading to the development of auto-antibodies, immune complex deposition in the kidneys, and an interferon signature[263][264][265][266]. In the pristane-induced model, high circulating levels of type I IFN are produced by immature Ly6C^{hi} monocytes recruited to the peritoneum after pristane administration[267], and the type I IFN production is mediated exclusively by TLR7 signalling[268]. Aldara is a cream containing the TLR7 agonist IMQ, and induces lupus-like symptoms in mice when applied topically three times a week for a month or more, with TLR7 playing a critical role[269]. The absence of TASL in both of these models ameliorated SLE, with decreased levels of auto-antibodies, decreased ISG expression and decreased splenomegaly. However, whether TASL plays a role in a genetically-determined murine model of lupus has not yet been investigated.

In this work I have focused on the B6.MRL-Fas^{Lpr}/J model. These mice are homozygous for the spontaneous mutation in the murine lymphoproliferation (*Lpr*) locus, which encodes a non-functional form of FAS, the apoptosis-inducing receptor[270]. This leads to defective FAS-mediated apoptosis, and mice with this mutation develop spontaneous lymphadenopathy, with a striking increase in the population of double negative (DN) $\text{CD3}^+\text{CD4}^-\text{CD8}^-$ T cells, as well as severe multi-organ inflammation[271]. B cells play a critical role in the development of autoimmunity in these mice, as do CD4^+ T cells, but the *Lpr* mutation must be in both lymphoid and non-lymphoid populations for the full phenotype to

develop[272][273]. Both males and females are affected in this model, although disease is more severe in females than in males, but some observations can still be made about whether there are sexually dimorphic effects of *Tasl*.

5.1.1 Chapter aims

In this chapter I have aimed to answer the questions:

- Does TASL deficiency ameliorate features of SLE in a murine model?
- Does the impact of TASL differ between females and males?
- Does TASL play a role in the production of ABCs?

5.2 Results

5.2.1 Effect of *Tasl* knockout on splenomegaly and lymphadenopathy

To determine whether TASL was necessary for the development of lupus, I crossed TASL^{KO} mice with the B6.MRL-Fas^{Lpr}/J congenic murine model of lupus, thereby generating B6.MRL-Fas^{Lpr}/J × TASL^{KO} mice (hereafter referred to as MRL^{Lpr} × TASL^{KO}). These mice were aged to 20 weeks to allow features of disease to develop, and were then compared to B6.MRL^{Lpr} mice (hereafter referred to as MRL^{Lpr} mice), or to WT C57BL/6 controls.

I found that the significant splenomegaly and lymphadenopathy typically seen in MRL^{Lpr} was completely absent in MRL^{Lpr} × TASL^{KO} mice (Fig. 5.1A). Substantial splenomegaly and lymphadenopathy was observed in MRL^{Lpr} mice, but in MRL^{Lpr} × TASL^{KO} mice, these secondary lymphoid organs were the same size and mass as WT mice in both sexes (Fig. 5.1B-C).

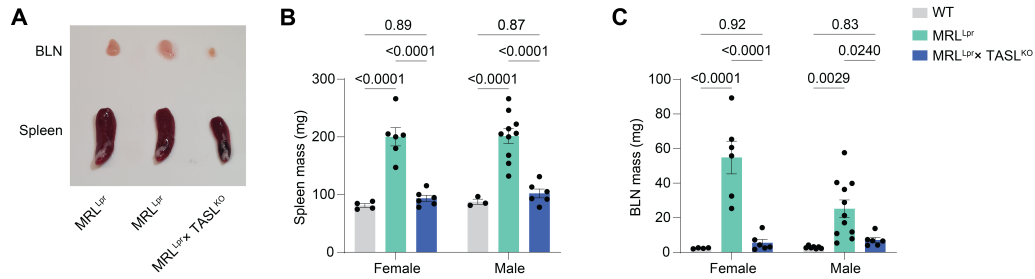


Figure 5.1: Absence of TASL decreases splenomegaly and lymphadenopathy (A) Representative image of spleen and brachial lymph node (BLN) from female MRL^{Lpr} and MRL^{Lpr} × TASL^{KO} mice. (B-C) Quantification of mass of (B) spleen and (C) BLN from female WT (n=4), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=6) mice and male WT (n=3), MRL^{Lpr} (n=10) and MRL^{Lpr} × TASL^{KO} (n=6) mice.

Statistical significance was calculated using a two-way ANOVA with Šidák's multiple testing correction. Data are representative of and pooled from three independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.2.2 *Tasl* deficiency prevents spontaneous GC development in lupus

MRL^{Lpr} mice develop GCs in the absence of immunisation or infection, termed spontaneous GCs. Endolysosomal signalling is critical for their development, and I therefore investigated whether deficiency of *Tasl* also prevented their formation. I found that in both sexes, MRL^{Lpr} × TASL^{KO} mice had the same number of GCBCs as WT mice, compared to MRL^{Lpr} mice which had large numbers of GCBCs (Fig. 5.2A-B).

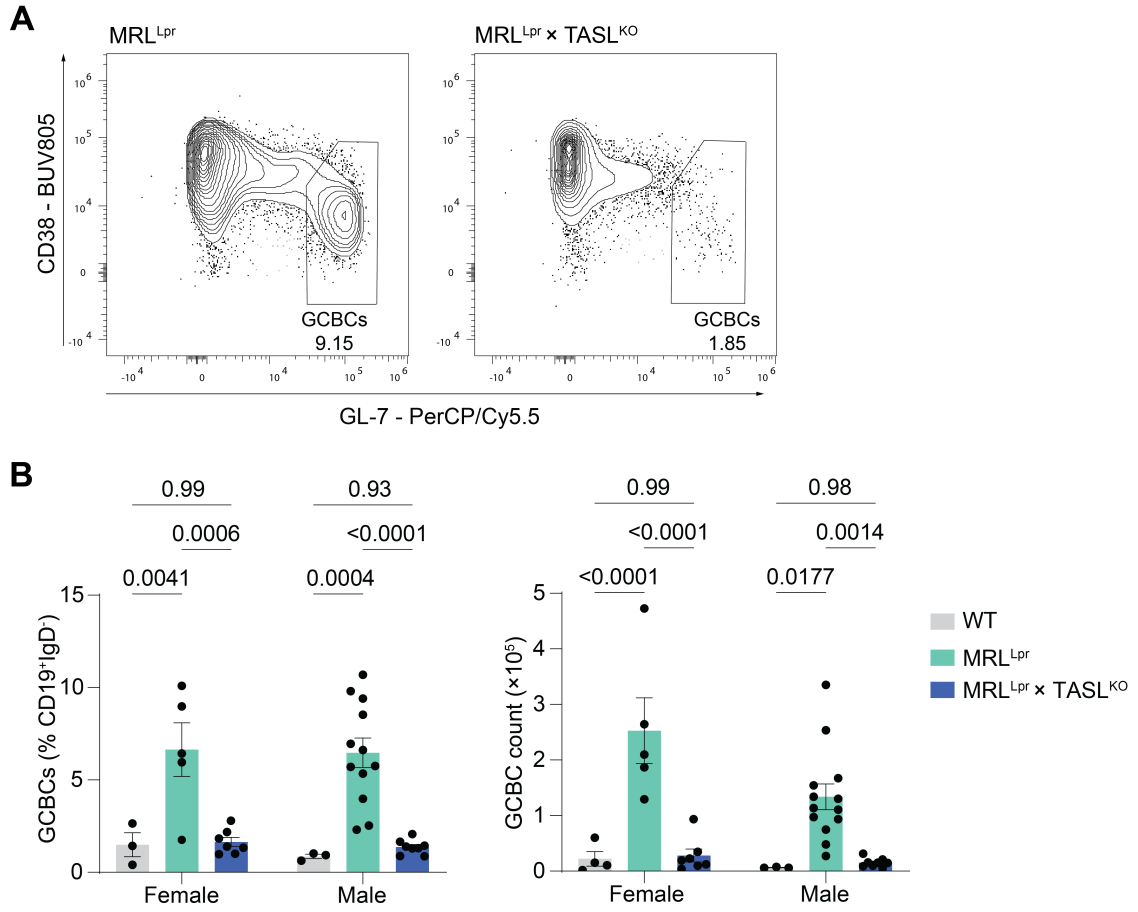


Figure 5.2: *Tasl* deficiency prevents spontaneous GC development in lupus (A) Representative gating of CD19⁺IgD⁻CD38⁻GL-7⁺ germinal centre B cells (GCBCs) in spleens of female MRL^{Lpr} and MRL^{Lpr} × TASL^{KO} mice. (B) Quantification of GCBC frequency and count in female WT (n=4), MRL^{Lpr} (n=5) and MRL^{Lpr} × TASL^{KO} (n=7) mice and male WT (n=3), MRL^{Lpr} (n=12) and MRL^{Lpr} × TASL^{KO} (n=7) mice. Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from three independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.2.3 The Tfh population is reduced in MRL^{Lpr} × TASL^{KO}

As the number of GCBCs was reduced to that of WT mice in MRL^{Lpr} × TASL^{KO}, I next examined the number of Tfh cells, which reside in the light zone of the germinal centre where B cells must present antigen to Tfh cells in order to receive signals to survive and proliferate. B cell-T cell cognate interactions have been shown to be required for autoantibody production in lupus[274]. I found that, similarly to numbers of GCBCs, Tfh cell populations were reduced in the MRL^{Lpr} × TASL^{KO}

mice to the same levels seen in WT mice, compared to significantly expanded populations in both male and female MRL^{Lpr} mice (Fig. 5.3A-B).

