THE ROLE OF β2-GLYCOPROTEIN I-REACTIVE T CELLS IN THE DEVELOPMENT OF SYSTEMIC LUPUS ERYTHEMATOSUS

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ABSTRACT

This thesis examines the role of β2-glycoprotein I (β2GPI)-reactive T cells in the development of systemic lupus erythematosus (SLE). SLE is a prototypic model for B cell epitope spread in autoimmunity. Autoantibodies to numerous molecularly distinct self-antigens emerge in a sequential manner over several years, leading to disease manifestations. Among the earliest autoantibodies to appear are those targeting phospholipids and phospholipid-binding proteins, particularly β2-glycoprotein I (β2GPI). Our laboratory has developed a model of SLE in which mice immunized with β2GPI and lipopolysaccharide (LPS) display a remarkably similar pattern of autoantibody emergence to that seen in human SLE, as well as SLE-like kidney disease. Here we use this model to investigate whether epitope spread to SLE autoantibodies is associated with a unique or limited β2GPI-reactive T cell response. We ask whether MHC class II haplotype, and its associated T cell epitope restriction, impacts epitope spread to SLE autoantibodies. Furthermore, we investigate the origin of β2GPI-reactive T cells initiating this epitope spread. We hypothesize that binding of β2GPI to necroptotic cells presents the immune system with a "scaffold" of cellular self-antigens in a pro-inflammatory and immunogenic context, leading to a robust β2GPI-reactive T cell response. Splenocytes from β2GPI/LPS-immunized mice with different MHC class II haplotypes were used to determine β2GPI-reactive T cell epitopes, using a peptide library spanning the entire sequence of human β2GPI. One β2GPI-reactive T cell epitope (LYRDTAVFECLPQHAMFG) in Domain III appeared to be a dominant epitope, since it was recognized in β2GPI/LPS-immunized mice with different MHC class II haplotypes, as well as in SLE-prone MRL/*lpr* mice. We next showed that β2GPI binds to necroptotic, as well as apoptotic, L929 cells but that necroptotic, not apoptotic, cells enhance pro-inflammatory cytokine (TNF-α) secretion by activated macrophages and dendritic cells *in vitro*. Necroptotic cells promoted MHC class II and costimulatory molecule expression in immature dendritic cells, leading to an enhanced CD4 T cell response to β2GPI *in vitro*. Finally, we show that mice deficient in *Ripk3* (receptor-interacting serine/threonine-protein kinase 3), and hence necroptosis, show poor induction of SLE. In summary, we propose that factors enabling a β2GPI-reactive T cell response may predispose individuals to the development of SLE autoantibodies independent of their MHC class II haplotype. Furthermore, our findings suggest that necroptotic cells provide both self-antigens and pro-inflammatory signals that may be sufficient to overcome immune tolerance and induce SLE.

RÉSUMÉ

Cette thèse examine le rôle des lymphocytes T réactifs spécifiques à la β2-glycoprotéine I (β2GPI) dans le développement du lupus érythémateux disséminé (LED). LED est une maladie auto-immunitaire et un modèle pour la propagation des épitopes des cellules B dans le contexte auto-immunitaire. Les auto-anticorps dirigés contre de nombreux auto-antigènes apparaissent de manière séquentielle pendant plusieurs années, menant à des manifestations pathologiques. Parmi les premiers auto-anticorps à apparaître sont ceux qui ciblent contre les phospholipides et aussi les protéines qui se lient aux phospholipides, particulièrement la β2-glycoprotéine I (β2GPI). Notre laboratoire a développé un modèle de LED où les souris sont immunisées avec la β2GPI et le lipopolysaccharide (LPS) qui présente un profil d'émergence des auto-anticorps remarquablement similaire à celui observé dans le LED chez les humains, ainsi qu'une maladie rénale similaire. Pour nos recherches, nous utilisons ce modèle pour déterminer si la propagation des épitopes aux auto-anticorps LED est associée à une réponse de lymphocytes T réactifs spécifiques à la β2GPI est unique ou limitée. Nous cherchons à déterminer si l'haplotype du CMH de classe II (MHC II), et la restriction d'épitope des lymphocytes T associée à celui-ci, affectent la propagation des épitopes aux auto-anticorps LED. De plus, nous étudions l'origine des lymphocytes T réactifs spécifiques à la β2GPI dans l'initiation de cette propagation des épitopes. Nous émettons l'hypothèse que la liaison de la β2GPI aux cellules nécroptotiques présente au système immunitaire une "plateforme" d'auto-antigènes cellulaires dans un contexte pro-inflammatoire et pro-immunitaire, menant à une réponse vigoureuse des lymphocytes T réactifs spécifiques à la β2GPI. Nous avons d'abord isolé des splénocytes de souris immunisées qui possèdent des haplotypes du CMH de classe II différents, et puis déterminé la spécificité des épitopes des lymphocytes T réactifs spécifiques à la β2GPI en utilisant une banque de peptides couvrant la séquence entière de la β2GPI humaine. Un épitope dans la β2GPI (LYRDTAVFECLPQHAMFG) dans le troisième domaine semble être un épitope dominant, car il est reconnu chez des souris immunisées ayant des haplotypes du CMH de classe II différents, ainsi que chez des souris MRL/*lpr* sujettes au LED. Nous montrons ensuite que la β2GPI peut se lier aux cellules nécroptotiques et que les cellules nécroptotiques, mais non pas apoptotiques, améliorent la sécrétion de cytokines pro-inflammatoires (TNF-α) par des macrophages et des cellules dendritiques (DCs) activés *in vitro*. De plus, les cellules nécroptotiques augmentent l'expression de la molécule du CMH de classe II et les molécules de la costimulation par les DCs

immatures, ce qui mène à une réponse accrue des lymphocytes T CD4 réactifs spécifiques à la β2GPI. Enfin, nous montrons que les souris déficientes en *Ripk3*, et donc la nécroptose, possèdent une induction défectueuse de LED. En résumé, nous proposons que les facteurs permettant une réponse des lymphocytes T réactifs à la β2GPI peuvent prédisposer des individus au développement d'auto-anticorps LED indépendamment de leur haplotype CMH de classe II. Ces études suggèrent également que les cellules nécroptotiques fournissent à la fois des auto-antigènes et aussi des signaux pro-inflammatoires qui peuvent être suffisants pour surmonter la tolérance immunitaire et induire le LED.

CONTRIBUTION OF AUTHORS

The work described in chapters 1, 2, 3 and 4 of this thesis is published as follows:

Chapter 1: **Section 1.5.3.2** is adapted from Rauch, J., **Salem, D**., Subang, R., Kuwana, M., and Levine, J.S. β2-Glycoprotein I-reactive T cells in autoimmune disease. *Frontiers in Immunology* 2018, in press.

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The studies in Chapter 2 were performed in collaboration with colleagues and collaborators as follows: Rebecca Subang assisted in the immunizations and bleeding of mice. Yuka Okazaki performed studies on PBMCs and human β2GPI-reactive T cell clones from autoimmune patients. Dr. Patrick Laplante assisted in the editing of the manuscript. Dr. Jerrold Levine was involved in experimental design, writing and editing of the manuscript. Dr. Masataka Kuwana provided recombinant β2GPI proteins and assisted in the writing and editing of the manuscript. Dr. Joyce Rauch was involved in experimental design, writing and editing of the manuscript. I performed immunizations of mice, all murine T cell isolation and epitope mapping experiments, murine ELISAs, and wrote and edited the manuscript.

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Kuwana provided the recombinant β2GPI proteins and assisted in the writing and editing of the manuscript. Dr. Jerrold Levine was involved in experimental design, writing and editing of the manuscript. Dr. Joyce Rauch was involved in experimental design, writing and editing of the manuscript. I performed immunizations of mice, all murine T cell isolation, phenotyping and epitope mapping experiments, murine ELISAs, and wrote and edited the manuscript.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

The studies done in this thesis contributes original knowledge to the field of the pathogenesis of systemic lupus erythematosus disease. The specific contributions are as follows:

1. We have shown that mice, independent of MHC class II haplotype (H-2^b, H-2^d, and H-2^k), develop high titers of SLE autoantibodies upon immunization with β2GPI and LPS.

2. We have shown that β2GPI-reactive T cells are associated with epitope spread to multiple SLE autoantibodies in induced and spontaneous models of SLE.

3. We identified MHC class II haplotype (H-2^b, H-2^d, and H-2^k)-restricted β2GPI-T cell epitopes in induced and spontaneous models of SLE.

4. We have shown that β2GPI-reactive T cells from an autoimmune patient recognize the same peptide (23) as H-2^b-bearing mice. Peptide 23 recognition was restricted to an SLE-associated HLA class II allele DRB1*0403.

5. We identified a potential immunodominant epitope in Domain III (peptide 31) of β2GPI, which is recognized by T cells in induced and spontaneous SLE, across different MHC class II haplotypes.

6. We have shown that β2GPI-reactive splenic T cells from murine SLE favor pro-inflammatory $CD4^+$ Th phenotypes: a Th1 phenotype in induced SLE and a Th17 phenotype in spontaneous SLE [MRL/*lpr* mice].

7. We have shown that necroptotic and apoptotic L929 cells bind β2GPI, which was detected by murine monoclonal anti-β2GPI antibodies. However, necroptotic cells favored pro-inflammatory cytokine release and activation of antigen presenting cells, whereas apoptotic cells were shown to be immunosuppressive.

8. We have shown that necroptotic cells, as opposed to apoptotic cells, promote pro-inflammatory cytokine release and activate antigen presenting cells, with subsequent activation of β2GPI-reactive T cells *in vitro.*

9. We have shown that mice deficient in *Ripk3*, and therefore necroptosis, show poor induction of SLE as evidenced by decreased autoantibody production and lack of kidney disease

10. We provide evidence that necroptotic cells can serve as an autoantigenic stimulus that could lead to activation of β2GPI-reactive T cells and promote B cell epitope spread.

ABBREVIATIONS

ACR	American College of Rheumatology
APC	antigen presenting cells
aPL	anti-phospholipid antibody(ies)
APS	anti-phospholipid syndrome
ADP	adenosine diphosphate
AP	alkaline phosphatase
Apaf-1	Apoptotic protease activating factor-1
ATP	adenosine triphosphate
B6	C57BL/6
BAFF	B cell activating factor
Bcl-6	B cell lymphoma 6
β2GPI	β2-glycoprotein I
BMDC	bone marrow-derived dendritic cell
BMDM	bone marrow-derived macrophage
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cpm	counts per minute
CD	cluster of differentiation
CXCR	C-X-C chemokine receptor type
CFA	complete Freund's adjuvant
CL	cardiolipin
CMV	cytomegalovirus
CO_2	carbon dioxide
DAMP	danger associated molecular pattern
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DN	double negative
DNA	deoxyribonucleic acid
dsDNA	double stranded-deoxyribonucleic acid

DTT	dithiothreitol
EBV	Epstein-Barr virus
EBNA-1	Epstein-Barr virus nuclear antigen-1
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FADD	Fas-associated protein with death domain
Fas	first apoptosis signal
FBS	fetal bovine serum
Fcgr	Fc-gamma receptor
FITC	fluorescein isothiocyanate
FoxP3	forkhead box protein 3
FLIP	FLICE inhibitory protein
FVa	Factor Va
GM-CSF	granulocyte macrophage-colony stimulating factor
GSK	GlaxoSmithKline
GWAS	genome-wide association studies
H&E	hematoxylin-eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour
HSA	human serum albumin
HLA	human leukocyte antigen
HMGB1	high mobility group box protein-1
IBD	inflammatory bowel disease
Ig	immunoglobulin
IgG	immunoglobulin G
IL	interleukin
IFN	interferon
IRI	ischaemia-reperfusion injury
i.v.	intravenous
kDa	kilodalton

KO	knockout
LA	lupus anticoagulant
LogFC	log fold change
lpr	lymphoproliferation
LPS	lipopolysaccharide
MalBP	maltose-binding protein
MCTD	mixed connective tissue disease
M-CSF	macrophage-colony stimulating factor
MEM	minimal essential medium
MFI	mean fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
Mlkl	mixed lineage kinase domain-like
mL	milliliter
MRL/*lpr*	MRL/MpJ-*Tnfrsf6*^{lpr}
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
NaCl	sodium chloride
NaN_3	sodium azide
Nec-1	necrostatin-1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NMDAR	N-methyl-D-aspartate receptor
ng	nanogram
OD	optical density
oxLDL	oxidized low density lipoprotein
PAPS	primary anti-phospholipid syndrome
PAS	periodic acid-Schiff
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cell

PE	phycoerythrin
pg	picogram
pH	potential of hydrogen
PL	phospholipid(s)
PMA	phorbol12-myristate 13-acetate
PP2A	serine threonine protein phosphatase 2A
PS	phosphatidylserine
QTL	quantitative trait loci
RA	rheumatoid arthritis
RNA	ribonucleic acid
RNP	ribonucleoprotein
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
Ripk	receptor-interacting serine/threonine-protein kinase
ROR-γt	RAR-related orphan receptor gamma t
SAPS	secondary anti-phospholipid syndrome
SCR	short consensus repeat
SD	standard deviation
SE	standard error
SLE	systemic lupus erythematosus
SIRS	systemic inflammatory response syndrome
Sm	Smith antigen
SmB	small nuclear ribonucleoprotein-associated protein B
snRNP	small nuclear ribonucleoprotein
Syk	spleen tyrosine kinase
T1D	type 1 diabetes
TBS	tris-buffered saline
TCR	T cell receptor
Th	T helper
TGF-β	transforming growth factor-beta
Tfh	T follicular helper

TMB	3,3',5,5'-Tetramethylbenzidine
TNF	tumor necrosis factor
TLR	toll-like receptor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
Treg	T regulatory
U1-70K	U1 small nuclear ribonucleoprotein 70 kDa
µg	microgram
µL	microliter
U	units
UV	ultraviolet
WT	wild type
ZAP70	zeta(ζ)-associated protein kinase 70 kDa
zVAD-fmk	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone
7-AAD	7-aminoactinomycin D

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL FEATURES OF SLE

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that affects over one in 1000 Canadians and results in multitude of clinical phenotypes and premature mortality[1]. Due to the heterogeneity of the disease, diagnosing SLE and discovering new treatments has been challenging. The American College of Rheumatology (ACR) has developed 11 criteria for the classification of SLE, four of which must be present at any time during the patient's history[2,3]. These criteria include clinical features affecting different organ systems, as well as immunological features frequently seen in patients with SLE (**Figure 1.1**). SLE is more prevalent in females, with a 9:1 female to male ratio of disease incidence, and is more common in African American, Hispanic, and Asian populations[4]. Mortality in SLE occurs in only 10% of cases within 10 years of diagnosis[5]. Nevertheless, this disease often results in life-long suffering due to infections, cardiovascular disease, renal failure, and financial burden (cost of immunosuppressive treatment and hospitalization)[6].

The etiology of SLE remains poorly defined since every patient can express a distinct set of symptoms, but it is clear that both genetic and environmental factors are involved in the development of SLE[7]. Over 40 genetic loci have been shown to be associated with development of SLE according to several genome-wide association studies (GWAS)[8]. Among those genes, polymorphisms in major histocompatibility complex (MHC) and certain human leukocyte antigen (HLA) alleles have been shown to be strongly associated with SLE development[9,10]. Nevertheless, studies demonstrate only a 25% concordance rate of SLE in monozygotic twins, depicting a genetic association but also an obvious underlying environmental influence[4,11]. Hormones, ultraviolet (UV) radiation, drugs, and viral and bacterial infections have all been suggested to affect the progression of SLE[10]. While the precise mechanisms involving these factors remain elusive, it is clear that both genetics and environment play a role in the development of SLE. It is, furthermore, clear that patients with SLE develop autoantibodies to cellular surface and nuclear antigens[10].

1.2 AUTOANTIBODIES IN SLE

SLE patients typically develop autoantibodies to cellular self-antigens in a sequential manner that precedes the diagnosis of SLE[12]. This was shown in an elegant study by Arbuckle et al.[12], who studied autoantibodies in the sera from members of the United States armed forces both at baseline (upon enlisting) and every year following enlistment. It was clear that certain autoantibodies appeared first, up to nine years prior to any clinical manifestation was observed, while other autoantibodies developed in the later phase of the disease[12]. Among the earliest autoantibodies to appear were anti-phospholipid antibodies (aPL)[12], which are primarily autoantibodies that recognize phospholipid-binding proteins that bind to phospholipids). Antibodies against double stranded DNA (dsDNA) and ribonucleoproteins (RNPs), like Ro (SS-A), La (SS-B), and Smith antigen arose shortly after. The process of this "spread" in the immune response to multiple autoantibody specificities is most commonly referred to as "epitope spread", and is also seen in other autoimmune diseases such as in type 1 diabetes (T1D) and multiple sclerosis (MS)[12-15]. This is a mechanism by which the immune response to a single antigen spreads to multiple epitopes, either on the same antigen (intramolecular) or on a different antigen (intermolecular) within the same macromolecular complex[16].