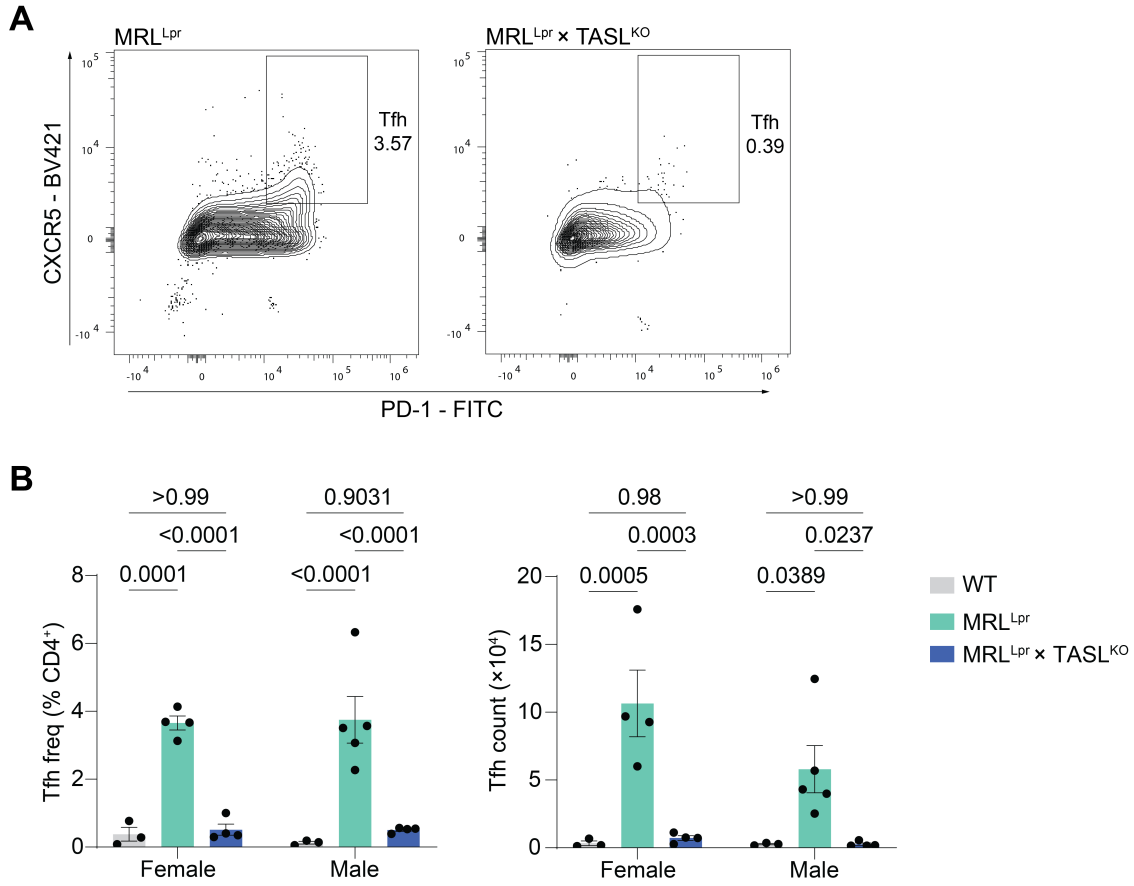


Figure 5.3: Tfh population is reduced in MRL^{Lpr} × TASL^{KO} mice

(A) Quantification of GCBC frequency and count in female WT (n=3), MRL^{Lpr} (n=4) and MRL^{Lpr} × TASL^{KO} (n=4) mice and male WT (n=3), MRL^{Lpr} (n=7) and MRL^{Lpr} × TASL^{KO} (n=6) mice. (B) Quantification of Tfh frequency and count in female WT (n=3), MRL^{Lpr} (n=4) and MRL^{Lpr} × TASL^{KO} (n=4) mice and male WT (n=3), MRL^{Lpr} (n=7) and MRL^{Lpr} × TASL^{KO} (n=6) mice.

Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from three independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.2.4 Spontaneous GCs are absent in BLNs in MRL^{Lpr} × TASL^{KO} mice

As MRL^{Lpr} mice have significant lymphadenopathy, and altered immune cell populations and spontaneous GCs in their BLNs, I then investigated whether spontaneous GCs were also absent in the BLNs of MRL^{Lpr} × TASL^{KO} mice. I found

that numbers of GCBCs and Tfh cells were significantly decreased in both female and male MRL^{Lpr} × TASL^{KO} mice, with the counts of these cell types being almost zero, matching WT mice (Fig. 5.4A-B).

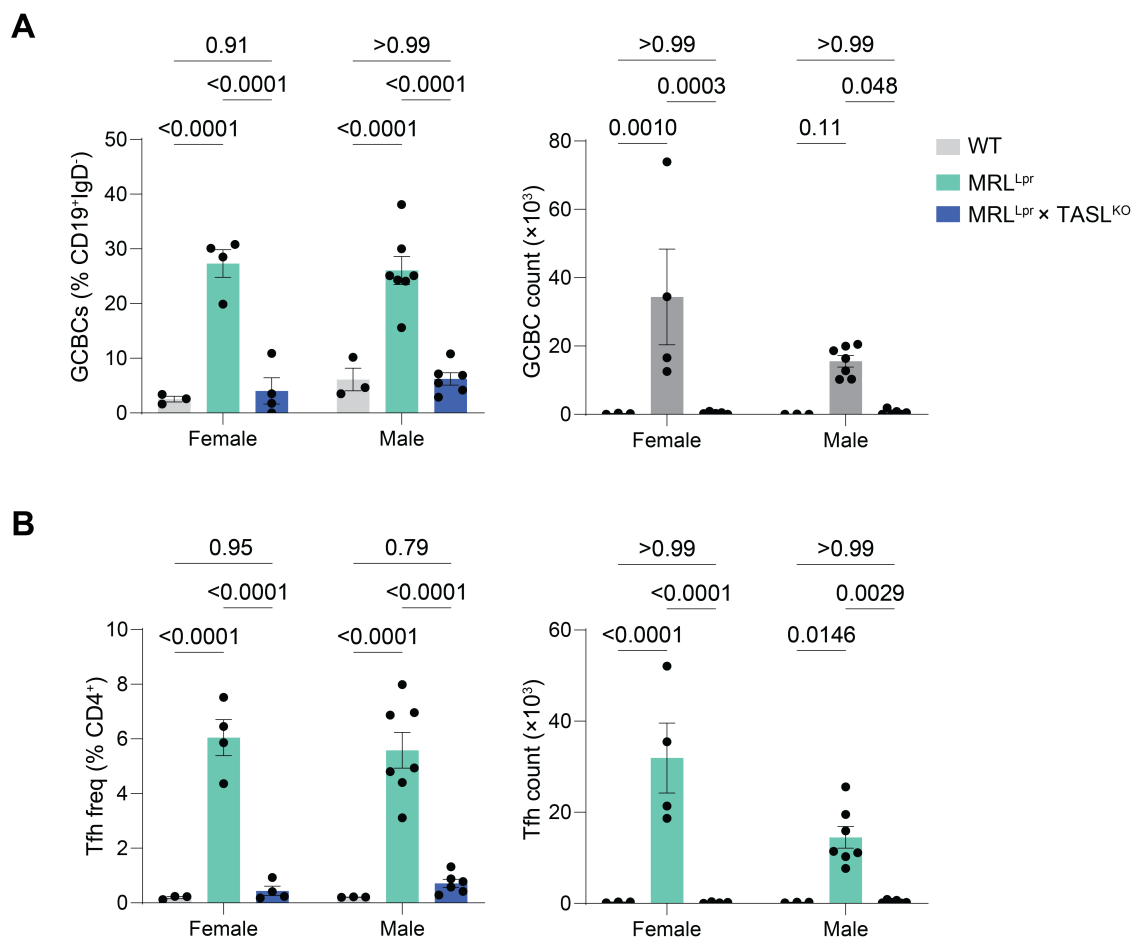


Figure 5.4: Spontaneous GCs are absent in BLNs in MRL^{Lpr} × TASL^{KO} mice (A) Representative gating of CD4⁺CXCR5⁺PD-1^{hi} T follicular helper (Tfh) cells in spleens of female MRL^{Lpr} and MRL^{Lpr} × TASL^{KO} mice. (B) Quantification of Tfh frequency and count in female WT (n=3), MRL^{Lpr} (n=4) and MRL^{Lpr} × TASL^{KO} (n=4) mice and male WT (n=3), MRL^{Lpr} (n=5) and MRL^{Lpr} × TASL^{KO} (n=4) mice.

Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from two independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.2.5 Impact of TASL on Tfr populations

Follicular regulatory T (Tfr) cells, a subset of regulatory T cells (Tregs), are also found in germinal centres, and can be distinguished by their high expression of *Foxp3* (Fig. 5.5A). Tfr cells inhibit interactions between GCBCs and Tfh cells, in

order to prevent excessive levels of inflammation, and mice lacking Tfr cells develop autoimmune disease[275]. In SLE patients, a decrease in the level of circulating Tfr cells, and a consequent increase in the ratio of circulating Tfh:Tfr cells, has been identified[276]. The numbers of Tfr cells, and an increased Tfh:Tfr ratio, also correlated with increased levels of anti-dsDNA auto-antibodies and disease severity, even though the Tfr cells present were still fully functional[277]. Transferring Tfr cells into the BXD2 mouse model of autoimmunity, decreased the size of GCs in these mice and decreased autoantibody production[278].

I found that, whilst the absolute count of Tfr cells was reduced in $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ compared with MRL^{Lpr} mice (Fig. 5.5B), in a similar manner to that of Tfh cells, the frequency of Tfr cells in the $\text{CD4}^+\text{CXCR5}^+\text{PD-1}^{hi}$ population was actually increased, corresponding to a decreased ratio of Tfh:Tfr cells (Fig. 5.5C-D). The ratio of Tfh:Tfr cells was similar in $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ to that in WT mice, suggesting the abnormalities in this ratio caused by autoimmunity were normalised.