The origin of SLE autoantibodies remains unclear. Increased autoantigen exposure triggered by enhanced cell death from infections or UV radiation is one theory[7,10]. Deficiency in the clearance of dying cells, such as apoptotic cells, is proposed as another reason for this exposure[17]. As shown by GWAS studies, many patients with SLE have polymorphisms in genes involved in the complement cascade or Fc receptors, which are both involved in the clearance of apoptotic cells (and hence, self-antigens)[9]. An increase in cell death, alone or combined with a defect in clearance, could potentially lead to these autoantigens being presented by the immune system and autoimmunity. Viral infections, such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), or parvovirus B19, could also provide an environmental trigger by enhancing presentation of these autoantigens in a pro-inflammatory context[18]. Ultimately, this would lead to a loss of immune tolerance towards these self-antigens and autoimmunity. Immune responses to viruses primarily involve a high level of production of type I interferons (IFN), like IFN-α, by plasmacytoid dendritic cells (pDCs)[19]. IFN-α is known to increase responsiveness of pDCs to RNA and DNA-containing immune complexes through toll like receptors (TLR) 7 and 9[19]. EBV has also been suggested to provide a trigger in SLE through molecular mimicry, a phenomenon

in which an immune response to a foreign antigen (e.g., a viral antigen) results in recognition of a self-antigen (due to sequence similarities with a foreign antigen)[20,21]. For example, the amino acid sequences of the EBV nuclear antigen-1 (EBNA-1) closely resembles the sequences of human ribonucleoproteins small nuclear ribonucleoprotein-associated protein B (SmB) and Ro, resulting in potential cross reactivity with these self-antigens[20,21]. Finally, B and T cells in some patients with SLE have impaired signalling functions that lead to increased recognition of autoantigens, and subsequent autoantibody production[7,10].

Generally, SLE autoantibodies have high affinity for their antigen, are somatically mutated, and are mostly IgG[10]. This indicates that T helper (Th) cells are required for class switching in B cell germinal centers. These autoantibodies are directly involved in tissue pathology and often deposited in sites of organ damage[22]. Anti-dsDNA autoantibodies are thought to be highly specific to SLE, and are associated with the development of renal disease, known as lupus nephritis[23]. Immune complex deposition in the glomeruli of the kidneys is thought to recruit lymphoid and myeloid cells, and induce glomerular and interstitial nephritis[23]. In contrast, anti-Ro autoantibodies have been closely associated with development of congenital heart disease and cutaneous lupus[24]. There are autoantibodies of other specificities (e.g., anti-N-methyl-D-aspartate receptor antibody [NMDAR]) that show selective association with different pathologies[25] (e.g., neuropsychiatric manifestations[26]). For all of these autoantibodies, the source of autoantigen is largely thought to be dying cells, mainly apoptotic cells[10]

1.2.1 β2-Glycoprotein I (β2GPI) in SLE

aPL are present in 20-30% of SLE patients and are among the earliest autoantibodies detected in the years preceding the diagnosis of SLE[12]. aPL comprise a group of autoantibodies recognizing phospholipids and/or phospholipid-binding proteins. aPL were first discovered in patients with syphilis (*Treponema pallidum*) in 1906, using the Wassermann complement-fixation test[27,28]. However, the precise antigen was not identified as cardiolipin (CL) until 1941, when positive sera were shown to react to extracts of bovine hearts. In mammalian and plant cells, CL is almost exclusively found in the mitochondrial membrane, while, in bacterial cells, CL is also found in cell membrane[29]. SLE patients positive for aPL were often misdiagnosed

with syphilis, despite not showing any clinical symptoms[30]. More sensitive immunoassays for aPL were developed in the early 1980's, which led to the diagnosis of patients with anti-cardiolipin (aCL) syndrome, eventually termed anti-phospholipid syndrome (APS)[31,32]. APS is identified by the detection of aPL and a clinical outcome associated with aPL, such as vascular thrombosis or pregnancy morbidity[33]. APS can be detected in the absence of other autoimmune disease (primary APS [PAPS]) or can be accompanied by SLE (termed secondary APS [SAPS]). aPL production can also be seen in patients with bacterial and viral infections (e.g., syphilis), but reactivity mainly toward the phospholipid rather than the phospholipid-binding protein[34]. In contrast to "infectious aPL," patients with autoimmune disease (i.e., APS) are primarily reactive to phospholipid-binding proteins (alone or complexed with phospholipids)[35]. One of the major phospholipid-binding proteins targeted in APS and SLE is β2-Glycoprotein I (β2GPI)[36].

β2GPI, also known as apolipoprotein H, is a 50 kDa protein found in mammalian plasma (in humans, at a concentration of ~200 μg/mL)[37]. It is mainly produced in the liver and placenta, but its function in the physiological state remains poorly understood. β2GPI is believed to play a role in coagulation, functioning both as a positive and negative regulator of this process under certain conditions[37]. This is due to the ability of β2GPI to regulate the activity of several factors in the coagulation cascade, such as Factor Va (FVa), FXI, and FXII. In addition, β2GPI has been shown to interact with negatively charged molecules, including DNA, heparin, oxidized low-density lipoprotein (OxLDL), and lipopolysaccharide[37,38]. In fact, its functions are a consequence of its ability to bind to negatively charged particles. It is thought that β2GPI sequesters circulating negatively charged molecules known to trigger the intrinsic coagulation cascade. Plasma from mice lacking β2GPI have impaired thrombin generation *in vitro,* but these mice have otherwise intact coagulation[39]. In addition, β2GPI-deficient mice have lower fecundity rates than wildtype littermates. This suggests that β2GPI is not essential for reproduction, but may be involved in early embryonic development or implantation[39]. Interestingly, humans who have undetectable serum levels of β2GPI due to a frameshift mutation in the gene were not found to be at risk of thrombosis and have normal hematological profiles[40].

The β2GPI polypeptide consists of 345 amino acid residues, including a 19-amino acid leader sequence that is cleaved upon secretion from the cell[41]. The protein is divided into five

highly conserved short consensus repeats (SCR), called Domains I-V[37]. Domain V differs from the other domains in that it contains a stretch of 14 positively charged residues and a flexible hydrophobic loop[42]. This allows for stable binding to anionic phospholipids, like cardiolipin and phosphatidylserine, and insertion into the lipid bilayer. Domains III-IV contain five glycosylation sites, which protect the glycoprotein from proteolysis. In the circulation, Domain I interacts with Domain V, creating a circular or closed conformation[42]. When Domain V binds to phospholipid, Domains I-II protrude away from the plasma membrane into the extracellular space. creating a distinct fish hook-like structure (Figure 1.2). Domain I-binding antibodies recognize epitopes in the exposed region of β2GPI, and are strongly associated with the development of thrombosis in patients with APS in SLE patients[42-44].

1.3 MURINE MODELS OF SLE

The heterogeneous clinical manifestations of SLE, as well as the multigenic and environmental contributions to development of the disease, make it a challenging disease to model experimentally. That being said, there are multiple murine models of SLE that can be divided into two categories: spontaneous and induced models. Each model has its advantages and weaknesses, but none of these models reflect all of the characteristics seen in patients with SLE. Nevertheless, all SLE murine models are accompanied by the production of autoantibodies, lymphocyte activation, and tissue injury, most often glomerulonephritis.

1.3.1 Spontaneous Models of SLE

There are several spontaneous models of SLE in which the genetics of the mice predispose them to developing “SLE-like” disease. These models allow us to study the underlying genetic factors of disease, but provide little understanding of the environmental triggers. However, these models have confirmed certain quantitative trait loci (QTLs) associated with susceptibility to SLE, and enabled the discovery of SLE heritability genes[45].

1.3.1.1 NZB/W F1

The first SLE model was discovered in 1966 by crossing non-autoimmune New Zealand Black (NZB) and White (NZW) mice[46]. Their F1 progeny produced high levels of anti-dsDNA IgG, lymphoadenopathy, and nephritis at 5-6 months of age, leading to kidney failure and death at 10-12 months[47]. Accidental backcrossing of these mice with NZW generated the NZM2410 strain, which develops a more severe and earlier SLE-like disease. Notably, three susceptibility loci *(Sle1-3)* discovered in NZM2410 strain have been linked to susceptibility to SLE in human GWAS studies. These loci contain genes with certain polymorphisms responsible for susceptibility, but most of these genes have not yet been mapped[45]. Through generating congenic strains on a non-autoimmune background, like C57BL/6 (B6) mice, the mechanism of action of these loci has been determined. *Sle1* locus was associated with loss of tolerance to chromatin through activation of autoreactive B cells and $CD4^+$ T cells, and reduction in the number of regulatory $FoxP3^+$ T (Treg) cells[45,48]. Two genes affected in this locus, *Fcgr2b* (Fc-gamma receptor 2B) *and Cr2* (complement receptor 2), have also been shown to be associated with human SLE[49,50]. *Sle2* locus was shown to reduce the activation threshold of B1 cells, whereas the *Sle3* locus, through polymorphisms in the kallikrein (*Klk*) genes, is associated with decreased levels of activation-induced cell death in $CD4^+$ T cells[45,47]. While congenic B6 strains expressing any one of these loci do not develop SLE-like disease, co-expression of the three susceptibility loci, as in the B6.*Sle1*.*Sle2*.*Sle3* strain, recapitulates the disease seen in NZM2410 mice[48].

1.3.1.2 BXSB.*Yaa*

The BXSB.*Yaa* strain was derived from a cross between B6 females and SB/Le males, and severe lymphoproliferation and features of SLE were observed in male offspring[51]. These mice produce aPL and anti-nuclear antibodies, accompanied by severe glomerulonephritis[47]. The *Yaa* element, or Y-linked autoimmune accelerator, is caused by the translocation of the telomeric end of the X-chromosome onto the Y-chromosome. This causes a duplication of over 16 genes, increasing expression of these genes including *Tlr7*[47]. This *Yaa* element alone, however, is not sufficient for the development of autoimmunity since insertion of this element onto the B6 background does not cause disease[52]. The MHC locus ($H\text{-}2^{b/b}$) has been shown to play an important role in disease process in the BXSB strain, since BXSB.$H\text{-}2^{d/d}$ mice are protected while heterozygote mice ($H\text{-}2^{b/d}$) are not[53]. Therefore, MHC haplotype and presentation of

certain autoantigenic epitopes to T cells appear to be crucial for development of SLE in this model.

1.3.1.3 MRL/MpJ-*Tnfrsf6*^{lpr} (MRL/*lpr*)

The MRL/*lpr* strain is the most commonly used spontaneous murine model of SLE. Pathogenesis involves immune complex-mediated glomerulonephritis and accumulation of double negative (DN) ($CD4^-CD8^-$) T cells leading to lymphoadenopathy[47]. Unlike most spontaneous SLE strains, MRL/*lpr* mice produce the full spectrum of autoantibodies seen in human SLE, including: high levels of aPL (anti-β2GPI and anti-CL), anti-dsDNA, and most anti-ribonucleoprotein IgG autoantibodies. These autoantibodies appear in a sequential manner similar to human disease. This autoimmunity is partly attributed to the "*lpr* (lymphoproliferation) mutation", a mutation leading to insertion of an early transposable element in the apoptosis signal (Fas) receptor gene that inhibits its transcription and expression on the cellular surface[54,55]. Upon recognition of the Fas receptor by Fas-ligand (FasL), cell death is induced by apoptosis. The Fas receptor mutation inhibits apoptosis in T and B cells, resulting in severe lymphoproliferation and loss of immune tolerance to certain autoantigens. This mutation alone is not enough to induce glomerulonephritis, since expression of this gene in C3H or B6 mice induced some autoantibody production but had no major effect on proteinuria levels or renal pathology[56,57]. MRL/Mp (or MRL/+) strain is genetically identical to the MRL/*lpr* strain, but lacks the *lpr* gene. The MRL/+ strain is considered the control strain for MRL/*lpr* mice, but these mice are also autoimmune and develop a mild and delayed form of SLE-like disease.

Cross-breeding of MRL/*lpr* and B6.*lpr* mice has uncovered certain susceptibility loci (*lmb1-4*) in the MRL background, but these genes have not yet been fully characterized[58]. One affected gene in the *Lmb3* locus, codes for the protein coronin-1A, an actin cytoskeleton-regulating protein that has been implicated in T cell proliferation, activation, and apoptosis[59,60]. The MRL/*lpr* model has also provided insight into some of the possible sex-related mechanisms important in SLE. In contrast to NZM2410 mice, where mostly female mice develop SLE, both sexes develop disease in the MRL/*lpr* strain[47]. However, when females from either NZM2410 or MRL/*lpr* mice are ovariectomized, mice have reduced autoantibody titers and

glomerulonephritis[61,62]. When these mice are supplemented with estradiol (estrogen steroid hormone), the effects are reversed. Therefore, estrogen plays an important role in disease progression in these murine SLE models, and likely in women with SLE[63].

Eliminating different compartments of the immune system of the MRL/*lpr* background, either through genetic deletion or antibody-mediated depletion, has identified critical players driving disease. Depletion of key cytokines, like IFN-γ[64], IFN-α/β[65,66], IL-17a[67], and IL-21[68], in MRL/*lpr* mice reduced autoantibody levels and improved glomerulonephritis and survival. These studies provided insight into the different T cell populations involved in murine SLE.

The MRL/*lpr* murine model has also helped in understanding the role of B cells in SLE, as well as identifying novel therapeutic targets. Depletion of B cells by administration of anti-CD20 in MRL/*lpr* mice resulted in decreased autoantibody production and kidney pathology[69]. Similarly, inhibiting activation and survival of B cells using monoclonal antibodies to B cell activating factor (BAFF) receptor reduced proteinuria and glomerulonephritis, and increased survival in MRL/*lpr* mice[70]. These two findings have promoted the development of novel B cell therapeutic drugs. Monoclonal anti-CD20, known as Rituximab, is an important drug for treating rheumatoid arthritis and was also thought to be useful for treating SLE[71]. Although this drug was found to efficiently deplete circulating B cells, certain B cell populations in the spleen and bone marrow were more resistant to depletion, and so the Rituximab failed phase II/III trials in SLE patients[72]. Anti-BAFF antibody treatment (Belimumab), however, was shown to be efficient in reducing disease severity in SLE patients[73]. This was the first drug to meet clinical trial end points and is now a recommended treatment for SLE. Last but not least, B cells are not only autoantibody producing cells, but can also serve as antigen presenting cells (APCs). Specific MHC class II deletion in B cells of MRL/*lpr* mice, which ablates their antigen presenting abilities, reduced kidney pathology[74].

1.3.2 Induced Models of SLE

Induced models differ from spontaneous models in that they require an external trigger to initiate the disease process. In this manner, induced models enable the evaluation of

environmental factors that trigger SLE in a healthy individual. Since administration of the environmental trigger controls initiation of the disease, mechanisms involved at the very early stages of the disease process are more easily identified and monitored than in spontaneous models. Furthermore, these triggers can be evaluated in genetically altered, as well wild type, mice to determine the effect of a specific gene on the initiation or progression of disease. Thus, induced models can provide key information on both the genetics and environmental factors involved in SLE.

1.3.2.1 Pristane-induced model of SLE

In the pristane-induced model of SLE, non-autoimmune mice are injected intraperitoneally with an isoprenoid alkane called pristane to induce SLE-like disease[75]. Now produced synthetically as a mineral oil, pristane (2,6,10,14-Tetrametylpentadecane) was originally isolated from the livers of shark and was used to generate ascites in the peritoneal cavities of mice injected with hybridomas[47]. It was later discovered that mice injected with pristane, particularly the BALB/c strain, developed high levels of anti-nuclear, anti-dsDNA, and anti-ribonucleoprotein autoantibodies[75]. These mice also had immune complex deposition in the glomeruli of their kidneys, and developed nephritis and proteinuria[76]. Most inbred strains were found to be susceptible to developing this SLE model, and disease was more severe in females in SJL mice[77]. One weakness of the pristane-induced model is the length of time (6-10 months after immunization) required for the mice to develop SLE-like. In addition, anti-dsDNA antibodies are only detected in only 40% of pristane-injected mice[78].

Nevertheless, the pristane-induced model has been used to explore potential mechanisms in SLE. IL-12 and IFN-γ, but not IL-4, deficiency led to the reduction in autoantibody titers and improvement of kidney disease[79,80]. These findings highlight the importance of Th1 over Th2 cells in driving inflammation and pathology in this model. Furthermore, the pristane model was one of the first models to reveal the importance of type I interferons in SLE. IFNα receptor-1 (IFNAR1)- or IFNAR2-deficient mice were found to be protected from disease[81]. High levels of type I interferon affect the immune system in multiple ways. Immature peritoneal monocyte production of type I interferon was found to increase expression of TLR7 and TLR9 on B cells,

rendering these cells more sensitive to nucleic acid-containing immune complexes[82]. Type I interferon also prevents maturation of monocytes, which acts in a feedback loop resulting in recruitment of more monocytes. However, immature monocytes lack phagocytic capability, and so, fail to clear cellular debris and immune complexes[83]. Finally, type I interferon increases IL-12 production, which increases Th1 cell differentiation[81]. Together, type I interferon production leads to increased autoreactive B and T cell activation, autoantibody production and nephritis.

1.3.2.2 β2GPI/LPS-induced model of SLE

Autoantibodies recognizing β2GPI were first identified in patients with APS[42]. This led to the speculation that β2GPI might serve as an initiating antigen for inducing models of APS. In 1994, Blank et al.[84] induced APS in BALB/c mice by immunization with human β2GPI in the presence of complete Freund's adjuvant (CFA). The immunized mice produced IgG aPL, developed thrombocytopenia, and had high rates of fetal resorption, typical to what is seen in human APS patients with pregnancy morbidity.

Anti-β2GPI autoantibodies are also among the earliest autoantibodies to appear in SLE, and can be detected up to 10 years before disease onset[12,85]. Our laboratory hypothesized that β2GPI might serve as a useful immunogen to induce SLE in mice. This hypothesis was based on the ability of β2GPI to bind apoptotic cells, and potentially enable epitope spread to multiple SLE autoantigens expressed on these cells[86]. Levine et al.[86] showed that B6 and BALB/c mice immunized with human β2GPI in the presence of LPS develop high titers of IgG anti-β2GPI and anti-CL antibodies. In addition, prolonged immunization of the mice with β2GPI and LPS resulted in the production of multiple SLE specific autoantibodies, including anti-dsDNA, anti-Ro, anti-La, anti-Sm, and anti-nRNP autoantibodies, as well as SLE-like glomerulonephritis[86]. Glomerulonephritis was associated with complement and immune complex deposition in the glomeruli of the kidneys. Development of this SLE model was dependent on T cell co-stimulation, as immunized CD28-deficient mice did not develop aPL or SLE autoantibodies. Moreover, the SLE-like disease that developed in this induced model very closely resembled human SLE in that autoantibodies emerged in a sequential manner similar to that seen in SLE patients[12] . The emergence of multiple autoantibodies unrelated to the initial immunogen

suggests that epitope spread occurs in this model[86]. The proposed mechanism for epitope spread in the β2GPI/LPS induced model of SLE focuses on: (1) the ability of β2GPI to bind to apoptotic cells, which comprise a cellular scaffold of SLE autoantigens; and (2) the ability of β2GPI-reactive T cells to provide help to B cells reactive with SLE autoantigens other than β2GPI. In this model, a strong and persistent β2GPI-reactive T cell response develops following repeated immunization with β2GPI and LPS (Figure 1.3). Apoptotic cells and bodies, which express several autoantigens on their surface, are rich in anionic phospholipids and can bind β2GPI. B cells specific for various SLE-associated autoantigens can take up these β2GPI-bound apoptotic cells and present MHC class II-bound β2GPI peptides on their surface. These B cells can then receive help from β2GPI-reactive T cells, and secrete class-switched autoantibodies to these autoantigens[86]. In this way, β2GPI-reactive T cells have the potential to promote autoantibody production to a multitude of self-antigens expressed by dying cells.

In summary, murine models have been crucial in helping us understand mechanisms of disease progression in SLE. Although the etiology of disease is still undefined, we have a clearer picture of certain factors (environmental and genetic) that may lead to SLE. Most importantly, these murine models can provide us with new therapeutic as well as diagnostic and prognostic targets for treating SLE.

1.4 CELL DEATH AND SLE

Dying cells have long been implicated in the development of SLE because of their expression and potential release of autoantigens, and activation of autoreactive T and B cells[10,87]. Cell death is increased in patients with SLE possibly stemming from environmental triggers like exposure to UV radiation and infection[7,88,89]. Additionally, SLE patients often have polymorphisms in Fc receptor (*FCGR2A*) and complement genes (*C1Q, C2, C3, C4, CR2*), which affect clearance of dead cell debris[9,17]. Apoptosis is the best described mechanism of programmed cell death involved in SLE but others, such as autophagy and NETosis[90], have also been suggested.

1.4.1 Apoptosis

Apoptosis is a programmed cell death that can occur through the intrinsic or extrinsic pathway. The intrinsic pathway is triggered by oxidative and genomic damage, often resulting in loss of mitochondrial function[17]. The extrinsic pathway is initiated by ligation of death receptors, such as TNF, Fas, and TNF-related apoptosis-inducing ligand (TRAIL) receptors. This results in downstream activation of cysteine-aspartic proteases (caspases), nuclear condensation, exposure of anionic phospholipids in the outer membrane, cell shrinkage, and blebbing of the plasma membrane creating apoptotic bodies (Figure 1.4)[91]. Apoptotic cells and bodies, which contain autoantigens, are normally quickly taken up and cleared by phagocytic cells, like macrophages, monocytes, and DCs. Upon ingestion of apoptotic cells, phagocytic cells produce anti-inflammatory cytokines like IL-10 and transforming growth factor-beta (TGF-β), which are tolerizing signals to prevent activating autoreactive T and B cells[91]. It has been proposed that apoptotic debris are not readily cleared in patients with SLE. Baumann et al.[92] demonstrated that there was an accumulation of apoptotic debris on the surface of follicular DCs in germinal centers of SLE patients compared to healthy controls, which they suggest may lead to activation of autoreactive B and T cells that would normally die by negative selection. Failure or delay in apoptotic cell clearance can also lead to secondary necrosis of the dying cells, membrane permeabilization, and release of intracellular content. Release of autoantigens, as well as danger associated molecular patterns (DAMPs) like adenosine triphosphate (ATP) and high mobility group box protein-1 (HMGB1) could lead to the presentation of autoantigens in a pro-inflammatory environment and activation of autoreactive cells. Furthermore, some autoantigens, including DNA, RNA, and proteins, have been shown to be modified during apoptosis[93]. Cleavage by proteases and endonucleases, or altered post-translational modification including methylation, ubiquitination, phosphorylation, citrullination, acetylation, ADP-ribosylation, and transglutamination, have been shown to modify autoantigens during apoptosis. These modifications can potentially render the autoantigens to appear as foreign to the host, and, thus, immunogenic.

1.4.2 Necroptosis

In contrast to apoptosis, necrosis is characterized by sudden swelling of the cell and rupture of the cell membrane, leading to lysis and release of cytosolic content. Necrosis was originally thought to be a non-regulated process, but recent studies have demonstrated a type of programmed necrosis called necroptosis[94]. Necroptosis can be initiated through activation of TNFR1, Type I and II IFN receptors, certain TLRs (TLR3 and TLR4), and cytosolic viral RNA sensors[95]. Necroptosis seems to occur only when apoptosis is inhibited. During viral infection, certain viral proteins have been shown to inhibit apoptosis and promote necroptosis and lysis to infect neighboring cells. In some cases, FLICE inhibitory protein (FLIP) can also bind to caspase-8, an apoptotic initiator, and thus prevent apoptosis[94]. Downstream activation of receptor interacting serine/threonine protein kinases (RIPK) 1 and 3 activates mixed lineage kinase domain-like (MLKL) pseudokinase, which migrates to the plasma membrane and initiates cell membrane rupture (Figure 1.4). Necroptosis has been implicated in a number of inflammatory pathologies and diseases, including retinal degeneration, brain impact trauma, cerulein-induced pancreatitis, ethanol-induced liver injury, septic shock, multiple sclerosis, Huntington disease, and tumour progression[96].

To date, few studies have evaluated the role of necroptosis in SLE. Fan et al.[97] showed that B cells from SLE patients have increased expression of necroptosis-related genes and that co-activation of the B cell receptor (BCR) and TLR7 could promote B cell hyperactivation and necroptosis. Honarpisheh et al.[98] identified elevated mRNA expression levels of *Ripk1*, *Ripk3* and *Mlkl* in the spleens of MRL/*lpr* mice at 6-weeks of age, suggesting the importance of necroptosis in the initiation of disease. Finally, Corradetti et al.[99] showed that RIPK3 was not necessary for autoantibody production in pristane-induced and chronic graft versus host disease murine models of SLE. However, neither model developed nephritis, making it difficult to accurately determine the role of RIPK3 in disease development. Although an association for necroptosis with development of SLE has not been established, the massive DAMP release and the expression of autoantigens by necroptotic cells provide a compelling rationale for their involvement in the initiation of SLE.

1.5 T CELLS IN SLE

SLE is characterized by the production of high levels of IgG class-switched autoantibodies necessitating a T helper response. Autoreactive T cells are known to play two major roles in SLE: (1) providing T cell help to autoreactive B cells and enabling their production of autoantibodies, and (2) contributing to organ inflammation and tissue injury. The question remains as to how these autoreactive T cells escape central and peripheral tolerance. Furthermore, it is still unclear how a T cell initiates an immune response to a non-protein antigen. It has been noted that many of the non-protein autoantigens (e.g. DNA, RNA, phospholipid) targeted in SLE form complexes *in vivo* with protein antigens[10]. This has led to speculation that a T cell response to the protein portion of the complex may provide T cell help to the non-protein component via intermolecular epitope spread. Nevertheless, the antigen specificity of autoreactive T cells driving epitope spread and inducing disease in SLE remains unknown.

1.5.1 Characteristics of T cells in SLE

T cells found in human SLE have a lower threshold of activation than those found in nonautoimmune individuals, partly due to an abnormal expression of TCR-associated molecules[100,101]. The TCR works in conjunction with CD3, a co-receptor consisting of various transmembrane subunits that initiate early signaling events necessary for T cell activation. In some patients with SLE, the ζ-chain of CD3 is replaced by a homologous FcγR chain, which inhibits ζ-associated protein kinase 70 kDa (ZAP70) recruitment and allows spleen tyrosine kinase (Syk) to bind[101]. This increases initial signaling events and is thought to permit activation of T cells responding to low avidity autoantigens not otherwise recognized by normal T cells. The modification in CD3ζ is due to increased levels and activity of the serine threonine protein phosphatase 2A (PP2A)[102], which inhibits CD3ζ and increases expression of FcγR proteins[101]. PP2A has also been shown to inhibit IL-2 expression, and promote IL-17 expression. Reduced IL-2 levels are strongly associated with a decrease in Treg cell number and suppressive function in patients with SLE[103]. Furthermore, pharmacologic inhibition *in vitro* of Syk in MRL/*lpr* mice or in T cells from SLE patients prevents kidney pathology in MRL/*lpr* mice and normalizes IL-2 production in human T cells[101,104]. Finally, T cells from SLE patients show higher surface expression of CD44, which increases adhesion and migration to organs like the kidneys[105].

1.5.2 T cell phenotypes in SLE

Three major Th cell phenotypes are involved in the development of SLE: Th1, Th17, and T follicular helper (Tfh) cells. Much of our knowledge of these T cell populations in disease progressions stems from studies in MRL/*lpr* mice. Th1 and Th17 cells, which produce high levels of IFN-γ and IL-17a respectively, contribute to the pro-inflammatory environment, activate myeloid cells, and infiltrate the kidneys of MRL/*lpr* mice[106]. Th17 cells play an important role in driving glomerulonephritis. IL-17a-deficient MRL/*lpr* mice have reduced Th17 cells in the glomeruli, which protects mice from disease[107]. The same is seen in IL-23R-deficient MRL/*lpr* mice, since IL-23 drives Th17 differentiation[108,109]. IL-17a in the kidneys of MRL/*lpr* is produced by Th17 cells, as well as DN and gamma-delta (γδ) T cells, and recruits monocytes and neutrophils to sites of inflammation[110]. SLE patients also have increased numbers of Th17 cells, as well as high levels of serum IL-17a[111-113]. In SLE patients with severe nephritis, Th1 cells are predominant [114]. Tfh cells, which express the cytokine IL-21, are pivotal in providing help to B cells and inducing class-switched antibody production in MRL/*lpr* mice and human SLE[115]. Tfh cells typically express CXC chemokine receptor type 5 (CXCR5), which allows for homing to germinal centers to exert their function on follicular B cells[116]. In MRL/*lpr* mice and patients with SLE, Tfh cells have been identified in the circulation and in nephritic tissue, and contribute to IgG production and disease[115,117]. They are known as circulating or extrafollicular Tfh cells.

1.5.3 Antigen specificity of T cells in SLE

1.5.3.1 U1-70K-reactive T cells

Studies conducted by Muller et al.[118] were among the first to identify the antigen specificity of $CD4^+$ T cells in SLE. These investigators identified a T cell population that recognizes an epitope on U1 small nuclear ribonucleoprotein 70 kDa (U1-70K) in MRL/*lpr* mice[119]. They determined that T cells from 7-week old MRL/*lpr* mice were reactive to a single epitope, peptide 131-151. These T cells expressed high levels of activation markers, including CD69 and CD44,

and produced IL-2 but not IFN-γ in response to the peptide. Peptide 131-151 was also recognized by IgG antibodies from MRL/*lpr* mice. T cells from NZB/W F1 mice were also later found to recognize the same peptide on U1-70K[120]. A modified version of U1-70K peptide 131-151, containing phosphorylated serine 140 (P140), was more immunogenic than unmodified U1-70K[121]. Administration of P140 intravenously in 4-week old MRL/*lpr* mice increased survival and reduced proteinuria, as well as anti-dsDNA antibody titers in mice[121]. U1-70K 131-151 peptide was also found to be recognized by T cells from patients with SLE or mixed connective tissue disease (MCTD)[122,123]. The phenotype of peptide 131-151-reactive T cells was identified by Kattah et al.[123], who showed that MRL/*lpr* T cells stimulated with peptide 131-151 expressed the Th17 lineage-specific transcription factor RAR-related orphan receptor gamma t (ROR-γt) and produced IL-17a. These studies led to P140 (pharmaceutical name: rigerimod or Lupuzor™) being tested as a treatment for SLE; it is currently in phase 3 clinical trials. Although the mechanism of action of the peptide was found to be more immunomodulatory rather than immunosuppressive in patients with SLE, the drug does show promise. P140 was found to inhibit uptake of autophagic cells by B cells, thereby reducing autoantigen processing and presentation to autoreactive T cells[118].

1.5.3.2 β2GPI-reactive T cells

Interest in β2GPI-reactive Th cells developed in the late 1990's to early 2000's[124-127], about 10 years after the discovery that β2GPI, and not phospholipid, was the antigen recognized by aPL. Specifically, anti-cardiolipin antibodies (anti-CL) subset of aPL were shown to react with β2GPI bound to cardiolipin or other anionic phospholipids[35,36]. Most published studies on human β2GPI-reactive T cells include both primary and secondary APS patients, as well as SLE patients without APS. One of the first studies on β2GPI-reactive T cells in SLE was published by Visvanathan et al.[125]. These authors studied the response of peripheral blood mononuclear cells (PBMCs) to native plasma-derived β2GPI in 24 aPL-positive (anti-CL or lupus anticoagulant positive) individuals, seven aPL-negative individuals with various autoimmune diseases (including SLE), and 15 healthy controls. PBMC responses to β2GPI were observed only in the aPL-positive group, and specifically in patients with APS (eight out of 18, four with SLE and

four with primary APS) and were characterized by a selective expansion of $CD4^+$ T cells producing IFN-γ, but not IL-4 (Th1-like response).

In later studies, the focus of studies on β2GPI-reactive T cells shifted to mapping their epitope specificity in patients with APS and SLE, as well as determining the HLA class II alleles associated with this response. Hattori et al.[126] studied the PBMC responses in patients with APS (five SLE, seven primary APS) and SLE patients without APS (*n*=13), as well as healthy controls (*n*=12). Most (91%) individuals with PBMC responses to β2GPI ("responders") expressed HLA-DR53-associated alleles (DRB1*04, *07, or *09), as compared to 47% of "non-responders". The domain specificity of the CD4 T cell proliferative response to recombinant β2GPI was assessed in six patients positive for anti-β2GPI antibodies (three primary APS, two SLE with APS, one SLE without APS), and all recognized an epitope within Domains IV and/or V. Patients with the DRB1*0901; DQB1*0303 haplotype also recognized an epitope within Domains III/IV, while T cells from patients not expressing this haplotype recognized only Domains IV/V. Finally, T cells from one primary APS patient recognized Domains I/II as well as Domain IV/V.