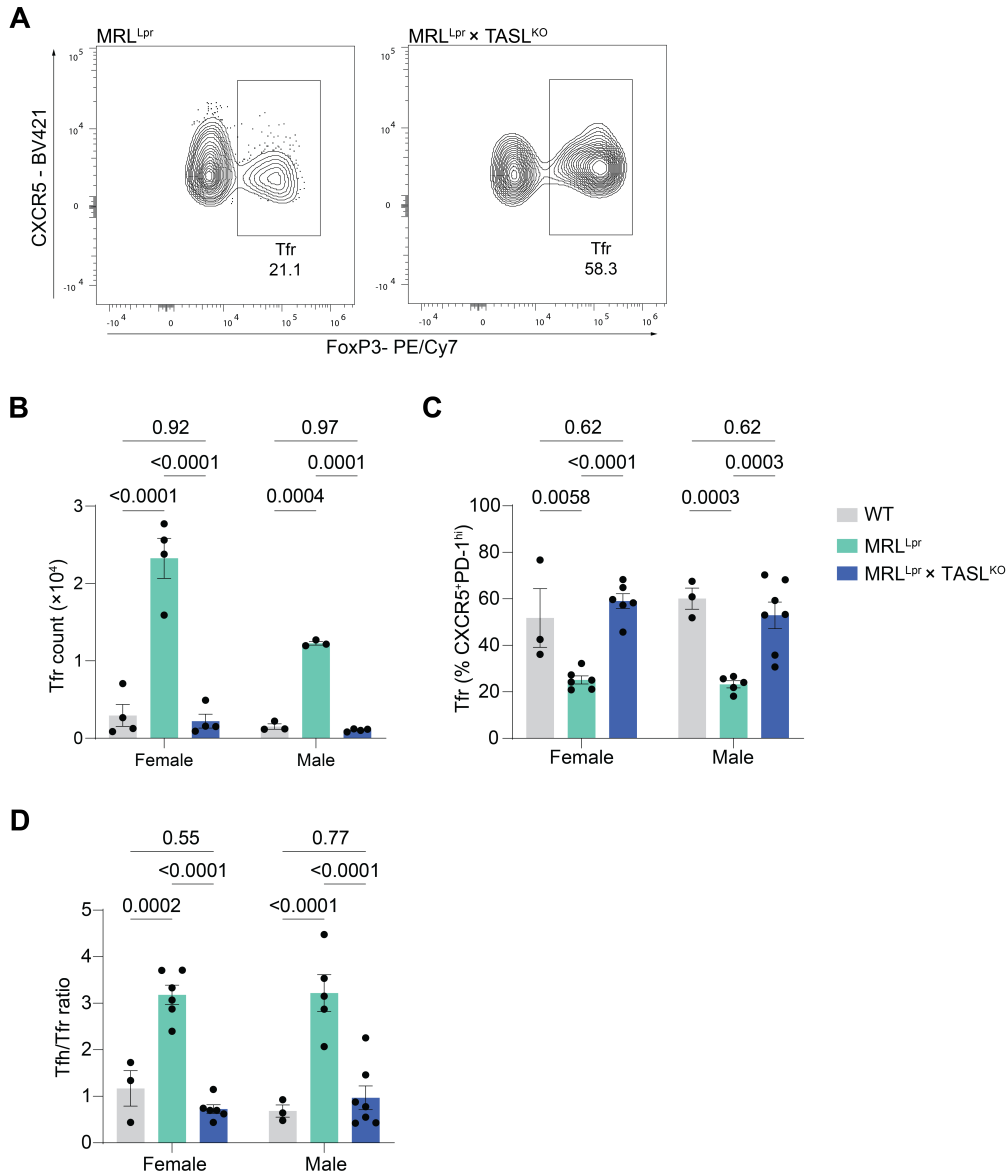


Figure 5.5: Impact of TASL on Tfr populations

(A) Representative gating of CD4⁺CXCR5⁺PD-1^{hi}FoxP3⁺ T follicular regulatory (Tfr) cells in spleens of female MRL^{Lpr} and MRL^{Lpr} × TASL^{KO} mice. (B) Quantification of Tfr count in female WT (n=4), MRL^{Lpr} (n=4) and MRL^{Lpr} × TASL^{KO} (n=4) mice and male WT (n=3), MRL^{Lpr} (n=3) and MRL^{Lpr} × TASL^{KO} (n=4) mice. Data are representative and pooled from two independent experiments. (C) Quantification of Tfr frequency in female WT (n=3), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=6) mice and male WT (n=3), MRL^{Lpr} (n=5) and MRL^{Lpr} × TASL^{KO} (n=7) mice. Data are representative and pooled from three independent experiments. (D) Ratio of T follicular helper (Tfh) cells to Tfr cells in the spleens of female WT (n=3), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=5) mice and male WT (n=3), MRL^{Lpr} (n=5) and MRL^{Lpr} × TASL^{KO} (n=7) mice. Data are representative and pooled from three independent experiments.

Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from three independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.2.6 TASL is required for development of expanded plasmablast populations in lupus

I next examined whether the number of plasmablasts was altered in $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ mice. Plasmablasts are known to be expanded in MRL^{Lpr} mice, and produce high levels of autoantibodies[17]. I observed that the proportion and number of plasmablasts was significantly decreased in $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ mice (Fig. 5.6A-C). This reduction was of a far greater magnitude in female mice than in males.

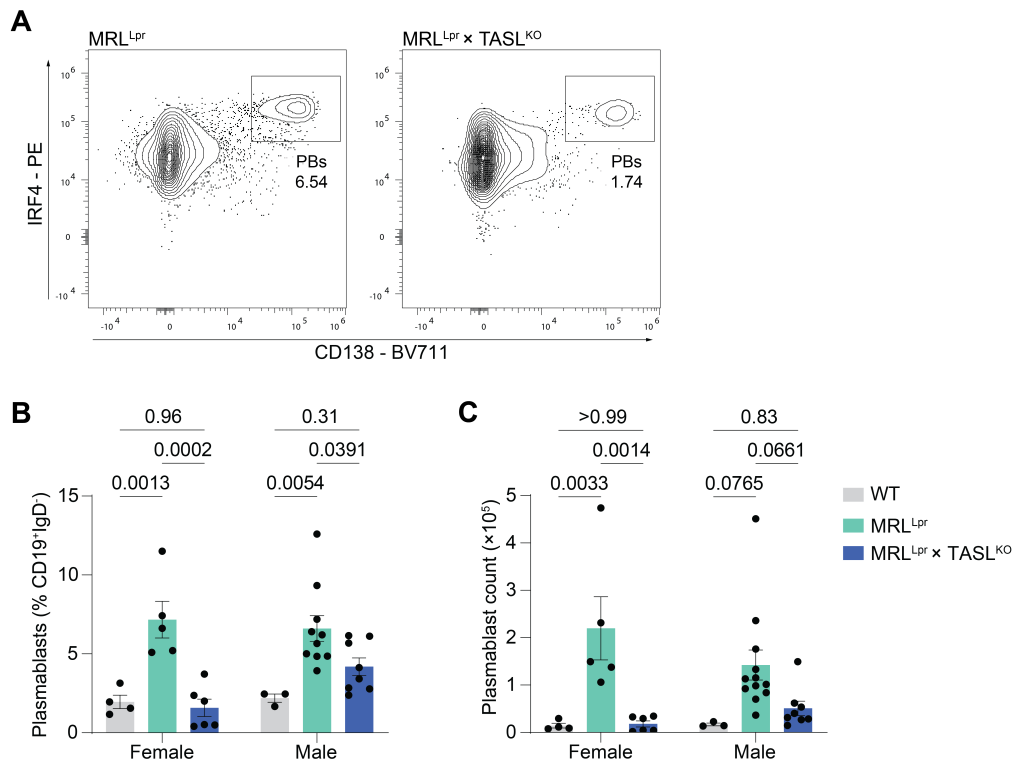


Figure 5.6: TASL is required for development of expanded plasmablast populations in lupus

(A) Representative gating of $\text{CD19}^+\text{IgD}^-\text{CD138}^+\text{IRF4}^+$ plasmablasts in spleens of female MRL^{Lpr} and $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ mice. (B) Quantification of plasmablast frequency and (C) count in female WT (n=4), MRL^{Lpr} (n=5) and $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ (n=6) mice and male WT (n=3), MRL^{Lpr} (n=12) and $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ (n=8) mice.

Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from three independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

5.2.7 BM PCs are decreased in female TASL deficient mice

I then isolated bone marrow from $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ mice to see whether the number of long-lived bone marrow plasma cells (BM PCs) was decreased. BM PCs have been shown to be responsible for producing up to 80% of circulating Igs[279][280], and the frequency of BM PCs is increased in MRL^{Lpr} mice[281]. The proportion and number of BM PCs was decreased by around two-thirds in female $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ mice compared with MRL^{Lpr} mice, but was not changed in males (Fig. 5.7A-B).

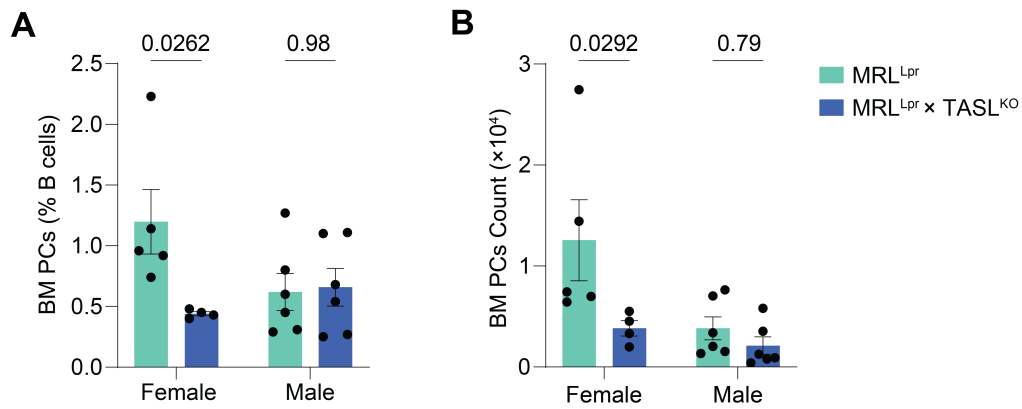


Figure 5.7: BM PCs are decreased in female $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ mice Quantification of (A) frequency and (B) count of bone marrow plasma cells (BM PCs) from female MRL^{Lpr} (n=5) and $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ (n=4) mice and male MRL^{Lpr} (n=6) and $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ (n=6) mice.

Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from two independent experiments. Data are presented as mean $\pm$ S.E.M. Each point represents a single mouse.

5.2.8 Impact of *Tasl* knockout on CD4^+ T cell activation in MRL^{Lpr} mice

T cell populations are also altered in SLE. I therefore used flow cytometry to analyse activation markers and identify different subsets of CD4^+ T cells (Fig. 5.8A). CD62L (also known as L-selectin) enables T cells to enter lymph nodes and is expressed highly on naive T cells, whereas CD44 is an activation marker expressed by effector T cells. I found that the number of effector ($\text{CD62L}^{lo}\text{CD44}^{hi}$) T cells was decreased in female $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ mice, whereas there were increased numbers of naive

(CD62L^{hi}CD44^{lo} T cells (Fig. 5.8B-C). This change was only seen in female mice, although there was a trend in the same direction in males.