To further analyze the epitope specificity and functional capacity of the T cells in these patients, Arai et al.[128] generated CD4 T cell clones from three patients with APS (two primary APS, one SLE with APS). The majority (six out of seven) of the β2GPI-specific T cell clones recognized a peptide encompassing amino acid residues 276 to 290 (KVSFFCKNKEKKCSY) of β2GPI in the context of the DRB4*0103 allele (DR53). Interestingly, this peptide spans the major phospholipid-binding site of β2GPI. All of the β2GPI-reactive T cell clones produced IFN-γ and had a Th1-like cytokine expression profile. While the majority (10 of 12) of the β2GPI-specific T cell clones stimulated autologous peripheral blood B cells to produce anti-β2GPI antibodies *in vitro*, IFN-γ was not involved in B cell activation by these clones. Instead, stimulation was dependent on T cell production of IL-6 and CD40-CD40L interaction. Finally, Ito et al.[127] identified $CD4^+$ T cells from patients with SLE and secondary APS recognizing peptide 244-264 in Domain V. Peptide recognition was restricted to multiple HLA class II alleles including DRB1*04:03 and DRB4*01:03, and was also linked to a Th1-like phenotype. These

data indicate the presence of a possible HLA DR53-restricted immunodominant epitope on β2GPI in patients with APS and SLE that may be associated with development of disease.

Most β2GPI-reactive T cells derived from PBMCs do not respond to native β2GPI, but respond well to bacterially expressed recombinant β2GPI fragments and to chemically reduced β2GPI. These findings suggest that the generation of β2GPI T cell epitopes requires unfolding or structural modification of β2GPI. Kuwana et al.[129] demonstrated that anionic phospholipid may be involved in the generation of T cell epitopes (often referred to as "cryptic epitopes") not generated through processing of native β2GPI. They showed that DCs or macrophages pulsed with vesicles containing anionic phospholipid and β2GPI, but not β2GPI or phospholipid alone, induced a response in human T cell lines specific for the Domain V epitope (276-290) in an HLA-DRB4*0103-restricted manner. A later study showed that the same epitope can be generated *in vivo* by monocytes through FcγRI-mediated uptake of negatively charged particles (e.g., phosphatidylserine-containing vesicles or apoptotic cells) that have bound β2GPI in the presence of IgG anti-β2GPI antibodies[130]. β2GPI bound to OxLDL or activated platelets also induced β2GPI-specific T cell responses[130]. These data suggest that disease-relevant T cell epitopes in β2GPI may arise as a consequence of antigen processing of anionic phospholipid-bound β2GPI.

1.6 OBJECTIVES AND RATIONALE

SLE is a clinically heterogeneous autoimmune disease characterized by the presence of autoantibodies to multiple self-antigens and pathology involving different organ systems[7,12]. Despite the clinical heterogeneity of SLE and the multiple autoantigens targeted in SLE patients, autoantibodies emerge in a specific sequence, with anti-β2GPI being among the first to appear[12]. This finding raises a number of questions, including whether β2GPI-reactive T cells can promote the production of multiple SLE-related autoantibodies through a mechanism referred to as "epitope spread", and whether T cells targeting particular immunodominant epitopes within β2GPI are critical for the development of SLE. Investigating a role for β2GPI as an important autoantigen in SLE also addresses the question of the autoantigenic stimulus in SLE, which remains unclear. Dying cells represent an abundant source of self-antigens, and apoptosis has long been thought to play a key role in the pathogenesis of SLE[131]. However, apoptotic cells are usually anti-inflammatory and tolerogenic, making it unclear how these cells promote autoimmunity[132]. Necroptosis is a recently described form of regulated cell death associated with inflammatory pathologies, but little is known about necroptosis in SLE. Unlike apoptosis, cells rupture and release molecules that promote inflammation and immunogenicity[133]. Apoptotic cells have been shown to bind β2GPI, but it is not known whether necroptotic cells also bind this phospholipid-binding protein[134]. A link between β2GPI, necroptosis, and SLE induction would provide insight into whether necroptosis is implicated in SLE.

In our laboratory, we have previously shown that immunization of nonautoimmune mice with β2GPI, in the presence of LPS, induces the emergence of aPL and other SLE-related autoantibodies, as well as SLE-like disease[86]. These mice develop a strong and persistent T cell response to β2GPI[135]. However, little was known about the specificity of this β2GPI-reactive T cell response, such as whether the response is to a single epitope or to multiple distinct epitopes within β2GPI and whether these T cells are associated with disease pathogenesis. Moreover, β2GPI-reactive T cells have not been studied in other induced or spontaneous models of SLE, or comprehensively in patients with SLE.

We propose that development of a strong T cell response to β2GPI promotes epitope spread to multiple autoantibodies in SLE. We hypothesize that this T cell response is triggered by the presentation of β2GPI bound to necroptotic cells. Presentation of β2GPI in the context of pro-inflammatory signals should overcome immune tolerance and lead to epitope spread of the immune response to β2GPI. We further hypothesize that a limited number of MHC class II-restricted T cell epitopes within β2GPI promote the production of high levels of SLE-related autoantibodies and associated pathology.

The main objectives of my Ph.D. research were: (1) to determine the association of the β2GPI-reactive T cell response with SLE autoantibody development in mice bearing different MHC class II haplotypes; (2) to identify common β2GPI T cell epitopes in induced and spontaneous models of SLE; and (3) to determine whether β2GPI presented in the context of dying cells could provide the antigenic stimulus for the induction of SLE autoantibodies.

Table 1. American College of Rheumatology Criteria for the Diagnosis of Systemic Lupus Erythematosus (SLE).*

Criterion	Definition
Malar rash	A rash on the cheeks and nose, often in the shape of a butterfly
Discoid rash	A rash that appears as red, raised, disk-shaped patches
Photosensitivity	A reaction to sunlight that causes a rash to appear or get worse
Oral ulcers	Sores in the mouth
Arthritis	Joint pain and swelling of two or more joints
Serositis	Inflammation of the lining around the lungs (pleuritis) or inflammation of the lining around the heart that causes chest pain, which is worse with deep breathing (pericarditis)
Kidney disorder	Persistent protein or cellular casts in the urine
Neurologic disorder	Seizures or psychosis
Blood disorder	Anemia (low red-cell count), leukopenia (low white-cell count), lymphopenia (low level of specific white cells), or thrombocytopenia (low platelet count)
Immunologic disorder	Positive test for anti–double-stranded DNA, anti-Sm, or antiphospholipid antibodies
Abnormal antinuclear antibodies	Positive antinuclear-antibody test

* Four of the 11 criteria are needed for the formal diagnosis of SLE.

Figure 1.1: Table of American College of Rheumatology (ACR) criteria and definitions for the diagnosis of SLE. Diagnosis of SLE in patients requires the presence of at least four of the 11 criteria established by the ACR. Predominant criteria include the presence of a kidney disorder (lupus nephritis) and production of SLE-specific autoantibodies (classified as "an immunological disorder"). Tsokos, G.C. Systemic Lupus Erythematosus. *The New England Journal of Medicine* 2011, 365: 2110-2121[7]. Modified with permission from The New England Journal of Medicine, Copyright © 2011 Massachusetts Medical Society.

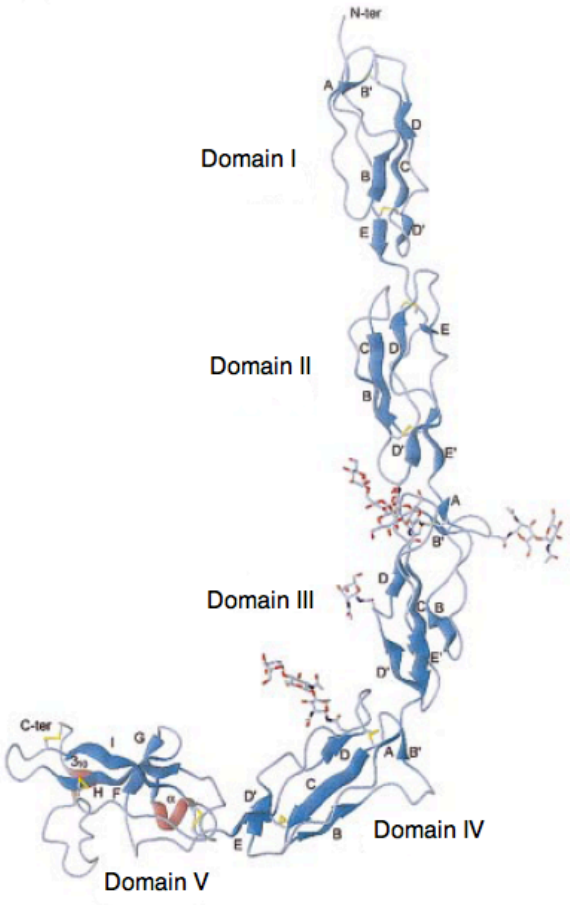

Figure 1.2. Ribbon structure of phospholipid-bound fish hook conformation of β2GPI, depicting Domains I-V. β2GPI consists of five highly conserved short sequence repeats (SCRs) known as Domains I-V. In the presence of anionic phospholipid, β2GPI is bound via its positively charged Domain V, leaving Domain I protruding away from the plasma membrane. This creates a fish-hook like structure. Modified from Schwarzenbacher, R., Zeth, K., Diederichs, K., Gries, A., Kostner, G. M., Laggner, P., and Prass, R. The crystal structure of human β2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. *The EMBO Journal* 1999, 18: 6228-6239[136]. Reproduced with permission from The EMBO Journal, Copyright © 1999 John Wiley and Sons.

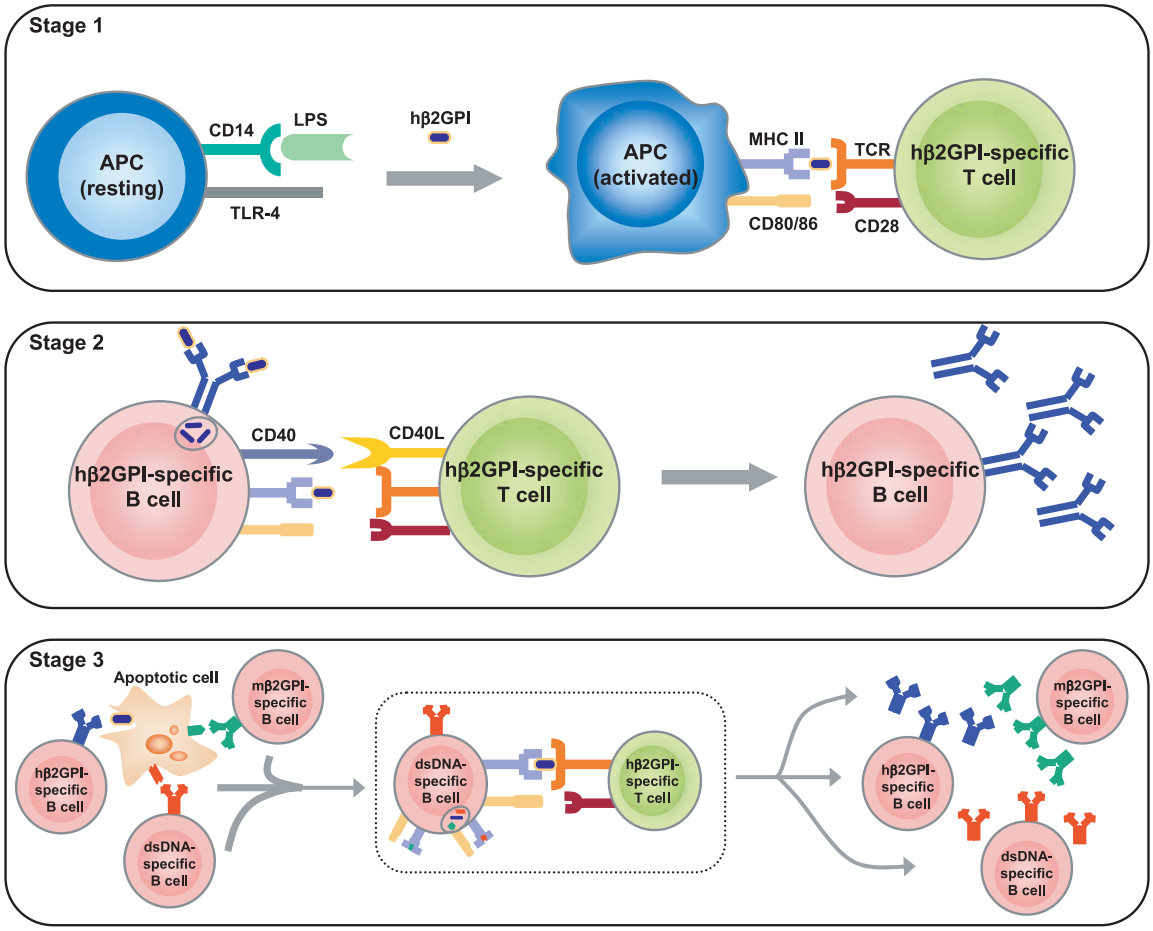

Figure 1.3. Proposed mechanism for epitope spread occurring in the β2GPI/LPS-induced model of SLE. In this induced model of SLE, mice are repeatedly immunized with an apoptotic cell-binding protein β2GPI and LPS, leading to production of multiple autoantibodies. The mechanism resulting in B cell epitope spread in this model is proposed to occur in three stages. *Stage 1:* Presentation of β2GPI by APCs is enhanced by LPS stimulation, which leads to a strong β2GPI-reactive T cell response. *Stage 2:* β2GPI-reactive T cells provide help to β2GPI-specific B cells and induce anti-β2GPI autoantibody production. *Stage 3:* B cells recognizing autoantigens that are expressed on the surface of apoptotic cells take up apoptotic cells, with β2GPI bound to their surface. β2GPI is presented on MHC class II molecules, enabling β2GPI-reactive T cells to provide help to these autoantigen-specific B cells and produce autoantibodies (e.g., anti-dsDNA). Modified from Levine, J., Subang, R., Nasr, S., Fournier, S., Lajoie, G., Wither, J., and Rauch, J. Immunization with an apoptotic cell-binding protein recapitulates the nephritis and sequential autoantibody emergence of systemic lupus erythematosus. *The Journal of Immunology* 2006, 177: 6504–6516[86]. Reproduced with permission from The Journal of Immunology, Copyright © 2006 American Association of Immunologists, Inc.

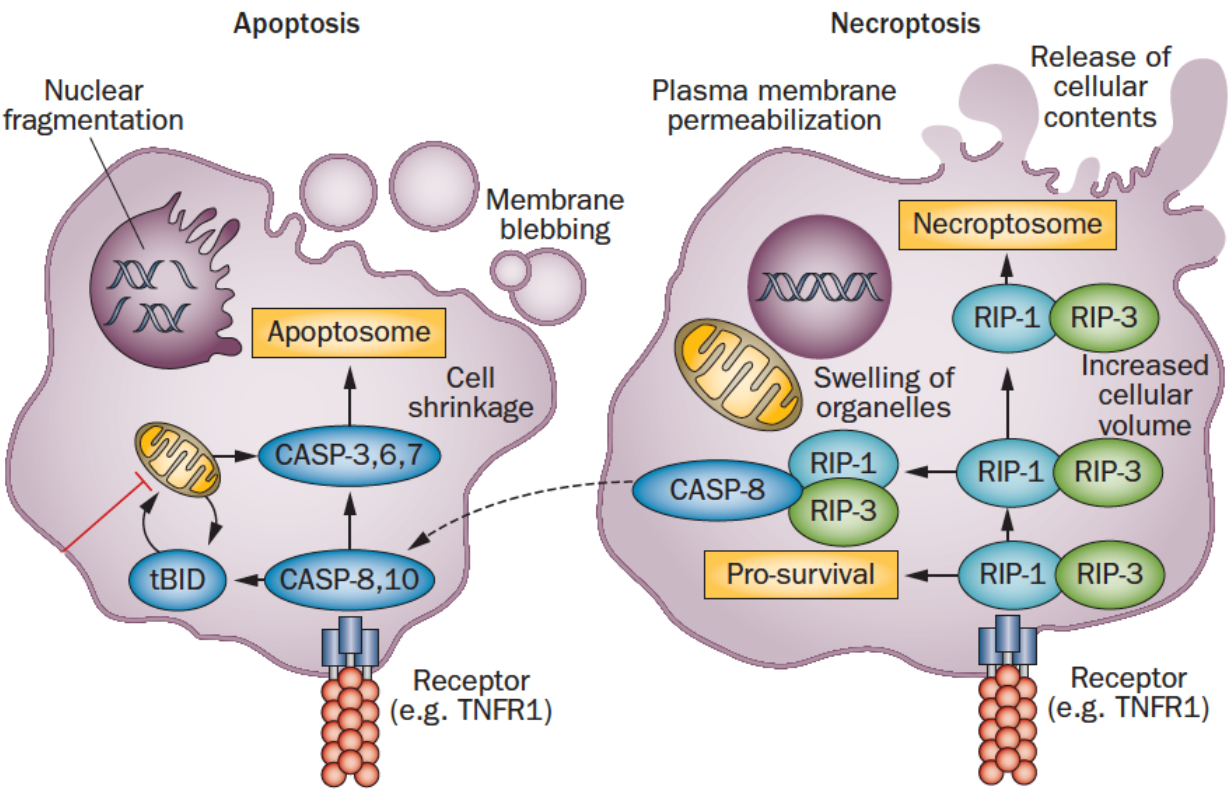

Figure 3. Apoptosis and necroptosis: morphology and signaling pathways. Apoptosis and necroptosis can both be triggered by activation of TNFR1. During apoptosis, the initiator caspase-8 is activated, and it activates the effector caspases-3, 6, and 7. This activation induces nuclear condensation, exposure of anionic phospholipids in the outer membrane, cell shrinkage, and blebbing of the plasma membrane, resulting in apoptotic bodies. When cytochrome c is released by mitochondrial damage, association with apoptotic protease activating factor-1 (Apaf-1) forms the apoptosome and can also initiate apoptosis. When caspase-8 is inhibited, necroptosis is initiated, which allows for the oligomerization and cross-phosphorylation of RIPK1 and RIPK3 kinases, forming the necroptosome (or necrosome). RIPK1/RIPK3 phosphorylate and activate MLKL, which migrates to the surface and induces rupturing of the plasma membrane. Increased osmotic pressure creates swelling of the cell and organelles, and eventual release of cellular contents through necrosis. Modified from Wree, A., Broderick, L., Canbay, A., Hoffman, H. M. and Feldstein, A.E. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. *Nature Reviews Gastroenterology & Hepatology* 2013, 10:627-636[137]. Reproduced with permission from Nature Reviews Gastroenterology & Hepatology, Copyright © 2013 Macmillan Publishers Limited.

CHAPTER 2

β2-GLYCOPROTEIN I-SPECIFIC T CELLS PROMOTE EPITOPE SPREAD TO LUPUS-RELATED AUTOANTIBODIES

2.1 PREFACE

In Chapter 2 of this thesis, we evaluate the epitope specificity of β2GPI-reactive T cells from different MHC class II haplotypes with induced SLE. We first looked at the epitope specificity using recombinant proteins containing different β2GPI domains in a β2GPI-reactive T cell hybridoma generated from B6 mice with induced SLE. Findings revealed that the T cell hybridoma recognized an epitope on Domain I-II of β2GPI. Therefore, a peptide library encompassing the Domain I-II region was generated and subsequent epitope mapping of *ex vivo* T cells was done using this library. We examined whether a unique or limited β2GPI-reactive T cell response was associated with epitope spread to SLE autoantibodies. Furthermore, we evaluated the β2GPI epitope specificity of T cells isolated from a patient with autoimmune disease.

β2-GLYCOPROTEIN I-SPECIFIC T CELLS ARE ASSOCIATED WITH EPITOPE SPREAD TO LUPUS-RELATED AUTOANTIBODIES

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Running title: T cells and epitope spread to lupus-related autoantibodies

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Abbreviations: anti-CL, anti-cardiolipin; anti-β2GPI, anti-β2-glycoprotein I; anti-dsDNA, anti-double stranded DNA; aPL, anti-phospholipid antibodies; β2GPI, β2-glycoprotein I; SLE, systemic lupus erythematosus.

Keywords: animal model; autoimmune disease; epitope mapping; major histocompatibility complex (MHC); T-cell; β2-glycoprotein I; autoantibodies; epitope spread; systemic lupus erythematosus.

Background: Systemic lupus erythematosus (SLE)-related autoantibodies are of unknown origin, but target multiple apoptotic cell-derived antigens.
Results: T cell responses to multiple epitopes on β2-glycoprotein I (β2GPI), an apoptotic cell-binding protein, were associated with SLE-related autoantibody production.
Conclusion: Distinct β2GPI-reactive T cell responses are associated with SLE-related autoantibodies.
Significance: Factors enabling β2GPI-reactive T cell responses may predispose individuals to SLE.

2.2 ABSTRACT

Systemic lupus erythematosus (SLE) is a prototypic model for B cell epitope spread in autoimmunity. Autoantibodies to numerous and molecularly distinct self-antigens emerge in a sequential manner over several years, leading to disease manifestations. Among the earliest autoantibodies to appear are those targeting the apoptotic cell-binding protein β2-glycoprotein I (β2GPI). Notably, mice immunized with β2GPI and lipopolysaccharide (LPS) display a remarkably similar pattern of autoantibody emergence to that seen in human SLE. Here we use this model to investigate whether epitope spread to SLE-related autoantibodies is associated with a unique or limited β2GPI-specific T cell response. We ask whether MHC class II haplotype, and its associated T cell epitope restriction, impacts epitope spread to SLE-related autoantibodies. We found that β2GPI/LPS-immunized mice produced similar SLE-related autoantibody profiles regardless of their β2GPI T cell epitope specificity or MHC class II haplotype. While β2GPI T cell epitope specificity was clearly determined by MHC class II haplotype, a number of different β2GPI T cell epitopes were associated with epitope spread to SLE-related autoantibodies. Notably, one β2GPI T cell epitope (peptide 23 [NTGFYLNGADSAKCT]) was also recognized by T cells from an HLA-DRB1*0403^{+} autoimmune patient. These data suggest that the generation of a β2GPI-reactive T cell response is associated with epitope spread to SLE-related autoantibodies, independent of epitope specificity or MHC class II restriction. Based on these findings, we propose that factors enabling a β2GPI-reactive T cell response may predispose individuals to the development of SLE-related autoantibodies independent of their MHC class II haplotype.

2.3 INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease in which autoantibodies to self-antigens, particularly cellular components, appear in a consistent and sequential pattern[1,2]. Autoantibodies to the plasma protein, β2-glycoprotein I (β2GPI), either alone (called "anti-β2GPI") or bound to anionic phospholipid (called "anti-cardiolipin [anti-CL]") are among the earliest to appear. Other SLE-associated autoantibodies, such as anti-double stranded DNA

(anti-dsDNA), anti-Sm, and anti-nuclear ribonucleoprotein antibodies, emerge somewhat later[1,3]. The accumulation of multiple diverse SLE-related autoantibodies leads eventually to clinically evident disease[1].

While much is known about the targets and specificities of SLE-related autoantibodies, far less is understood about their etiology. Antibodies to β2GPI are generally included in the category of "anti-phospholipid antibodies (aPL)", and are present in approximately 20-30% of patients with SLE. Patients who are aPL-positive not only develop other SLE-related autoantibodies earlier than aPL-negative individuals, but also appear to have a more severe clinical outcome[3]. The early appearance of aPL, and their association with a premature onset of other SLE-related autoantibodies, suggests that these autoantibodies, or the mechanism leading to their formation, may be an initiating event for epitope spread to multiple other autoantibodies in SLE.

Consistent with this theory, we have shown that nonautoimmune mice immunized with β2GPI, in the presence of a strong innate immune activator (e.g., lipopolysaccharide [LPS]), produce SLE-related autoantibodies in a sequential manner recapitulating that seen in human SLE and develop overt SLE-like glomerulonephritis[4]. We have proposed that the strong and persistent T cell response to β2GPI observed in these mice[5] is responsible for B cell epitope spread to multiple SLE-related autoantibodies[4]. β2GPI binds to apoptotic cells[6], which express many SLE-associated autoantigens[7,8], and it is this property of β2GPI that we believe underlies the ability of β2GPI-specific T cells to promote inter-molecular spread to other SLE autoantigens[4,9].

Here, we take advantage of the influence of MHC class II background on T cell epitope specificity to test the hypothesis that generation of a β2GPI-specific T cell response enables epitope spread to SLE-related antibodies. Using our model of induced SLE, we first produced a strong T cell response to β2GPI in several nonautoimmune murine strains of varying MHC class II haplotype. We then determined the epitope specificity of the resulting β2GPI-reactive T cell response, and whether MHC class II haplotype and its associated β2GPI T cell epitope restriction

impacted epitope spread to SLE-related autoantibodies. Finally, we investigated whether β2GPI T cell epitopes are shared between murine and human individuals.

Our findings demonstrate that a T cell response to β2GPI alone is associated with B cell epitope spread to SLE-related autoantibodies. Although the epitope specificity of the β2GPI-specific T cell response was determined by the individual's MHC class II haplotype, multiple β2GPI T cell epitopes were associated with the production of SLE-related autoantibodies. One β2GPI T cell epitope was shared by both H-2^b-bearing mice and an HLA-DRB1*0403^+ autoimmune patient, suggesting that the induced β2GPI-specific T cell response mimics that in autoimmune disease. Together, our data indicate that B cell epitope spread to SLE-related autoantibodies can occur in the context of multiple MHC class II haplotypes and their correspondingly restricted T cell epitopes. We propose that generation of a β2GPI-reactive T cell response may represent a critical initiating event permitting B cell epitope spread and leading ultimately to the production of the full range of SLE-related autoantibodies.

2.4 EXPERIMENTAL PROCEDURES

2.4.1 Mice and immunization

Specific pathogen-free female C57BL/6 and BALB/c mice (8-12 weeks of age) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Female C3H/HeN and some BALB/c mice were generously provided by Dr. Salman Qureshi and Dr. Samuel David, respectively. Female 129S1/SvImJ, B6.C-H2^d/bByJ, and C.B10-H2^b/LilMcdJ mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Mice were maintained and bred according to Canadian Council on Animal Care (CCAC) guidelines, and maintained on food and water *ad libitum*. Animal experiments were approved by the McGill University Animal Care Committee.

C57BL/6, BALB/c, and C3H/HeN mice were immunized with 20 μg β2GPI and 10 μg LPS, as described previously[5]. Mice were injected every two weeks, and bled for serum 10 days following the second and third immunizations. For the T cell studies, the number of

immunizations required was determined by the levels of anti-β2GPI antibodies observed. C57BL/6 mice received four immunizations (fourth immunization was with half the dose of β2GPI and LPS); BALB/c mice received three immunizations; and C3H/HeN mice received two immunizations. C3H/H3N mice were immunized only twice as they did not survive a third immunization. Their antibody levels (post-second immunization) were similar to those of C57BL/6 and BALB/c mice following three immunizations. Two (of four) 129S1 mice died after the third immunization; the post-second immunization serum was used for those mice. Premature death in the C3H/HeN and some 129S1 mice may have been due to an accelerated antibody response to the immunogen.

2.4.2 Reagents

Unless stated otherwise, all reagents were obtained commercially from the following sources and used without further purification: human β2GPI (≥95% pure; Crystal Chem, Downers Grove, IL); LPS (*E. coli*-derived, serotype O111:B4) (List Biological Laboratories, Campbell, CA); bovine heart CL (Avanti Polar Lipids, Alabaster, AL); *Escherichia coli* DNA (dsDNA) (Worthington Biochemical Corporation, Lakewood, NJ); Ro (SS-A), La (SS-B), Smith antigen (Sm), and nuclear ribonucleoprotein (nRNP) from Immunovision (Springdale, AR); recombinant IL-2, rat anti-mouse IL-2, biotinylated rat anti-mouse IL-2, mouse interferon-γ (IFN-γ) ELISA set (BD OptEIA kit), 3,3',5,5'-Tetramethylbenzidine (TMB) substrate reagent set (BD OptEIA kit), and hamster anti-mouse CD3e from BD Biosciences (Mississauga, ON); alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG and AP-conjugated streptavidin from Southern Biotech (Birmingham, AL); and p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO).

2.4.3 β2GPI recombinant fragments and synthetic peptides

Recombinant maltose-binding protein (MalBP) fusion proteins encoding the following regions of human β2GPI were used as antigens for T cell stimulation, as previously described[10]. These fusion proteins, which have been described previously[11] included (Figure 1A): GP-F, encoding the entire amino acid sequence of β2GPI (amino acid residues 1-326); GP-1, encoding

Domains I and II (amino acid residues 1-133); GP-2, encoding Domains III and IV (amino acid residues 119-254); and GP-3, encoding Domains IV and V (amino acid residues 182-326). MalBP was also prepared and used as a control antigen.

Twenty-six 15-mer peptides, with 10-residue overlap, spanning domains I and II of human β2GPI were synthesized and their purity determined by high performance liquid chromatography (Sigma-Aldrich). The peptides were dissolved in 200 µl of dimethyl sulfoxide (DMSO), and further diluted in 0.01 M phosphate-buffered saline, pH 7.3 (PBS) in 500 µl stocks. Peptide stock solutions in DMSO and PBS were stored at -70°C. The peptides were added to the antigen presentation assays described below at a final concentration of 10 µg/ml in PBS.

2.4.4 Cell culture

Unless stated otherwise, all cells were cultured in DMEM medium (4.5 g/L glucose, 110 mg/mL sodium pyruvate), containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% L-glutamate, 1% HEPES, 1% non-essential amino acids, and 0.1% 2-mercaptoethanol (medium and supplements were from Life Technologies Inc., Burlington, ON), hereafter referred to as complete DMEM. Splenic T cells from immunized mice were isolated using an EasySep T cell kit (StemCell Technologies, Vancouver, BC) and were cultured in complete DMEM containing β2GPI-depleted FBS. FBS was depleted of β2GPI using a HiTrap Heparin HP column (GE Healthcare, Piscataway, NJ) to eliminate the potential influence of bovine β2GPI. β2GPI-depleted FBS was unable to support binding of a bovine β2GPI-dependent murine monoclonal antibody to cardiolipin by ELISA (data not shown). Human T cell clones were cultured in RPMI 1640 supplemented with 10% β2GPI-depleted FBS, 2 mM L-glutamine, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 50 U/mL penicillin, and 50 µg/mL streptomycin at 37ºC (5% CO_2).

2.4.5 Generation of β2GPI-specific T cell hybridoma (C3hB-1.5)

The C3hB-1.5 T cell hybridoma was generated from a C57BL/6 mouse that had received four biweekly intravenous immunizations with human β2GPI (20 µg) on Day 1 and LPS (10 µg)

on Day 2, as previously described[4,5], and a fifth injection of β2GPI alone (20 μg) two weeks prior to the fusion experiment. Isolated splenic CD4+ T cells (EasySep T cell kit) were plated at 10^6 cells/well, and incubated with human β2GPI (15 μg/ml) (37°C, 10% CO2) in the presence of naive C57BL/6 splenocytes (4 x 10^6 cells/well) as APCs. IL-2 (20 μg/ml) was added to the culture on Day 5. On Day 11, the cultured T cells (2.9 x 10^6) were fused with 10^7 BWα-/β-cells, as previously described (12). The resulting hybridomas were screened against human β2GPI and human serum albumin (HSA). T cell hybridomas that responded to human β2GPI, but not HSA, were kept and subcloned by limiting dilution. C3hB-1.5 is a subclone that responded strongly to human β2GPI, but showed no response to HSA.

2.4.6 Domain and epitope specificity of β2GPI-specific T cell hybridoma (C3hB-1.5)

Domain specificity of the C3hB-1.5 T cell hybridoma was determined using recombinant protein fragments of human β2GPI (GP-1, GP-2, GP-3, and GP-F), or MalBP as a negative control. Twenty microliters of recombinant protein fragments (10 μg/ml in PBS, final concentration), or commercial human β2GPI (10 or 20 μg/ml, final concentration), were added to murine APCs (C57BL/6 splenocytes; 2 x 10^6 cells/mL; 50 μl/well) in triplicate wells. C3hB-1.5 T cell hybridoma cells (2 x 10^6 cells/mL; 50 μl/well) were then added to the wells. C3hB-1.5 T cell hybridoma cells stimulated with anti-mouse CD3e antibody (5 μg/mL in PBS) served as a positive control. Supernatants were screened for IL-2 by ELISA, as previously described[13]. Results are expressed as the mean IL-2 concentration (ng/ml) of triplicate samples, as determined from a standard curve using recombinant IL-2.

The epitope specificity of the C3hB-1.5 T cell hybridoma was determined using the assay described above for domain specificity, except that peptides (10 μg/ml, final concentration) were used in place of recombinant fragments.