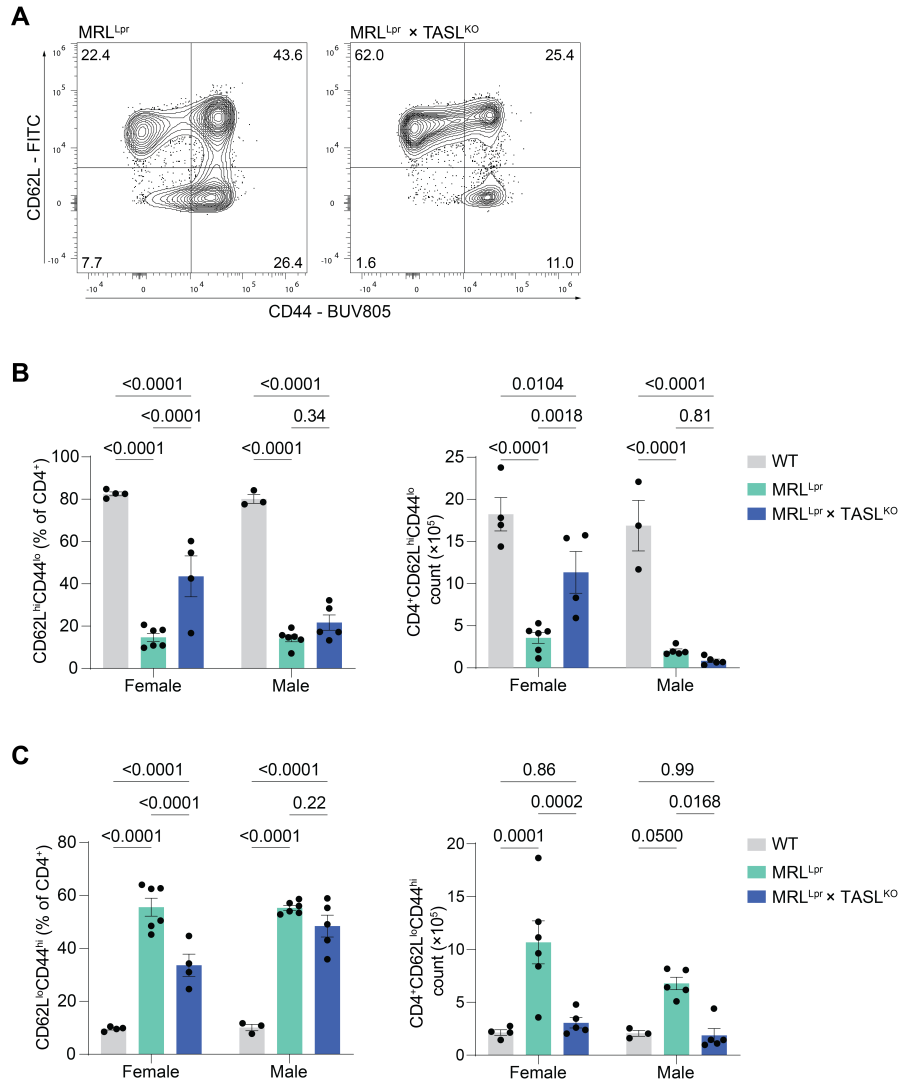


Figure 5.8: TASL deficiency alters CD4⁺ T cell populations

(A) Representative flow cytometric gating for identification of CD4⁺ populations based on CD62L and CD44 staining in spleens of female MRL^{Lpr} and MRL^{Lpr} × TASL^{KO} mice. (B) Quantification of frequency and count of CD4⁺CD62L^{hi}CD44^{lo} T cells in female WT (n=4), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=4) mice and male WT (n=3), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=5) mice. Data are representative and pooled from two independent experiments. (C) Quantification of frequency and count of CD4⁺CD62L^{lo}CD44^{lo} T cells in female WT (n=4), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=4) mice and male WT (n=3), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=5) mice. Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from two independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.2.9 CD8⁺ T cell populations are significantly altered in female *Tasl*-deficient mice

I then examined whether CD8⁺ populations were similarly altered in the absence of TASL. I found that in female mice naive CD8⁺CD62L^{hi}CD44^{lo} T cells were four times less frequent amongst total CD8⁺ T cells in MRL^{Lpr} mice as in WT mice (Fig. 5.9A). In female MRL^{Lpr}×TASL^{KO}, naive CD8⁺ T cells were significantly increased compared to MRL^{Lpr} mice, and were almost at WT levels. However, in males this was not the case, and numbers of naive CD8⁺ T cells were similar between MRL^{Lpr} and MRL^{Lpr}×TASL^{KO} mice. A similar pattern was seen in activated, CD8⁺CD62L^{lo}CD44^{lo} T cells, which were significantly increased in MRL^{Lpr} mice compared to WT mice, but this abnormality was not present in female MRL^{Lpr}×TASL^{KO} mice (Fig. 5.9B). In males, there was no significant difference between the MRL^{Lpr} and MRL^{Lpr}×TASL^{KO} mice.

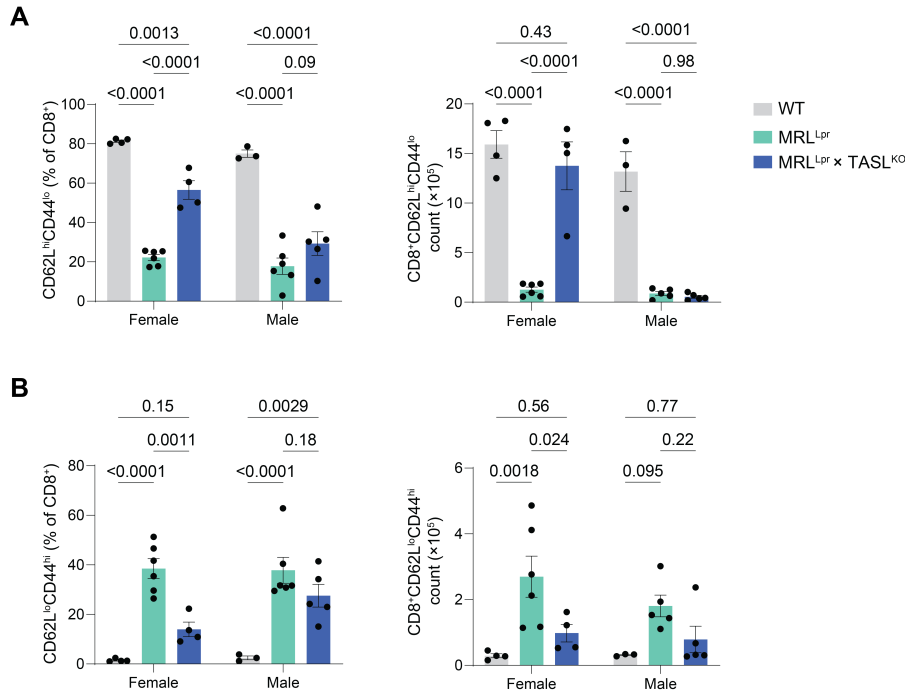


Figure 5.9: CD8⁺ T cell populations are altered in the absence of TASL

(A) Quantification of frequency and count of CD8⁺CD62L^{hi}CD44^{lo} T cells in female WT (n=4), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=4) mice and male WT (n=3), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=5) mice. Data are representative and pooled from two independent experiments. (B) Quantification of frequency and count of CD4⁺CD62L^{lo}CD44^{lo} T cells in female WT (n=4), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=4) mice and male WT (n=3), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=5) mice. Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from two independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.2.10 *Tasl* deficiency decreases the DN T cell population

In SLE there is an expanded population of DN T cells[271][137], a population normally present at only very low levels in health[127]. I found that the frequency of DN T cells was significantly reduced in female MRL^{Lpr} × TASL^{KO} mice, to the same level as that in WT mice (Fig. 5.10A-B). However the decrease in DN T cells in male mice was not significantly different.

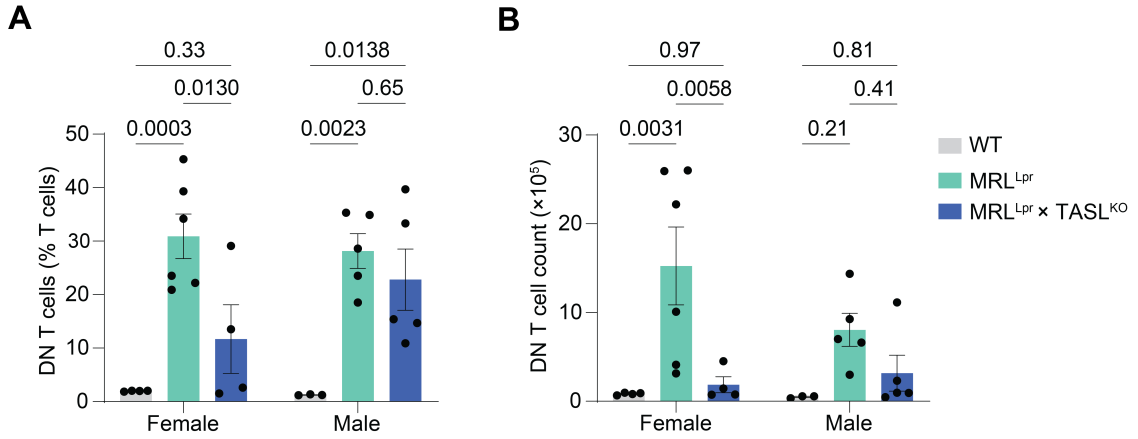


Figure 5.10: *Tasl* deficiency decreases DN T cell population Quantification of (A) frequency and (B) count of TCRb⁺CD8⁻CD4⁻ T cells in spleens of female WT (n=4), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=4) mice and male WT (n=3), MRL^{Lpr} (n=5) and MRL^{Lpr} × TASL^{KO} (n=5) mice.

Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from two independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.2.11 pDC activation is decreased in the absence of *TASL*

I then looked at the number of pDCs in the spleen, and found that neither the proportion or count of pDCs were significantly different in MRL^{Lpr} mice or MRL^{Lpr} × TASL^{KO} compared to WT mice. The number of pDCs was slightly decreased in MRL^{Lpr} × TASL^{KO} mice compared to MRL^{Lpr} mice (Fig. 5.11A-B).

However, despite only minor changes in the frequency and count, there was a significant difference in the number of activated CD86^{hi} pDCs, double in MRL^{Lpr} mice compared to MRL^{Lpr} × TASL^{KO} and WT mice (Fig. 5.11C).