2.4.7 Domain and epitope specificity of T cells from immunized mice

Strain-matched APCs (splenocytes from naïve C57BL/6, BALB/c, or C3H/HeN mice) were plated at 4 x 10^6 cells/well in complete DMEM containing β2GPI-depleted FBS. Recombinant

protein fragments of β2GPI or MalBP, commercial β2GPI, or Domain I-II peptides were added to the APCs in duplicate. Then, splenic T cells (10^6 cells/well) isolated from immunized C57BL/6, BALB/c, or C3H/HeN mice were added to the culture, and incubated for 48 hours (37°C, 5% CO_2). T cells stimulated with phorbol 12-myristate 13-acetate (PMA) (0.02 μg/ml in PBS) and ionomycin (1 μM in PBS) served as a positive control for maximal T cell activity. Cell supernatants were collected, and IFN-γ levels were quantified by ELISA. Results were expressed as the mean IFN-γ concentration (pg/ml) of duplicate samples, as determined from a standard curve using recombinant IFN-γ.

2.4.8 Detection of autoantibodies

Anti-β2GPI, anti-CL, anti-dsDNA, anti-Ro (SS-A), anti-La (SS-B), anti-Sm, and anti-nRNP antibodies were determined by ELISA, as previously described[4]. Antibodies to domains of β2GPI (anti-β2GPI domain antibodies) were determined in mouse sera by the following ELISA. ELISA-HB (high binding) plates (Greiner Bio-One, Monroe, NC) were coated with recombinant protein fragments of β2GPI (10 μg/ml in PBS with 0.02% azide [PBS-azide]) for 16 hours at 37°C. The coated plates were blocked with PBS-azide containing 0.5% gelatin and 10% FBS for 2 hours at 4°C, and washed three times with 0.01 M Tris-buffered saline, pH 7.4 (TBS). Sera were diluted 1/100 (unless otherwise noted) in PBS-azide containing 0.3% gelatin and 10% FBS (PBS-0.3% gelatin-10% FBS), and incubated in duplicate in coated wells for 3 hours at 25°C. Following three washes with TBS, AP-conjugated goat anti-mouse IgG, diluted 1/1000 in PBS-azide containing 0.4 % bovine serum albumin [BSA]), was added and incubated for 16 hours at 4°C. Plates were washed with TBS, developed with p-nitrophenol phosphate, and the optical density at 405 nm (OD_{405}) read using an ELISA reader (Bio-Tek Instruments Inc., Winooski, VT). Murine hybridoma anti-β2GPI antibodies (with known domain specificity) served as positive controls.

Anti-β2GPI domain antibodies in patient serum were measured using an ELISA in which CL-coated plates were pre-incubated with purified human native β2GPI, GP-F, GP-1, GP-2, GP-3, and MalBP, as described previously (14). Serum samples, diluted at 1:100, were tested in triplicate, and the values represent the mean OD_{405} ± SD.

2.4.9 β2GPI-reactive human T cell clones

All patient samples were obtained following written informed consent, as approved by the Keio University Institutional Review Board. Human $CD4^+$ T cell clones (OM3, OM9, and OM13) reactive with GP-1 (Domains I and II) of β2GPI were derived from a patient (OM) with primary anti-phospholipid syndrome (APS). These T cell clones were generated from peripheral blood T cells by repeated stimulation with GP-F followed by limiting dilution, as described previously[10], and were selected based on specific recognition of GP-1 in a HLA-DR-restricted manner and availability. The HLA-DR alleles of the patient OM include DRB1*1502, DRB1*0403, DRB4*0103, and DRB5*0101.

2.4.10 T cell proliferation assay

Antigen-specific T cell proliferation in peripheral blood T cells and β2GPI-reactive $CD4^+$ T cell clones was assayed as described previously[10,11]. For peripheral blood T cells, peripheral blood mononuclear cells (PBMCs), isolated from heparinized venous blood by Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) density-gradient centrifugation, were cultured with or without antigen in 96-well flat-bottomed culture plates for 7 days. GP-F, GP-1, GP-2, GP-3, MalBP, and tetanus toxoid (List Biological Laboratories, Campbell, CA) were used as antigens at a concentration of 5 μg/ml. Phytohemagglutinin (1 μg/ml) was used to ensure that the T cells were responsive. The T cell clones were cultured with irradiated autologous Epstein Barr virus-transformed B cells and antigen, including GP-F, GP-1, GP-2, GP-3, MalBP, and a series of synthetic peptides covering Domain I-II of β2GPI (5 μg/mL) for 3 days. L cells transfected with the DRA gene and one of the following DRB genes (DRB1*1501 [LDR2B)]; DRB1*0403 [B19]; DRB4*0103 [L17.8]; and DRB5*0101 [LDR2A]) were used as APCs in place of B cells to evaluate HLA-DR restriction. L cells were irradiated and incubated with synthetic peptides (5 μg/mL) for 2 hours before mixing with T cell clones. ^{3}H-thymidine (0.5 μCi/well) was added to the cultures during the final 16 hours of incubation. The cells were harvested, and ^{3}H-thymidine incorporation was measured in a Top-Count microplate scintillation counter (Packard, Meriden,

CT). All cultures were performed in triplicate, and values represent the mean of triplicate determinations.

2.4.11 Statistical analysis

Statistical significance was determined by a two-tailed unpaired non-parametric Mann-Whitney test using Prism 6.0 (GraphPad Software Inc., San Diego, CA). The minimal threshold for significance was $p < 0.05$.

2.5 RESULTS

2.5.1 A β2GPI-reactive T cell hybridoma from C57BL/6 (H-2^b) mice recognizes a peptide (23) from Domain II of β2GPI

We have previously shown that C57BL/6 mice immunized with β2GPI and LPS produce a strong T cell response to β2GPI[5]. As the first step to investigating the domain and epitope specificity of this T cell response, we evaluated a β2GPI-reactive T cell hybridoma (C3hB-1.5) derived from β2GPI/LPS-immunized C57BL/6 mice. Domain specificity was evaluated using recombinant protein fragments of human β2GPI: GP-1 (Domains I and II); GP-2 (Domains III and IV); and GP-3 (Domains IV and V) (Figure 1A). Full-length recombinant β2GPI (GP-F) served as a positive control, and the control fusion protein (MalBP) as a negative control. The β2GPI-specific T cell hybridoma (C3hB-1.5) recognized recombinant fragment GP-1 exclusively (Figure 1B), indicating recognition of a peptide within Domains I and II. Next, T cell hybridoma C3hB-1.5 was screened with a peptide library (26 15-mer peptides) that spanned the entire sequence of Domains I and II. The T cell hybridoma recognized a single peptide (#23 [NTGFYLNGADSAKCT]) located in Domain II of β2GPI (Figure 1B).

2.5.2 β2GPI/LPS-immunized C57BL/6 (H-2^b) mice show a dominant T cell response to a single peptide (23) from Domain II of β2GPI

We next investigated whether the domain and epitope specificity of the C57BL/6-derived β2GPI-specific T cell hybridoma (C3hB-1.5) is representative of primary T cells from these mice. Splenic T cells were isolated from C57BL/6 (H-2^b) mice immunized with human β2GPI and LPS, and evaluated for their response to human β2GPI or the different recombinant fragments of human β2GPI. C57BL/6-derived T cells showed strong recognition of GP-1, similar to that of the C3hB-1.5 T cell hybridoma, and also recognized GP-2 and GP-3 (Figure 2A). These T cells also responded to serum-derived human β2GPI and recombinant full-length β2GPI (GP-F), but not to the control recombinant protein MalBP. Since C57BL/6-derived T cells recognized GP-1, their epitope specificity was evaluated using the same peptide library comprising the GP-1 sequence as we used to evaluate the C3hB-1.5 T cell hybridoma. Similar to the C3hB-1.5 T cell hybridoma, T cells from β2GPI/LPS-immunized C57BL/6 mice recognized a single peptide (peptide 23) (Figure 2A). These findings suggest that C57BL/6 (H-2^b) mice immunized with β2GPI and LPS have a dominant T cell response to peptide 23 within Domain II of β2GPI.

2.5.3 Domain and epitope specificities of the β2GPI-reactive T cell response vary with MHC class II haplotype

To determine whether the T cell response to β2GPI is affected by MHC class II haplotype, we investigated the epitope specificity of the β2GPI-specific T cell response in mice with haplotypes other than H-2^b. Like C57BL/6 (H-2^b)-derived T cells, T cells from β2GPI/LPS-immunized BALB/c (H-2^d) mice recognized all recombinant fragments of β2GPI equally and responded to a single peptide within GP-1 (Figure 2B). However, T cells from BALB/c (H-2^d) mice responded to a different peptide within Domain I, peptide 7 (FSTVVPLKTFYEPGE), than that recognized by C57BL/6-derived T cells. We next evaluated T cells from β2GPI/LPS-immunized mice with a third haplotype (H-2^k). C3H/HeN (H-2^k) mice responded strongly to GP-2, but showed a minimal response to GP-3 and no response to GP-1 (Figure 2B). Consistent with their lack of response to GP-1, C3H/HeN-derived T cells did not recognize any peptides from GP-1 (Figure 2B). These data demonstrate that mice with different MHC class II haplotypes all produced a strong T cell response to β2GPI, but the domain and epitope specificity of the T cell response varied among these strains.

2.5.4 β2GPI T cell epitope specificity segregates with MHC class II haplotype

We wondered whether the differences in epitope specificity between β2GPI-reactive T cells from different mouse strains was determined by MHC class II haplotype or by other genetic differences between these strains. To ensure that MHC class II haplotype was the only variable assessed in these experiments, we used congenic strains of mice differing solely in MHC class II haplotype. To complement our earlier experiments, we selected mice in which the C57BL/6 (H-2^b) and BALB/c (H-2^d) MHC class II haplotypes had effectively been swapped: B6.C (C57BL/6 background with H-2^d haplotype) and C.B10 (BALB/c background with H-2^b haplotype). In both strains, T cell domain and epitope specificity was strikingly associated with MHC class II haplotype. Like BALB/c-derived T cells, T cells from β2GPI/LPS-immunized B6.C (H-2^d) mice recognized GP-1 (Domains I and II), and peptide 7 within Domain I (Figure 3A). Similarly, T cells from C.B10 (H-2^b) mice showed domain and epitope specificity resembling that seen in C57BL/6 mice (H-2^b). Like C57BL/6-derived T cells, C.B10-derived T cells recognized all domains of β2GPI, with specific recognition of peptide 23 within Domain II (Figure 3A). We also evaluated T cells from 129S1 mice, which have the same MHC class II haplotype (H-2^b) as C57BL/6 mice, but are otherwise unrelated to this strain. Similar to the other $H2^b$-bearing murine strains, T cells from β2GPI/LPS-immunized 129S1 mice showed epitope specificity for peptide 23 (Figure 3B). However, unlike C57BL/6- and C.B10-derived T cells, 129S1-derived T cells also recognized peptide 6 in Domain I of β2GPI. Together, these data demonstrate that β2GPI T cell epitope specificity is strikingly associated with MHC class II haplotype, but do not rule out a contribution from non-MHC class II genes within the MHC complex, such as those involved in antigen processing and presentation.

2.5.5 Multiple distinct MHC class II-restricted β2GPI T cell epitopes are associated with B cell epitope spread to SLE autoantibodies

As the specificity of the β2GPI T cell response differed between mice of different MHC class II haplotypes, we wondered whether the difference in T cell specificity and MHC class II haplotype would impact B cell epitope spread to SLE-related autoantibodies. We compared the

induction of SLE-related IgG autoantibodies in mice with different β2GPI T cell epitopes and MHC class II haplotypes: C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H/HeN (H-2^k). We first evaluated the antibody response to the immunizing antigen, human β2GPI, either alone (anti-β2GPI) or bound to CL (anti-CL). Notably, β2GPI/LPS-immunized mice from all three strains produced high levels (>1/1000 dilution) of anti-β2GPI ($p < 0.03$) and anti-CL ($p < 0.03$) antibodies, compared to LPS-immunized mice (Figure 4A). However, antibody titers differed among strains, particularly in comparing C3H/HeN mice with the other two strains. Following only two immunizations with β2GPI and LPS, C3H/HeN mice had antibody levels that were similar to those of C57BL/6 and BALB/c mice after three immunizations (Figure 4A). Between C57BL/6 and BALB/c mice, autoantibody titers were generally higher in BALB/c mice following three immunizations (Figure 4), but we have previously shown that these two strains produced similar levels of autoantibodies over the full course of immunization[4].

We next looked at whether the β2GPI/LPS-immunized mice developed other SLE-related autoantibodies (Figure 4B). Anti-dsDNA antibodies, which are considered highly specific for SLE, were found in all strains but C.B10 mice. Levels varied among strains, but were significantly elevated compared to the LPS-immunized strain controls in all strains except 129S1 ($p < 0.008$). In 129S1 mice, variability in the data shown in Figure 4B is due to the use of sera from different bleeds (post-second or post-third immunization), but both post-third immunization bleeds had elevated levels of anti-dsDNA antibodies (OD_{405}: 0.36, 1.17 versus 0.11 for the control). All strains had significantly elevated levels of anti-Ro (SS-A) antibodies ($p < 0.03$), and most (except CB.10, 129S1, and B6.C) had significantly elevated levels of anti-La (SS-B) antibodies ($p < 0.008$). Anti-Sm and anti-nRNP antibodies were observed in all strains except CB.10 and 129S1 ($p < 0.008$ for anti-Sm; $p < 0.02$ for anti-nRNP) (Figure 4B).

Together, these data indicate that multiple murine strains, despite having different β2GPI T cell epitope specificities and MHC haplotypes, all produce SLE-related autoantibodies following immunization with β2GPI/LPS. Inter-molecular B cell epitope spread occurred in all strains, suggesting that a strong β2GPI-specific T cell response, independent of its epitope specificity or MHC class II restriction, can support B cell epitope spread in this model.

To determine whether "intra-molecular B cell epitope spread" (i.e., between epitopes within β2GPI) had also occurred in these mice, we evaluated whether antibodies to β2GPI domains other than those recognized by the T cells were present (Figure 5). This is clearest among strains that did not show T cell responses to all domains. For example, B6.C (H-$2^{d.}$) mice, which had a T cell response predominantly to GP-1, produced antibodies to GP-2 and GP-3. Similarly, C57BL/6 (H-2^{b}) mice had a strong T cell response to GP-1, but the focus of the B cell response was to GP-2 and GP-3. These data indicate that intra-molecular, as well as inter-molecular, B cell epitope spread occurred in the presence of a β2GPI-specific T cell response across different MHC class II haplotypes.

2.5.6 β2GPI-reactive $CD4^{+}$ T cell clones derived from an autoimmune patient recognize the same peptide as H-2^{b}-bearing mice

To address whether the T cell epitope response that we observed in β2GPI/LPS-immunized mice also occurs in human autoimmune disease, primary cells (PBMCs) from a patient (OM) with APS were evaluated for proliferation to the same recombinant fragments of β2GPI. The clinical characteristics of this patient have been described previously[10]. T cells and antibodies from this patient were strongly reactive to GP-1, as well as GP-3 (11), but minimally reactive with GP-2 (Figures 6A and B). β2GPI-reactive $CD4^{+}$ T cell clones from this patient were used for epitope mapping. Two of the clones (OM9 and OM13) specifically recognized peptide 23 in Domain II, similarly to splenic T cells from C57BL/6 mice (H-2^{b}) (Figures 7A and B). To identify the HLA-DR molecules that present peptide 23 to these T cell clones, L cell transfectants expressing single HLA-DR molecules were used as APCs (Figure 7C and D). Both OM9 and OM13 responded to peptide 23 presented selectively by DRB1*0403^{+} L cells. These findings indicate that peptide 23 is a dominant epitope within Domain II of β2GPI for β2GPI-reactive T cells from this APS patient. The fact that the same T cell epitope specificity occurs as an induced response in H-2^{b}-bearing mice and spontaneously in an HLA-DRB1*0403^{+} autoimmune patient suggests that the induced β2GPI-specific T cell response in our model mimics that in autoimmune disease.

2.6 DISCUSSION

We have previously proposed that development of a β2GPI-reactive T cell response is a critical early event in the initiation of SLE-like autoantibodies and subsequent disease in mice immunized with β2GPI and LPS[4,5]. Here, we test the hypothesis that generation of a β2GPI-directed T cell response, independent of its epitope specificity, enables B cell epitope spread to SLE-related antibodies. We demonstrate that mice with different MHC class II haplotypes are all capable of developing a strong T cell response to β2GPI, as well as antibodies to β2GPI, cardiolipin, Ro (SS-A), and, in most cases, anti-dsDNA. Although T cells from strains with different MHC class II haplotypes displayed very different epitope specificities for β2GPI, the autoantibody profiles of MHC-differing mice looked similar. T cells from β2GPI/LPS-immunized H-2^b-bearing mice recognized a peptide (NTGFYLNGADSAKCT; peptide 23) within Domain II., while T cells from H-2^d-bearing mice responded to a peptide (FSTVVPLKTFYEPGE; peptide 7) within Domain I. T cells from mice of a third MHC class II haplotype (C3H/HeN mice [H-2^k]) failed to recognize either Domain I or II. Overall, our data demonstrate that SLE-related autoantibodies emerge in mice having a strong β2GPI-reactive T cell response, irrespective of MHC class II haplotype and corresponding T cell epitope specificity.

Our findings imply that, regardless of epitope specificity, β2GPI-reactive T cells are able to provide the T cell help needed for B cell epitope spread to multiple SLE-related autoantibodies. In mice, the autoantibody response to β2GPI, in the presence of a potent innate stimulus (LPS), occurs within weeks after the first immunization with β2GPI[4]. However, the production of anti-CL, anti-dsDNA, and other SLE-related autoantibodies requires multiple immunizations with β2GPI and LPS[4]. In the current study, the number of immunizations was determined by the titer of anti-β2GPI antibodies. Once a relatively high titer (>1/5000) of anti-β2GPI antibodies was observed, mice were used for T cell studies. In our current study, C57BL/6 mice received four immunizations, but BALB/c and C3H/HeN mice received three and two immunizations, respectively. Interestingly, C3H/H3N mice did not survive a third immunization (data not shown), and this premature death in C3H/HeN and some 129S1 mice (see "Experimental Procedures") was associated with an accelerated antibody response to β2GPI and LPS. The reason for the premature death in these mice is currently under investigation, but premature death

was observed only when β2GPI plus LPS were administered as immunogen, and not when either was administered alone.

In patients with SLE, autoantibodies to β2GPI also occur relatively early, while other SLE-related autoantibodies (e.g., anti-dsDNA, anti-Sm, and anti–nRNP antibodies) appear later[1]. Together with our murine model of induced SLE-related autoantibodies[4], these findings suggest that there is epitope spread in the autoantibody response from β2GPI to other SLE-related autoantigens (e.g., dsDNA). Epitope spread between these molecules ("inter-molecular epitope spread") presumably requires the physical association of β2GPI with these autoantigens. The apoptotic or dying cell represents a physiological scaffold upon which such association can occur.

Apoptotic cells express multiple SLE-related autoantigens[7,8], and thus provide an ideal "scaffold" for epitope spread of the autoantibody response from one autoantigen to another. β2GPI binds to apoptotic cells through interaction with phosphatidylserine[6,15,16] or Ro 60[17] exposed on the surface of these cells. Thus, a B cell specific for other SLE-related autoantigens (e.g., dsDNA) would recognize its cognate antigen on the apoptotic cell surface and therefore ingest this apoptotic cell. The ingesting B cell would then present peptide fragments from both cell-bound β2GPI and other apoptotic cell-derived proteins in the context of MHC class II. The key point is that a B cell presenting β2GPI peptides on its surface could receive help from β2GPI-reactive T cells independent of its autoantigen specificity (e.g., anti-dsDNA)[4]. In our model, we use human β2GPI as our immunogen. In addition to being the source of the T cell epitopes that we have identified, the injected β2GPI may also bind to apoptotic cells in the immunized mice. Notably, the human β2GPI-derived epitopes recognized by T cells in human β2GPI/LPS-immunized mice are closely related to comparable sequences within murine β2GPI, as illustrated by a high degree of homology between the sequences (80% and 66.7% identity for peptide 7 and peptide 23, respectively) (Figure 8). We do not yet know whether murine β2GPI plays a role in the epitope spread observed in our induced model. However, it is clear from our previous studies[4] that epitope spread to antibodies recognizing murine β2GPI does develop in human β2GPI/LPS-immunized mice.

Here, we show that β2GPI-reactive T cells with distinct epitope specificity and MHC class II restriction all permit B cell epitope spread to SLE-related autoantibodies. Notably, all of the nonautoimmune strains examined here (expressing three different MHC class II haplotypes) developed a similar profile of SLE-related autoantibodies. The extent of B cell epitope spread to different domains of β2GPI varied among strains, but did not appear to affect the degree of epitope spread to other autoantigens. Variation in the kinetics and intensity of the autoantibody response among strains may relate to non-MHC class II-related genes, and this area is currently under investigation.

Although SLE-related autoantibodies emerge in a remarkably similar pattern and sequence prior to the onset of clinical SLE disease[1], the mechanism responsible for epitope spread in these patients is not known. Of note, the role of β2GPI-reactive T cells has not yet been adequately evaluated in human and murine SLE. In fact, the limited number of studies of β2GPI-reactive T cells in both mice[18-20] and autoimmune patients[10,11,21-23] has focused primarily on the association of these cells with thrombosis or atherosclerosis (a clinical finding in patients with APS). The majority of human β2GPI-reactive CD4+ T cell clones evaluated in these studies recognized Domain V. and epitope mapping focused solely on this domain[10,11,22]. Moreover, as most of these human studies showed the presence of β2GPI-reactive CD4+ T cells only in patients having aPL, their association with other SLE-related autoantibodies was not evaluated[10,11,22,23]. Notably, a recent study found β2GPI-reactive PBMC responses in 32% of SLE patients, compared to 25% in primary APS (PAPS) and none in control subjects[21].

In the current study, we have focused on human $CD4^+$ T cell clones recognizing epitopes within Domains I and II in order to address whether the T cell epitope response that we observed in β2GPI/LPS-immunized mice also occurs in human autoimmune disease. We selected human $CD4^+$ T cell clones recognizing a recombinant fragment containing Domains I-II in order to compare their epitope specificity with the T cell hybridoma and primary cells from our immunized mice, which showed predominant recognition of Domains I-II. Two human $CD4^+$ T cell clones (OM9 and OM13) from a patient with APS recognized Domains I-II, and peptide 23 within that recombinant fragment of β2GPI. This epitope specificity is identical to that recognized by both the murine T cell hybridoma C3hB-1.5 and primary T cells from β2GPI/LPS-

immunized mice bearing an H-2^b haplotype (C57BL/6, 129S1, and C.B10 mice). Notably, the human T cell clones responded to peptide 23 presented in the context of a single HLA-DR allele, DRB1*0403. Identification of HLA-DRB1*0403 as the allele capable of presenting peptide 23 to β2GPI-reactive T cells is interesting, as this MHC allele has been shown to be strongly associated with the presence of aPL (both anti-CL and anti-β2GPI) in a European cohort of SLE patients[24]. Together with our findings in mice, these data suggest that the T cell response to β2GPI is restricted by MHC class II haplotype in both humans and mice. Moreover, the finding of a shared epitope specificity to β2GPI (peptide 23 in Domain II) in an HLA-DRB1*0403-positive patient and H-2^b haplotype-bearing mice indicates the relevance of our induced model for human autoimmune T cell specificities. Further studies are required to investigate whether the presence of β2GPI-reactive T cells is associated with epitope spread to multiple SLE autoantibodies in patients with SLE.

In summary, we have shown that a strong T cell response to β2GPI is associated with B cell epitope spread to SLE-related autoantibodies in β2GPI/LPS-immunized mice having different MHC class II haplotypes. While the specific β2GPI T cell epitopes recognized by the different mouse strains were directly linked to MHC class II haplotype, epitope spread to SLE-related autoantibodies occurred in all of the strains developing a strong β2GPI-reactive T cell response, regardless of β2GPI-reactive T cell epitope specificity. The dominant T cell epitope recognized by β2GPI-reactive T cells from mice with an H-2^b haplotype (e.g., C57BL/6 mice) was also recognized by T cells from a patient with APS, and was restricted by a MHC class II allele that has been genetically associated with the presence of autoantibodies to β2GPI and CL in SLE. These findings suggest that generation of a strong β2GPI-reactive T cell response, regardless of epitope specificity, is a common and decisive step in the initiation of SLE-related autoantibodies across multiple MHC class II backgrounds. We hypothesize that a T cell response to an apoptotic cell-binding protein, like β2GPI, allows B epitope spread of the autoimmune response to other SLE-related autoantigens expressed on the apoptotic cell surface.

2.7 ACKNOWLEDGEMENTS

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2.8 REFERENCES

1. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA *et al.* Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* 2003; **349**: 1526-1533.

2. Tsokos GC. Systemic lupus erythematosus. *N Engl J Med* 2011; **365**: 2110-2121.

3. McClain MT, Arbuckle MR, Heinlen LD, Dennis GJ, Roebuck J, Rubertone MV *et al.* The prevalence, onset, and clinical significance of antiphospholipid antibodies prior to diagnosis of systemic lupus erythematosus. *Arthritis Rheum* 2004; **50**: 1226-1232.

4. Levine JS, Subang R, Nasr SH, Fournier S, Lajoie G, Wither J *et al.* Immunization with an apoptotic cell-binding protein recapitulates the nephritis and sequential autoantibody emergence of systemic lupus erythematosus. *J Immunol* 2006; **177**: 6504-6516.

5. Tolomeo T, Rico De Souza A, Roter E, Dieude M, Amireault P, Subang R *et al.* T cells demonstrate a Th1-biased response to native beta2-glycoprotein I in a murine model of anti-phospholipid antibody induction. *Autoimmunity* 2009; **42**: 292-295.

6. Price BE, Rauch J, Shia MA, Walsh MT, Lieberthal W, Gilligan HM *et al.* Anti-phospholipid autoantibodies bind to apoptotic, but not viable, thymocytes in a beta 2-glycoprotein I-dependent manner. *J Immunol* 1996; **157**: 2201-2208.

7. Casciola-Rosen LA, Anhalt G & Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J Exp Med* 1994; **179**: 1317-1330.

8. Qian Y, Wang H & Clarke SH. Impaired clearance of apoptotic cells induces the activation of autoreactive anti-Sm marginal zone and B-1 B cells. *J Immunol* 2004; **172**: 625-635.

9. Levine J, Subang R, Setty S, Cabrera J, Laplante P, Fritzler M *et al.* Phospholipid-binding proteins differ in their capacity to induce autoantibodies and murine systemic lupus erythematosus. *Lupus* 2014; **23**: 752-768.

10. Arai T, Yoshida K, Kaburaki J, Inoko H, Ikeda Y, Kawakami Y *et al.* Autoreactive CD4(+) T-cell clones to beta2-glycoprotein I in patients with antiphospholipid syndrome: preferential recognition of the major phospholipid-binding site. *Blood* 2001; **98**: 1889-1896.

11. Hattori N, Kuwana M, Kaburaki J, Mimori T, Ikeda Y & Kawakami Y. T cells that are autoreactive to beta2-glycoprotein I in patients with antiphospholipid syndrome and healthy individuals. *Arthritis Rheum* 2000; **43**: 65-75.

12. McRae BL, Kennedy MK, Tan LJ, Dal Canto MC, Picha KS & Miller SD. Induction of active and adoptive relapsing experimental autoimmune encephalomyelitis (EAE) using an encephalitogenic epitope of proteolipid protein. *J Neuroimmunol* 1992; **38**: 229-240.

13. Behar SM, Podrebarac TA, Roy CJ, Wang CR & Brenner MB. Diverse TCRs recognize murine CD1. *J Immunol* 1999; **162**: 161-167.

14. Kaburaki J, Kuwana M, Yamamoto M, Kawai S, Matsuura E & Ikeda Y. Phospholipid-dependent anti-beta 2-glycoprotein I (beta 2-GPI) antibodies and antiphospholipid syndrome. *Intern Med* 1996; **35**: 105-110.

15. Balasubramanian K, Chandra J & Schroit AJ. Immune clearance of phosphatidylserine-expressing cells by phagocytes. The role of beta2-glycoprotein I in macrophage recognition. *J Biol Chem* 1997; **272**: 31113-31117.

16. Hunt JE, Simpson RJ & Krilis SA. Identification of a region of beta 2-glycoprotein I critical for lipid binding and anti-cardiolipin antibody cofactor activity. *Proc Natl Acad Sci U S A* 1993; **90**: 2141-2145.

17. Reed JH, Giannakopoulos B, Jackson MW, Krilis SA & Gordon TP. Ro 60 functions as a receptor for beta(2)-glycoprotein I on apoptotic cells. *Arthritis Rheum* 2009; **60**: 860-869.

18. Blank M, George J, Barak V, Tincani A, Koike T & Shoenfeld Y. Oral tolerance to low dose beta 2-glycoprotein I: immunomodulation of experimental antiphospholipid syndrome. *J Immunol* 1998; **161**: 5303-5312.

19. Buttari B, Profumo E, Capozzi A, Facchiano F, Saso L, Sorice M *et al.* Advanced glycation end products of human beta(2) glycoprotein I modulate the maturation and function of DCs. *Blood* 2011; **117**: 6152-6161.

20. George J, Harats D, Gilburd B, Afek A, Shaish A, Kopolovic J *et al.* Adoptive transfer of beta(2)-glycoprotein I-reactive lymphocytes enhances early atherosclerosis in LDL receptor-deficient mice. *Circulation* 2000; **102**: 1822-1827.

21. Conti F, Spinelli FR, Alessandri C, Pacelli M, Ceccarelli F, Marocchi E *et al.* Subclinical atherosclerosis in systemic lupus erythematosus and antiphospholipid syndrome: focus on beta2GPI-specific T cell response. *Arterioscler Thromb Vasc Biol* 2014; **34**: 661-668.

22. Ito H, Matsushita S, Tokano Y, Nishimura H, Tanaka Y, Fujisao S *et al.* Analysis of T cell responses to the beta 2-glycoprotein I-derived peptide library in patients with anti-beta 2-glycoprotein I antibody-associated autoimmunity. *Hum Immunol* 2000; **61**: 366-377.

23. Visvanathan S & McNeil HP. Cellular immunity to beta 2-glycoprotein-1 in patients with the antiphospholipid syndrome. *J Immunol* 1999; **162**: 6919-6925.

24. Galeazzi M, Sebastiani GD, Tincani A, Piette JC, Allegri F, Morozzi G *et al.* HLA class II alleles associations of anticardiolipin and anti-beta2GPI antibodies in a large series of European patients with systemic lupus erythematosus. *Lupus* 2000; **9**: 47-55.

2.9 FIGURES & LEGENDS

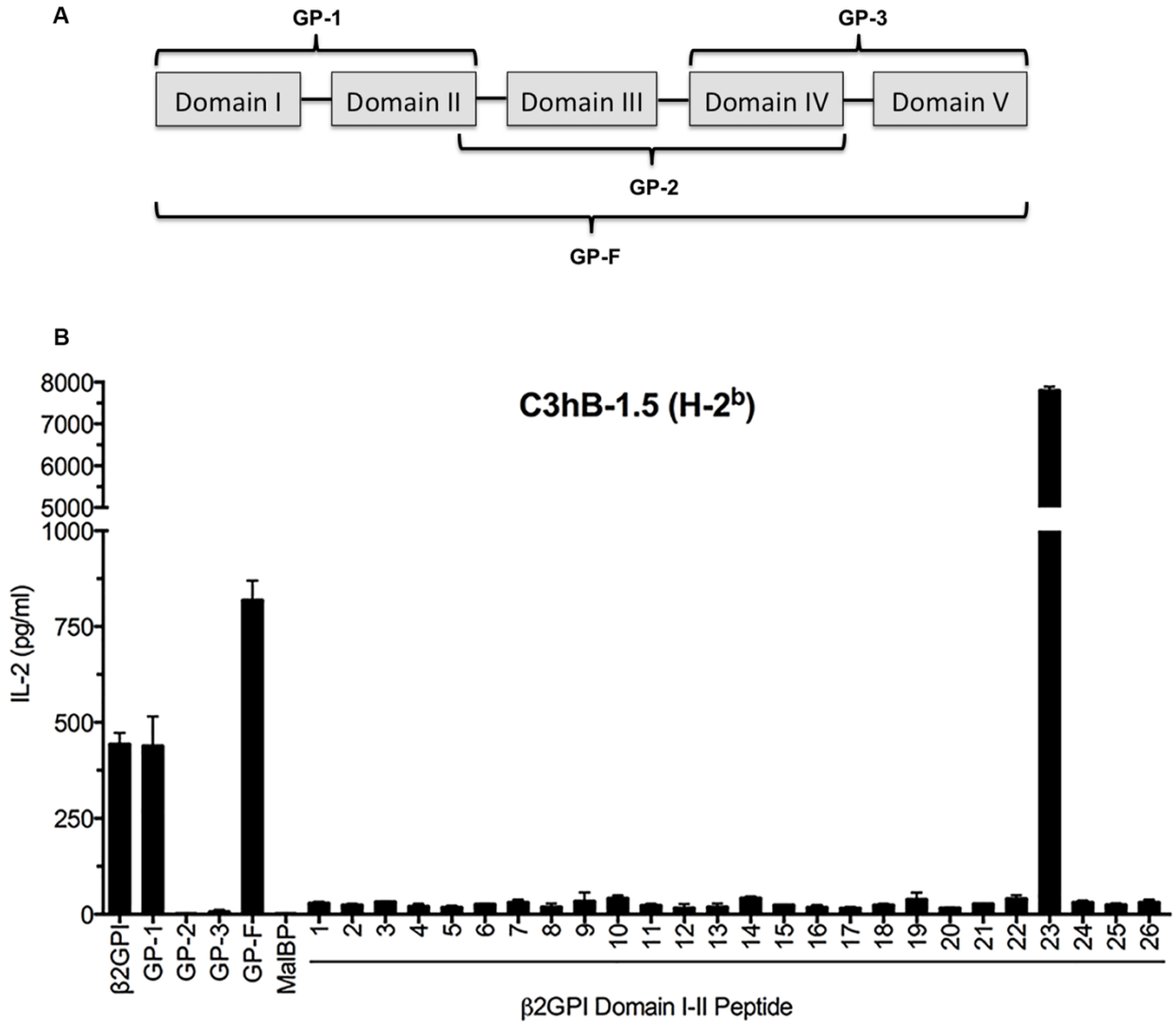

Figure 1. A β2GPI-reactive T cell hybridoma from C57BL/6 (H-2^b) mice recognizes a peptide (23) from Domain II of β2GPI. **A.** β2GPI recombinant fragments consisting of different combinations of domains were used to evaluate T cell epitope specificity. The recombinant fragments, shown schematically, were: GP-1 (Domains I-II), GP-2 (Domains III-IV), GP-3 (Domains IV-V), and GP-F (full length β2GPI). **B.** The C3hB-1.5 β2GPI-specific T cell hybridoma was evaluated for recognition of recombinant fragments of human β2GPI and twenty-six 15-amino acid peptides, with a 10-amino acid overlap, encompassing Domains I and II of β2GPI. Human β2GPI (20 μg/ml), recombinant fragments (20 μg/ml), or peptides (10 μg/ml) were incubated with C3hB-1.5 cells and C57BL/6-derived APCs for 24 hours, IL-2 concentration in the supernatant was measured by ELISA. Values represent the mean IL-2 concentration (ng/ml) + SE of triplicate samples, and the data shown are representative of 3 independent experiments.