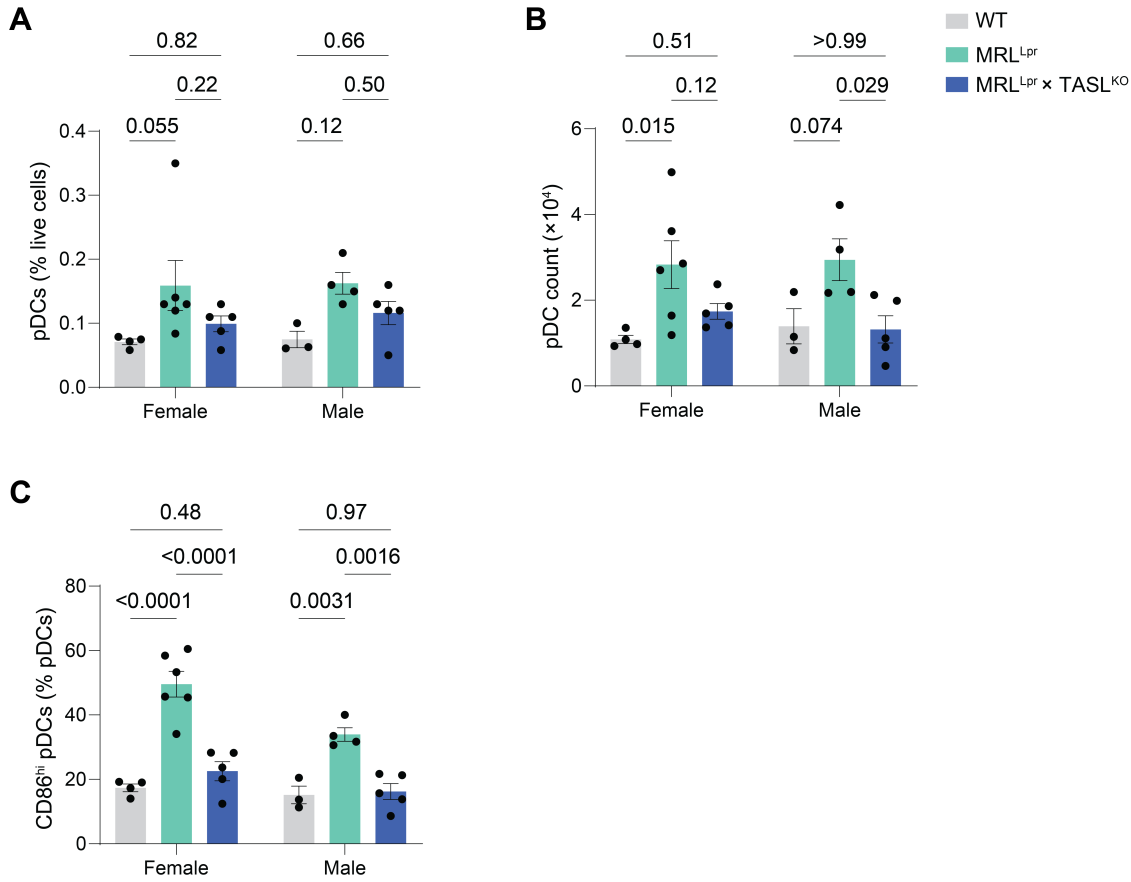


Figure 5.11: pDC activation is decreased in the absence of TASL Quantification of (A) frequency and (B) count of pDCs in spleens of female WT (n=4), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=5) mice and male WT (n=3), MRL^{Lpr} (n=4) and MRL^{Lpr} × TASL^{KO} (n=5) mice. (C) Quantification of CD86^{hi} pDCs as proportion of total pDCs in spleens of female WT (n=4), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=5) mice and male WT (n=3), MRL^{Lpr} (n=4) and MRL^{Lpr} × TASL^{KO} (n=5) mice. Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from two independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.2.12 ANA is absent in MRL^{Lpr} × TASL^{KO} mice

I next investigated whether there were changes in levels of circulating auto-antibodies in the absence of TASL. Anti-nuclear antibodies (ANAs) are a defining feature of SLE, so I used immunofluorescence to identify any changes in the levels of these. I did not observe any ANAs in the serum of MRL^{Lpr} × TASL^{KO} mice or WT mice, compared to high levels in MRL^{Lpr} mice (Fig. 5.12).

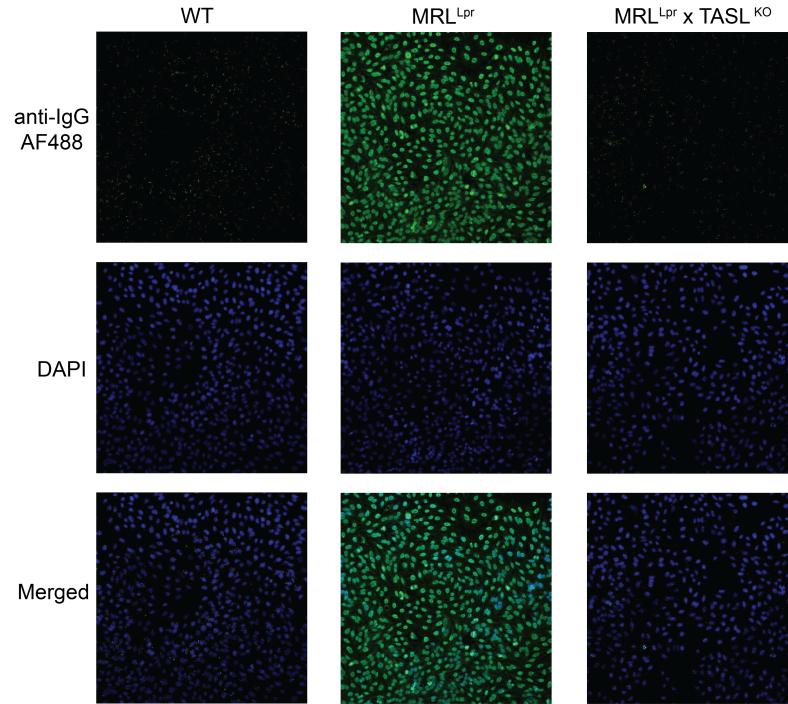


Figure 5.12: ANAs are absent in MRL^{Lpr} × TASL^{KO} mice Representative images of anti-nuclear antibodies (ANAs) in serum of 20-week old female WT, MRL^{Lpr} and MRL^{Lpr} × Tasl^{KO} mice, detected by immunofluorescence.

5.2.13 Lack of TASL prevents production of anti-dsDNA antibodies

The presence of anti-dsDNA antibodies is also a hallmark feature of SLE. I observed that the level of anti-dsDNA total IgG was significantly decreased in female MRL^{Lpr} × TASL^{KO} mice, but not in males (Fig. 5.13A). Due to the decreased ability of TASL^{KO} mice to produce IgG2c antibodies, and the prevalence of this isotype in autoimmunity, I also looked at levels of anti-dsDNA IgG2c. I found that this was also significantly reduced in female MRL^{Lpr} × TASL^{KO} mice, but again not in males (Fig. 5.13B).

I found that MRL^{Lpr} × TASL^{KO} mice did still have similar levels of anti-dsDNA IgM as MRL^{Lpr} mice (Fig. 5.13C), but anti-dsDNA IgM, whilst elevated in murine models of SLE, has been shown to be non-pathogenic[282].

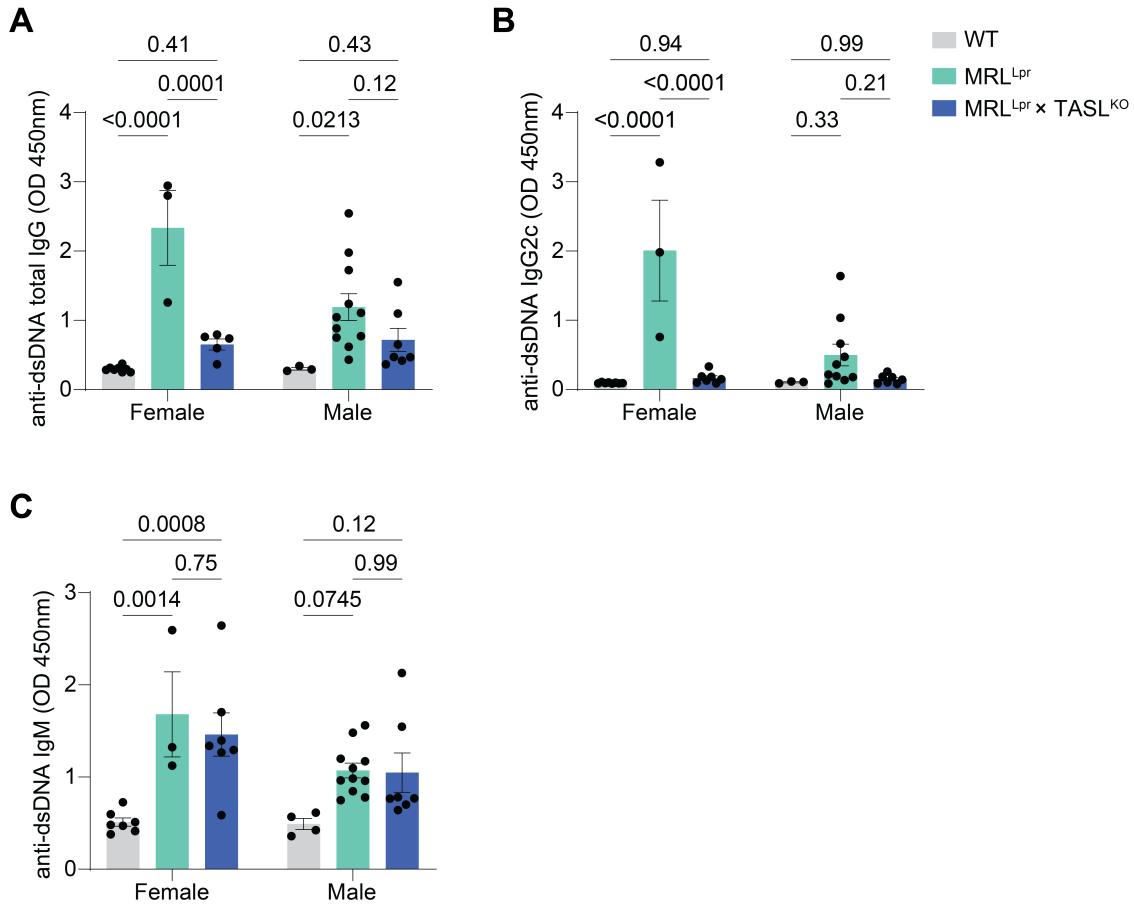


Figure 5.13: Lack of TASL prevents production of anti-dsDNA antibodies (A) ELISA quantification of total IgG anti-dsDNA auto-antibodies in serum of 20-week old female WT (n=8), MRL^{Lpr} (n=3) and MRL^{Lpr} × TASL^{KO} (n=5) mice and male WT (n=3), MRL^{Lpr} (n=11) and MRL^{Lpr} × TASL^{KO} (n=7) mice. (B) ELISA quantification of IgG2c anti-dsDNA auto-antibodies in serum of 20-week old female WT (n=8), MRL^{Lpr} (n=3) and MRL^{Lpr} × TASL^{KO} (n=7) mice and male WT (n=3), MRL^{Lpr} (n=10) and MRL^{Lpr} × TASL^{KO} (n=7) mice. (C) ELISA quantification of IgG2c anti-dsDNA auto-antibodies in serum of 20-week old female WT (n=7), MRL^{Lpr} (n=3) and MRL^{Lpr} × TASL^{KO} (n=7) mice and male WT (n=4), MRL^{Lpr} (n=11) and MRL^{Lpr} × TASL^{KO} (n=7) mice.

Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from two independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.2.14 Anti-Sm/RNP autoantibody levels are decreased in the absence of TASL

I also examined levels of the autoantibody anti-Sm/RNP, which is associated with high TLR7 expression in SLE patients[283]. In female MRL^{Lpr} × TASL^{KO} mice,

both total IgG and IgG2c anti-Sm/RNP levels were decreased to that seen in WT mice (Fig. 5.14A-B). In male mice, there were very low levels of anti-Sm/RNP antibodies even in MRL^{Lpr} mice, so no differences were observed.

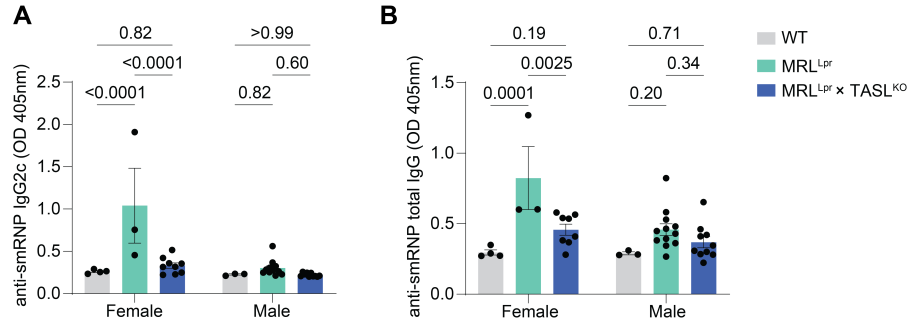


Figure 5.14: anti-Sm/RNP autoantibody levels are decreased in the absence of TASL (A) ELISA quantification of IgG2c anti-Sm/RNP auto-antibodies in serum of 20-week old female WT (n=4), MRL^{Lpr} (n=3) and MRL^{Lpr} × TASL^{KO} (n=9) mice and male WT (n=3), MRL^{Lpr} (n=12) and MRL^{Lpr} × TASL^{KO} (n=10) mice. (B) ELISA quantification of total IgG anti-sm/RNP auto-antibodies in serum of 20-week old female WT (n=4), MRL^{Lpr} (n=3) and MRL^{Lpr} × TASL^{KO} (n=8) mice and male WT (n=3), MRL^{Lpr} (n=12) and MRL^{Lpr} × TASL^{KO} (n=10) mice.

Statistical significance was calculated using a two-way ANOVA with Tukey's multiple testing correction. Data are representative of and pooled from two independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.2.15 Impact of *Tasl* knockout on ABC formation

ABCs are significantly expanded in mouse models of SLE and in SLE patients. In the previous chapter, I observed that the number of ABCs was dramatically decreased in aged TASL^{KO} mice. I therefore investigated whether ABCs were present in the spleen of MRL^{Lpr} × TASL^{KO} mice using flow cytometry (Fig. 5.15A). I found that in both male and female mice, the proportion and number of ABCs was significantly decreased in MRL^{Lpr} × TASL^{KO} mice, to the same levels as seen in WT mice (Fig. 5.15B-C).

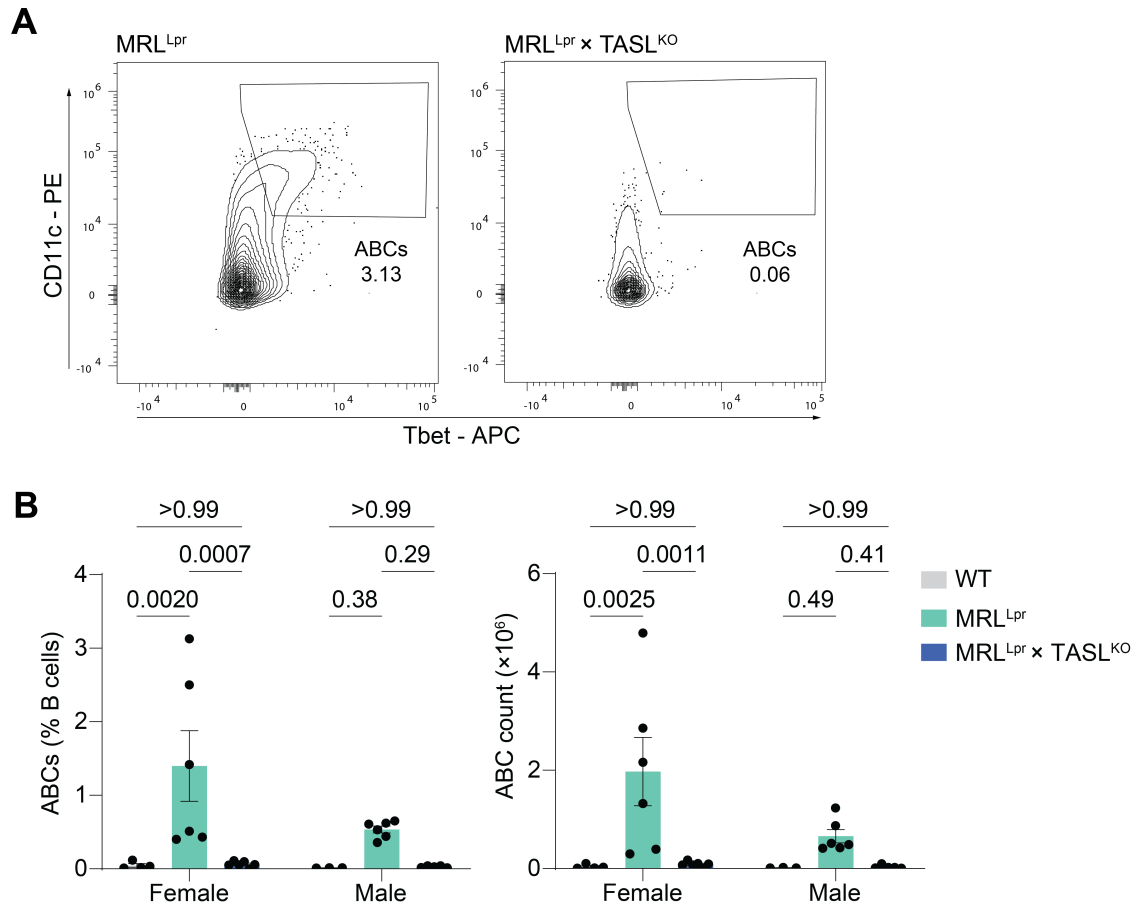


Figure 5.15: TASL deficiency prevents production of ABCs in autoimmunity (A) Representative gating of CD11b⁺CD11c⁺Tbet⁺ age-associated B cells (ABCs) from MRL^{Lpr} and MRL^{Lpr} × TASL^{KO} mice. (B) Quantification of ABC frequency and count from female WT (n=4), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=6) mice and male WT (n=3), MRL^{Lpr} (n=6) and MRL^{Lpr} × TASL^{KO} (n=5) mice. Statistical significance was calculated using a two-way ANOVA with Tukey's multiple comparisons test. Data are representative of and pooled from two independent experiments. Data are presented as mean ± S.E.M. Each point represents a single mouse.

5.3 Discussion

In summary, I found that the absence of TASL prevents the development of autoimmunity in B6.MRL^{Lpr} mice. *Tasl* deficiency prevented the development of spontaneous GCs, decreased the number of plasmablasts and plasma cells, and prevented the development of autoantibodies, as well as stopping the development of ABCs.

Spontaneous GCs were reduced to WT levels in MRL^{Lpr} × TASL^{KO} mice in both

male and females. This was reflected in decreased numbers both of GCBCs and Tfh cells, both of which are essential components of the GC reaction. There was also an increase in the proportion of Tfr cells, an important regulatory subset in the GC. This meant that although the absolute number of Tfrs was decreased in the MRL^{Lpr} × TASL^{KO} mice, due to the huge splenomegaly and increased number of GCs in the MRL^{Lpr} mice, the ratio of Tfh/Tfr cells was normalised to that seen in WT mice. This suggests that the dysregulation seen in GCs in MRL^{Lpr} mice was corrected by the absence of TASL. This fits with published results of IRF5-deficient mice, which also display a dramatic reduction in the number of spontaneous GCs[284].

The number of plasmablasts was also decreased in MRL^{Lpr} × TASL^{KO} mice, to a greater extent in female mice. The number of BM PCs was also significantly decreased in female MRL^{Lpr} × TASL^{KO} mice, whilst not being affected by *Tasl* deletion in males. This is particularly relevant as LLPCs in the bone marrow contribute to chronic inflammation in SLE, producing high levels of antibodies[279][280]. LLPCs have so far been shown to be resistant to treatment with current immunosuppressants[285], so understanding the molecular pathways that lead to their development is crucial. Conditional deletion of *Irf5* or treatment with an IRF5 inhibitor prevented the production of new LLPCs after they had been depleted using the drug bortezomib[286]. This suggests that IRF5 also plays a role in the development of LLPCs, matching my findings and supporting that targeting TASL may also be an effective way to deplete this population, thereby decreasing production of autoantibodies.

Corresponding to the decrease in plasmablasts and plasma cells, I also observed a significant reduction in levels of anti-dsDNA, anti-Sm/RNP antibodies and ANAs in MRL^{Lpr} × TASL^{KO} mice. This decrease was again more pronounced in female mice, however there were generally lower levels of autoantibodies in male MRL^{Lpr} mice, which could provide a partial explanation for this. Overall total IgG anti-dsDNA and anti-Sm/RNP were decreased, but due to the specific defect examined in the previous chapter in IgG2c production, I examined IgG2c anti-dsDNA and anti-Sm/RNP, and found that this isotype was almost completely absent in the MRL^{Lpr} × TASL^{KO}

mice. As IRF5 is important for class-switching to IgG2c[198], and also for the development of ABCs[199], which secrete high levels of IgG2c, the absence of IgG2c autoantibodies in the *Tasl*-deficient mice fits with its role in the IRF5 pathway.

ABCs were almost completely absent in the spleens of both $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ mice, matching the number seen in WT mice, compared to the highly elevated numbers of these cells seen in MRL^{Lpr} mice. This was observed in both male and female mice, although due to the lower numbers of ABCs in males, the trend did not reach significance. This result corresponds to the results from the previous chapter, where I identified that ABCs were dramatically decreased in aged TASL^{KO} mice, and ABCs were unable to develop *in vitro* from TASL^{KO} B cells. It also fits with previous work from other groups investigating IRF5, which demonstrated that IRF5 is critical for ABC development[199], so if this pathway cannot be activated due to the absence of *TASL*, then ABCs cannot develop.

ABCs are known to contribute to the development of autoimmunity, and also play a role in T cell activation[125]. Genetic depletion of ABCs decreased the activation of T cells, and increased the number of naive T cells present in MRL^{Lpr} mice[125], so the lack of ABCs in the $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ mice in my model could be contributing to the altered T cell activation observed.

I additionally identified that pDC activation was altered in $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ mice. pDCs are known to be increased in MRL^{Lpr} mice compared to C57BL/6 mice, although not as dramatically in other murine SLE models, such as NZB/W mice[287]. I did observe a slight increase in MRL^{Lpr} mice, but did not find this to be significantly different in the absence of *TASL*, except in male mice. At rest, pDCs express low levels of the co-stimulatory molecule CD86, and this is upregulated upon activation[288]. I found that the proportion of pDCs expressing high levels of CD86 was more than doubled in MRL^{Lpr} mice, but this proportion was reduced to WT levels in $\text{MRL}^{Lpr} \times \text{TASL}^{KO}$ mice in both males and females. This suggests that *Tasl* deficiency also impairs pDC activation. This highlights one of the limitations of this work, in that the model is a complete *Tasl* knockout, and therefore it cannot be determined whether the alterations in B cell function

and subtypes are cell-intrinsic or due to external factors. pDCs also express high levels of TASL[173], and other work has also demonstrated that *Tasl* deletion alters their activation, and decreases the amount of type I IFN that they secrete[182]. The decreased pDC activation and IFN production could therefore be impacting B cell activity, in addition to any cell intrinsic effects.

A striking reduction in DN T cells was also observed in the absence of TASL. In MRL^{Lpr} mice, a large amount of DN T cells accumulate and contribute to the splenomegaly and lymphadenopathy observed in these mice[132]. The reduction in DN T cells, along with GCs and B cells, could therefore be responsible for the reduced splenomegaly and lymphadenopathy in the MRL^{Lpr} × TASL^{KO} mice. DN T cells are also present in humans with SLE and other autoimmune conditions[289][131]. DN T cells are thought to arise from self-reactive CD8⁺ T cells that respond to self-antigens from uncleared debris, losing CD8 expression and becoming DN T cells[137]. Single-cell RNA-sequencing has revealed that the DN T cells actually consist of many different subsets, including cytotoxic DN T cells and DN T cells that perform innate immune functions[290]. Future work could therefore try to identify if any of these subsets were particularly depleted in the MRL^{Lpr} × TASL^{KO} model. As T cells do not express high levels of TASL, it is likely due to altered interactions with other cells and the altered inflammatory environment that the number of DN T cells is reduced in the mice. Similarly to other changes observed in this model, the decrease is only seen in female mice, and is not significant in males. There is some evidence that the reduction of marginal zone macrophages (MZMs) in SLE leads to the development of DN T cells, due to the resulting decreased clearance of cells and altered cytokine levels[290]. As macrophages do express TASL, there is therefore a chance that in the MRL^{Lpr} × TASL^{KO} mice there is altered MZM activity and this is leading to the reduction in DN T cell populations observed. Future work should therefore examine the activity of these cells and see if it is altered.

6

Discussion

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6.1 Sexually dimorphic impact of TASL

Across the *in vivo* experiments in this work, I consistently observed sexual dimorphism, both in response to immune challenge, and in the development of autoimmunity. There was a significant decrease in the number of plasmablasts in females in response to both T-dependent and T-independent antigens in TASL^{KO} mice, whereas in males only a trend in this direction was observed. There was also a much larger reduction in plasmablasts in female MRL^{Lpr} × TASL^{KO} mice than in males. These results fit with the sexual dimorphism in expression seen in human cells[166]. The risk SNP at the *TASL* locus associated with SLE, rs887369, acts as an expression quantitative trait locus only in females, where it leads to increased *TASL* expression, and this difference was amplified by stimulation with IFN.

I did not identify any sexual dimorphism in an *in vitro* setting when I stimulated isolated B cells. It could be of interest to investigate whether the addition of estradiol to culture medium changes the effect or amplitude of the impact of *TASL* knockout on B cell activation. B cells express high levels of the estrogen receptors $ER\alpha$ and $ER\beta$, and estrogen alters B cell activity, with a number of proposed impacts on autoimmunity, including altering the induction of B cell tolerance resulting in increased numbers of autoreactive B cells[291], and potentially affecting TLR7 and Type I IFN signalling in B cells[292][293]. Additionally, in individuals with two X chromosomes (XX), levels of estrogen affect the frequency of class-switched memory B cells, and estrogen blockade in transgender males, who have a karyotype of XX, decreases the frequency, and hormone replacement therapy (HRT) in postmenopausal females increasing frequency compared to postmenopausal females not on HRT[294]. Investigating whether sex hormones have an impact on the differences seen in the role of *TASL* in females and males could therefore provide useful insights.

TASL is also known to escape XCI, along with other immune-related genes, including TLR7[166]. Disrupting XCI by altering expression and activity of the long non-coding RNA *Xist*, which leads to XCI, in mice caused the development of autoimmune and inflammatory symptoms in female mice, with one of the features observed being increased numbers of ABCs, demonstrating that altered XCI can play a role in the development of autoimmunity[295]. Disruption of XCI caused altered expression of *Tasl* in macrophages and DCs in aged mice, but not in B cells, but altered autoantibodies and numbers of ABCs were still observed[295]. However, B cells were only examined generally, without looking at alterations in specific subsets. A more in-depth examination of B cells in this context, as well as examining the role of *TASL* in these other cell types is therefore important, to understand the cellular interactions leading to these changes. *Tlr7* expression was also significantly altered in macrophages, DCs and B cells from aged mice, showing that endolysosomal TLR signalling is a critical pathway in autoimmunity, and in leading to the sexual dimorphism of autoimmune disease, so is worth further investigation.

6.2 The relative role of TASL in the extrafollicular compared to the GC reaction

I found that TASL primarily seems to affect the extrafollicular response. Although the number of GCBCs was significantly decreased in both males and females in response to immunisation with a T-dependent antigen, NP-CGG, the functional output of the GC was not significantly impacted, with affinity maturation being unimpaired. In a competitive BM chimera setting, TASL^{KO} B cells also showed no disadvantage, contributing to GCs and plasmablast populations equally in comparison to WT cells. However, a role for TASL in the GC response cannot be completely ruled out.

In MRL^{Lpr} x TASL^{KO} mice, I also observed that the number of spontaneous GCs was decreased. The importance of this finding in the reduction of SLE symptoms and autoantibody levels is unclear, as recent evidence has shown that even in murine models where spontaneous GCs are present, they do not necessarily play a role in the development of autoimmunity[21]. Whilst there is evidence that TLR9 signalling inhibits spontaneous GC formation in SLE, B cell-intrinsic TLR7 signalling is required for the development of GCs in SLE[296], so the reduction in spontaneous GCs makes sense in light of the interference with the TLR7 signalling pathway by deleting *Tasl*.

TLR7 and TLR9 are both required for extrafollicular responses, and a B cell-specific absence of MyD88 causes a significant decrease in the number of plasmablasts in response to immunisation with virus-like particles [297]. The conjugation of CpG to antigen-coated beads led to increased antigen-specific plasmablast production via the extrafollicular pathway, with increased production of IgG2c antibodies[298]. TLR9 signalling via the IRF5 pathway is central to class-switching to IgG2c, and previously SLC15a4 has been shown to be required for IgG2c production[299], so the requirement for TASL in this process fits with previous research, and provides another link in the pathway.

6.3 Limitations

A critical question that this work did not address is the cell-specific functions of TASL. The single exon nature of *Tasl* makes the generation of a LoxP-flanked allele, that would allow conditional deletion, technically challenging. Whilst the *in vitro* work I carried out shows TASL plays a central role in B cell activation and signalling, I also found that pDC activation was altered. It is therefore not certain whether it is the role of TASL in pDCs or B cells that is predominant in these experiments.

Recently, Drobek et al discovered a homologue of TASL, termed TASL2[183]. They found that in certain circumstances there was some residual activity of IRF5, which they suspected to be due to TASL2. I used a knockout of TASL only in this work. To identify whether the lack of TASL fully inhibits IRF signalling, a double knockout of IRF5 and TASL could be investigated, to see whether there are any additional effects from this, or if the phenotype of these mice is identical to TASL knockout alone.

Additionally, I only investigated one murine model of SLE. To strengthen the conclusions, additional models of SLE should be examined, to see if TASL also plays a role in other models.

The lupus phenotype in the MRL-Fas^{Lpr} model is driven primarily by defects in apoptosis and abnormal lymphoproliferation[300] Although there is altered IFN signalling in the model, the phenotype is not principally driven by increased IFN signalling, and does not have an IFN signature. Consequently, there are limitations in the conclusions that can be drawn about the role of TASL in IFN pathways in this model, as the phenotype is at least partially independent of IFN and TLR signalling. Using a murine model where the lupus phenotype is more dependent on increased IFN signalling and an IFN signature is prevalent, such as the NZM model[301], could reveal further insight.

Analysing TASL in a murine model which is more severe in male mice, such as the BXSB/Yaa strain[300], could be interesting to see if TASL is similarly important in the development of SLE in that context. One of the contributing factors to development of SLE in the BXSB/Yaa model is the duplication of several genes,

including TLR7[302], which would also make this an interesting model due to the function of TASL in the TLR7 signalling pathway. Other TLR7 transgenic models could also be used to explore this, such as mice with the TLR7 gain-of-function mutation Tlr7^{Y264H} [77], to see if the absence of TASL is sufficient to prevent the onset of autoimmunity in this context.

To fully explore the sexually dimorphic role of TASL, lupus-susceptible mice with deletion of the male sex-determining gene *Sry* (XY^-) could be utilised[146], thereby helping to untangle whether sex hormones or X chromosome dosage are contributing to and differences between sexes.

6.4 Future work

Whilst this work has examined the role of TASL in autoimmunity, another interesting question is the role that TASL plays in the response to infection. Influenza activates TLR7, so would be a useful infection model to investigate the role of TASL in physiological immune responses. Influenza infection induces both an extrafollicular and a long-lasting GC response[303], and the ability of *Tasl*-deficient mice to control this infection, and produce long-lasting GCs, could help provide insight into the wider role of TASL in extrafollicular and GC reactions. ABCs also play an important role in response to infection, with their number hugely increased in response to both infection and vaccination for influenza, as well as after infection with other viruses including lymphocytic choriomeningitis virus (LCMV)[304]. Drobek et al found that mice in which both TASL and TASL2, a TASL homologue, were deleted, were unable to control chronic LCMV infection[183]. In contrast, mice with only deletion of TASL, whilst having decreased plasmablast and GC development, only showed a slight delay in clearance of the virus, and were still able to clear the infection successfully[183]. As deletion of TASL alone is sufficient to protect from SLE development, this is an intriguing finding, and targeting of TASL could be even more promising as a therapeutic approach if it can be inhibited without impairing broader responses to infection.

The results of bulk RNA-sequencing of *in vitro* stimulated B cells and *in vitro* assays showed increased antigen presentation by TASL^{KO} B cells in comparison to WT cells. Further investigation of whether antigen presentation is upregulated *in vivo* is therefore required. TLR7 or 9 stimulated B cells have been shown to play a crucial role in presenting antigen to initiate CD4⁺ T cell responses in the context of viral antigens[305]. Therefore, investigating the impact of TASL deficiency in response to viral infection, such as influenza, could reveal some insight into this potential role of TASL. Additionally, Epstein Barr Virus (EBV) is known to modulate TLR7 and TLR9 signalling[306], and TLR9 agonists have additionally been shown to alter the activity of B cells in response to EBV[307], therefore whether there is any interplay between EBV and TASL, and whether targeting of TASL could be beneficial in this context, is an interesting question that remains to be explored.

In addition to its role in autoimmunity, TASL is also a biomarker for poor prognosis in immunologically cold tumours, compared to in hot tumours where it is a biomarker for good prognosis[184]. Additionally, its expression correlates with good response to immunotherapy. Altered interactions between B cells and T cells could potentially provide an explanation for this, making the altered antigen presentation worth exploring in this context too. Whether TASL plays a role in the development of tumours, or if its absence leads to better or worse control of tumours, would also be of interest.

In this thesis I have examined the role of TASL by looking at the alterations in a model where it is absent. Using the technique of 'Clustered Regularly Interspaced Short Palindromic Repeat' (CRISPR) to specifically modify the *Tasl* locus with the SNP associated with SLE could reveal further insights about the role of this mutation in SLE susceptibility. A recent study by Lau et al. looked at the impact of the risk-associated SNP in an edited immortalised B cell line, and found that these cells produced increased amounts of IL-6 upon stimulation[182]. Investigating this genotype in an *in vivo* setting would therefore provide further insight into the alterations it causes on B cell activity.

Examining the impact of the risk SNP rs887369 in data from SLE patients could also provide some insight. Identifying whether this mutation is associated with particular features in SLE, such as increased number of ABCs, or a particularly high interferon signature, could help to further knowledge about the condition, as well as potentially help stratify patients for treatments.

As the IRF5 pathway plays an important role in the development of SLE, there has been a lot of interest in targeting it for treatment of autoimmune disease, but there have been significant challenges with this, such as off-target effects[189]. The discovery that TASL deletion seems to only affect activation of B cells specifically after endolysosomal TLR stimulation, matching other work that has looked at the role of TASL in these signalling pathways[168] makes targeting TASL a promising therapeutic target. For example, recently an inhibitor that locks the conformation of SLC15A4 such that TASL is unable to bind, leading to the subsequent degradation of TASL, has recently been tested *in vitro* and was found to block the TLR7-IRF5 inflammatory pathway[308]. Further testing of this inhibitor or other similar molecules, and whether they are effective *in vivo* could be an interesting avenue for exploration in SLE treatment.

6.5 Conclusion

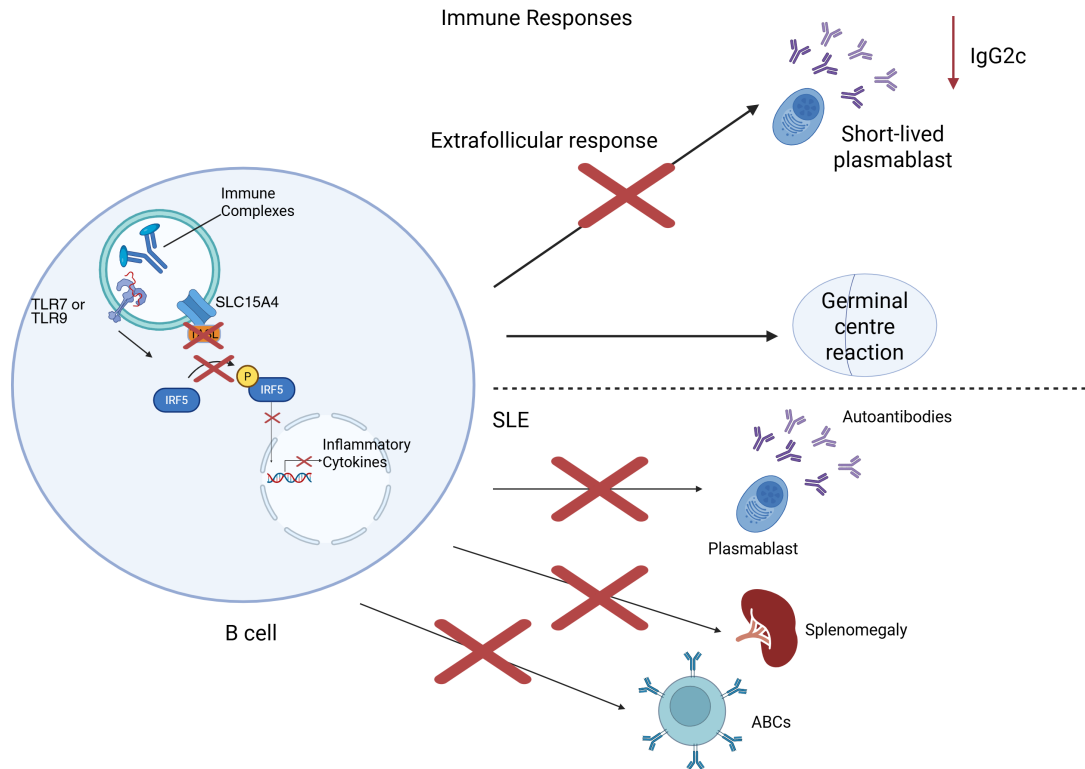


Figure 6.1: The role of TASL in immune responses and autoimmunity
 In this project I identified that in addition to its role in the IRF5 pathway, in the absence of TASL the extrafollicular response is severely impaired, and IgG2c antibodies are absent, but the germinal centre reaction is not significantly impacted. In autoimmunity, the absence of TASL prevents the development of lupus symptoms, preventing splenomegaly and lymphadenopathy, and leading to decreased numbers of plasmablasts, age-associated B cells (ABCs) and decreased production of autoantibodies. Figure created in <https://BioRender.com>

In conclusion, in this thesis I investigated the role of TASL, a newly identified protein that is genetically associated with SLE, and found that it does play an important role in the development of autoimmunity[309]. Deletion of *Tasl* prevented the onset of SLE in the MRL^{Lpr} murine model of SLE and the development of autoantibodies. The loss of TASL had a greater impact on female than male mice, supporting the hypothesis that it may play a role in the sexual dimorphism seen in SLE. I also found that TASL seems to mainly play a role in the extrafollicular B cell response, whilst having less of a functional impact on the GC response (Fig 6.1), and is critical for the formation of ABCs. The work presented in this thesis supports

investigation of TASL as a target for SLE treatment, as well as further examination into its role in sexual dimorphism in autoimmunity and humoral responses.

Appendices

A

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A.1 Table of Antibodies

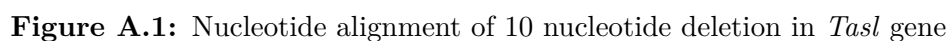
Antibody	Fluorochrome	Clone	Dilution (1/n)	Manufacturer	Cat no:
anti-B220	BV510	RA3-6B2	100	BioLegend	103248
anti-CD19	BV421	6D5	100	BioLegend	115538
anti-CD19	BV510	6D5	100	BioLegend	115546
anti-CD19	BV650	6D5	100	BioLegend	115541
anti-CD19	BV785	6D5	100	BioLegend	115543
anti-CD21/35	BV711	7E9	200	BioLegend	123435
anti-CD24	BV711	M1/69	200	BD Bioscience	563371
anti-BP-1 (CD249)	BUV737	6C3	100	BD Bioscience	741743
anti-IgD	BV650	11-26c.2a	400	BioLegend	405721
anti-IgD	AF700	11-26c.2a	400	BioLegend	405730
anti-CD43	PE	S11	200	BioLegend	143205
anti-CD23	BV786	BSB4	150	BD Bioscience	563988
anti-IgM	BUV395	II/41	150	BD Bioscience	743329
anti-CD69	BV711	H1.2F3	200	BioLegend	104537
anti-CD86	BV785	GL-1	100	BioLegend	105043
anti-CD138	BV711	281-2	250	BioLegend	142519
anti-CD4	BV605	RM4-5	150	BioLegend	100547
anti-CD8 α	PE/Cy7	53-6.7	100	BioLegend	100721
anti-CD8 α	BV711	53-6.7	200	BioLegend	100747
anti-CD38	BUV805	90/CD38	400	BD Bioscience	741955
anti-CXCR4	BUV737	2B11/CXCR4	50	BD Bioscience	741783
anti-CXCR5	BV421	L138D7	50	BioLegend	145511
anti-GL7	Pacific Blue	GL7	100	BioLegend	144614
anti-GL7	PerCP/Cy5.5	GL7	200	BioLegend	144609
anti-TCR β	BV510	H57-597	100	BioLegend	109233
anti-CD11c	PE	N418	200	BioLegend	117307
anti-CD11c	BV650	N418	100	BioLegend	117339
anti-CD11b	PerCP/Cy5.5	M1/70	200	BioLegend	101228
anti-CD62L	FITC	MEL-14	100	BioLegend	104405
anti-CD44	BUV805	IM7	200	BD Bioscience	741921
anti-I-A/I-E	AF647	M5/114.15.2	500	BioLegend	107618
anti-PDCA1	BV421	927	100	BioLegend	127023
anti-F4/80	FITC	BM8	100	BioLegend	123107
anti-PD1	FITC	29F.1A12	100	BioLegend	135213

Intracellular

Antibody	Fluorochrome	Clone	Dilution (1/n)	Manufacturer	Cat no:
anti-IRF4	AF488	3E4	100	BioLegend	646406
anti-IRF4	PE	3E4	100	Invitrogen	12-9858-82
anti-Tbet	APC	4B10	100	BioLegend	644814
anti-FoxP3	PE/Cy7	FJK-16s	100	Invitrogen	25-5773-80

A.2 *TASL*^{KO} mice generation

C57BL/6J-*Tasl*^{em3H}/H (*TASL*^{KO}) mice were generated by the Mary Lyon Centre at MRC Harwell, via a CRISPR/Cas9-induced mutation deleting 10 nucleotides in exon ENSMUSE00000697605 of the *Tasl* gene. The location of the 10 nucleotide deletion (Fig. A.1) and predicted protein alignment for the edited *Tasl* allele (Fig. A.2) can be found below.



[illegible]

Figure A.2: Predicted protein alignment with edited *Tasl* allele

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