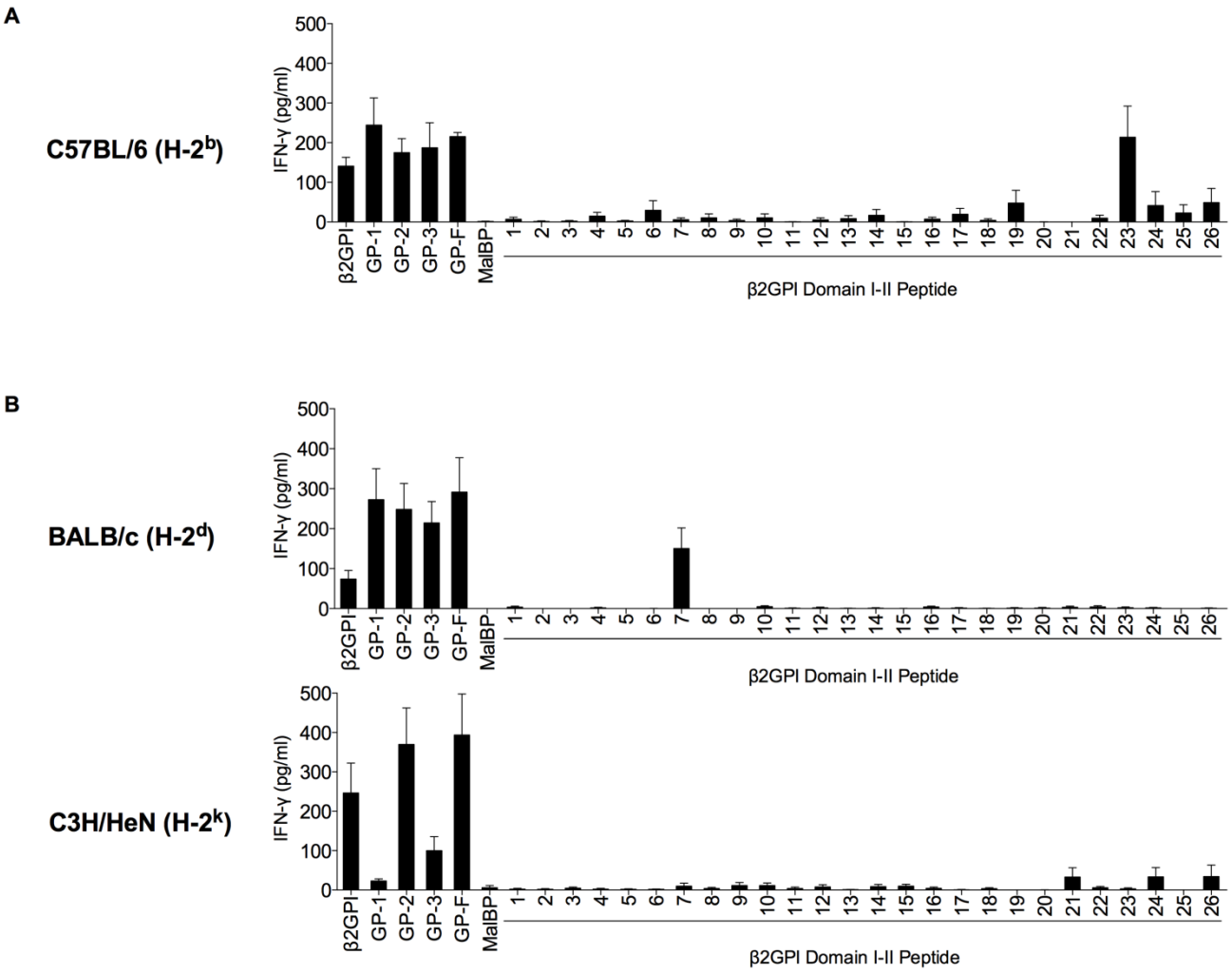

Figure 2. Domain and epitope specificities of the β2GPI-reactive T cell response vary with MHC class II haplotype. Splenic T cells from β2GPI/LPS-immunized mice (C57BL/6 [H-2^b] (A), and BALB/c [H-2^d] or C3H/HeN [H-2^k]) (B) were plated with strain-matched APCs. β2GPI, GP-1 (Domains I-II), GP-2 (Domains III-IV), GP-3 (Domains IV-V), GP-F (full length β2GPI), or MalBP (control fusion protein) was added to the culture at a concentration of 20 μg/ml, and individual peptides from Domains I-II were added at 10 μg/ml. Cells were incubated for 48 hours and IFN-γ production in the supernatant was measured by ELISA. Values represent the mean IFN-γ concentration (pg/ml) + SE of duplicate samples, and the data shown are pooled from 3 independent experiments.

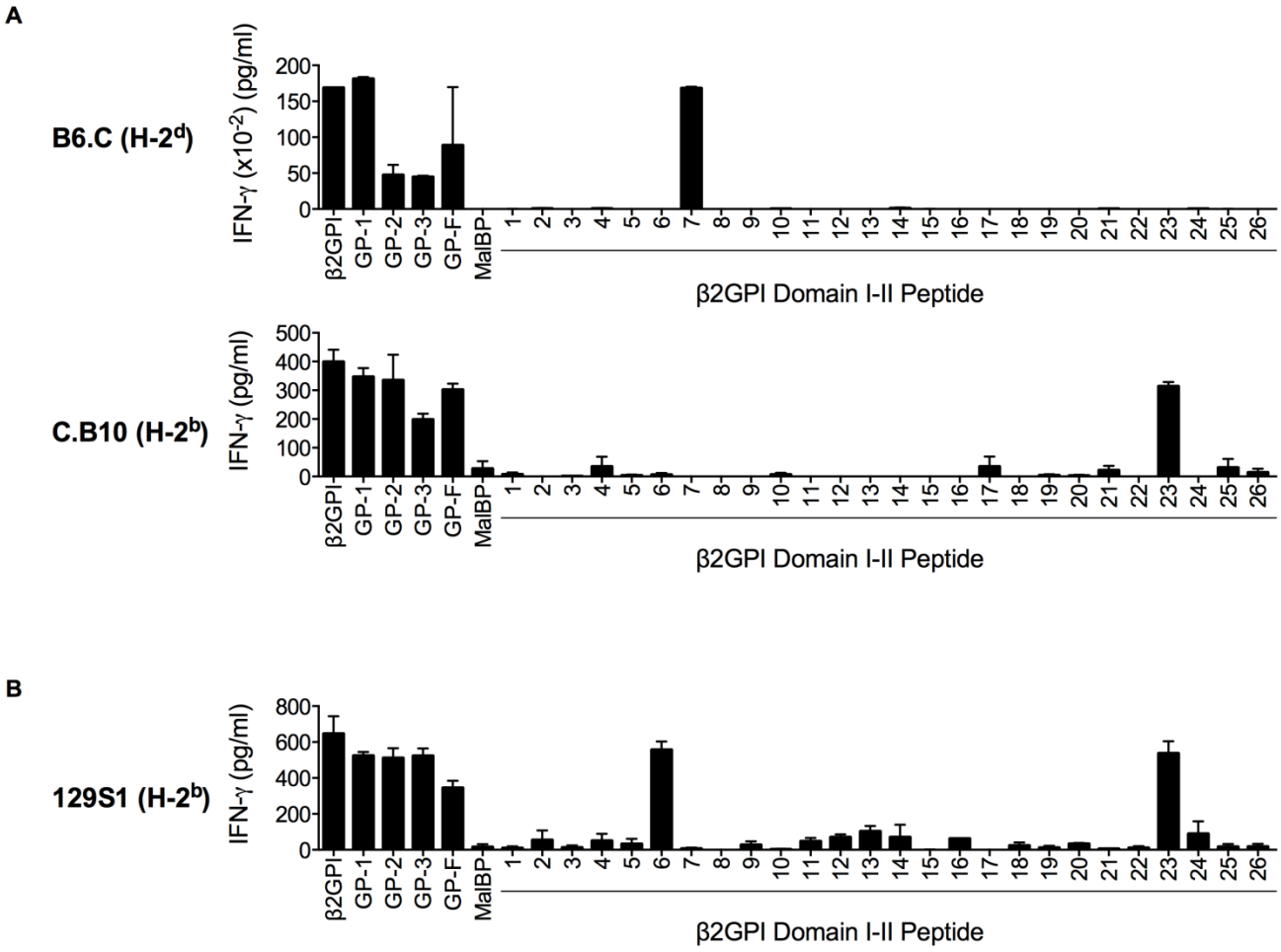

Figure 3. β2GPI T cell epitope specificity segregates with MHC class II haplotype. Splenic T cells from **(A)** B6.C (C57BL/6 with H-2^d) or C.B10 (BALB/c with H-2^b) mice, or **(B)** 129S1 (H-2^b) mice that had been immunized with β2GPI and LPS were plated with MHC class II haplotype-matched APCs. β2GPI, GP-1 (Domains I-II), GP-2 (Domains III-IV), GP-3 (Domains IV-V), GP-F (full length β2GPI), or MalBP (control fusion protein) was added to the culture at concentration of 20 μg/ml, and individual peptides from Domains I-II were added at 10 μg/ml. Cells were incubated for 48 hours and IFN-γ production in the supernatant was measured by ELISA. Values represent the mean IFN-γ concentration (pg/ml) + SE of duplicate samples, and the data shown are representative of 3 independent experiments.

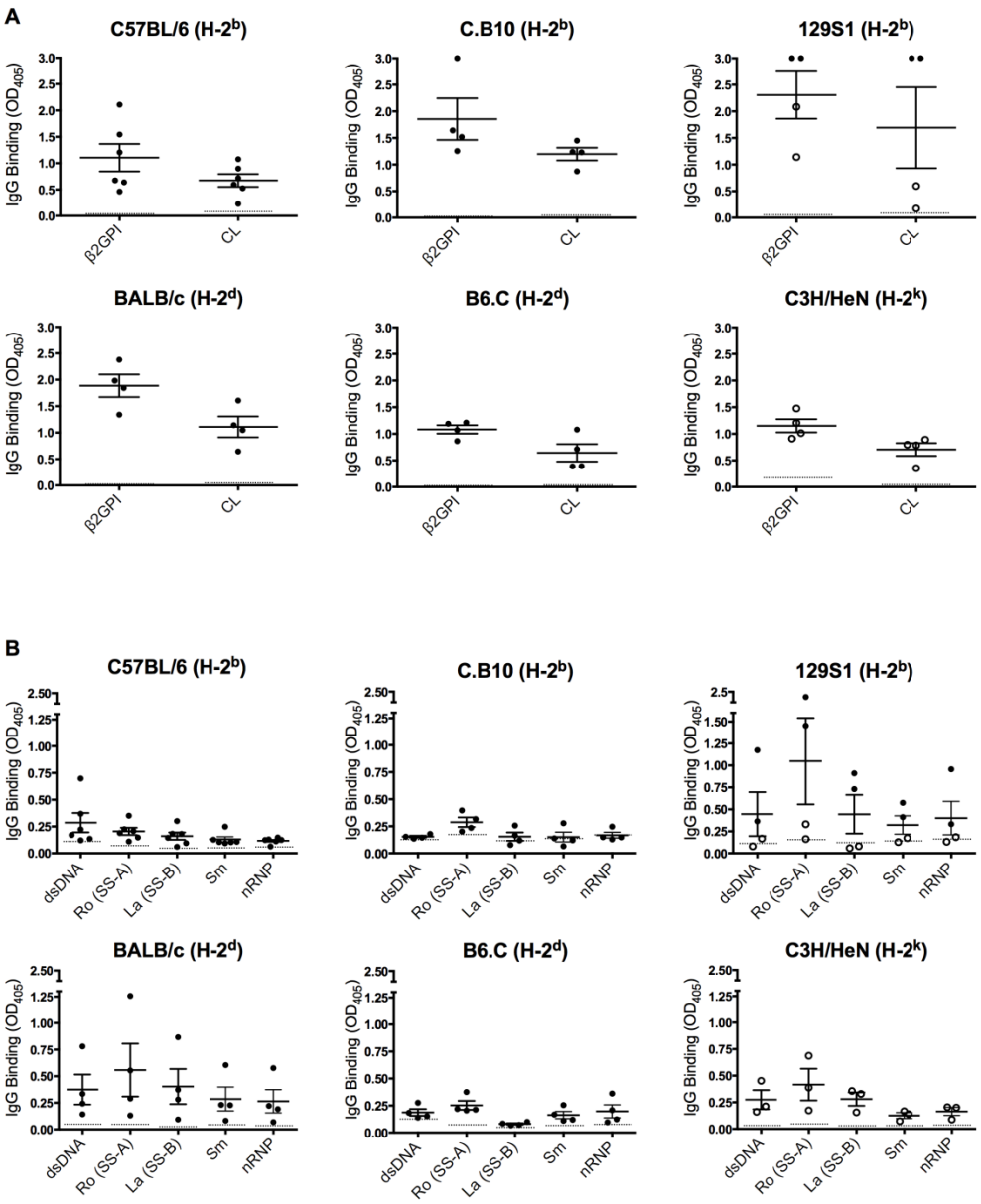

Figure 4. Multiple distinct MHC class II-restricted β2GPI T cell epitopes are associated with B cell epitope spread to SLE autoantibodies. Sera from β2GPI/LPS-immunized mice were tested for IgG antibodies to: **(A)** human β2GPI (1/5000 dilution) and cardiolipin (CL) (1/1000 dilution); and **(B)** dsDNA, Ro (SS-A), La (SS-B), Sm, and nRNP (1/50 dilution) by ELISA. The data shown for C57BL/6, C.B10, BALB/c, and B6.C mice are post-third immunization (black circles), while the data for all C3H/HeN and two 129S1 mice are post-second immunization (white circles) (see Methods - Mice and immunization). Sera from mice of the same strain, immunized with PBS and LPS, served as a negative control in these assays. The mean value for PBS/LPS-immunized mice (n =2 for each strain) is shown as a dotted line for each autoantibody assay. In certain cases, the mean value for the controls is very close to zero, and so may be difficult to distinguish from the x-axis. Each dot represents the mean IgG antibody binding (OD_{405} ± SE) of duplicate samples for an individual mouse (n = 3-6 mice/group), and the data shown are representative of 3 independent experiments.

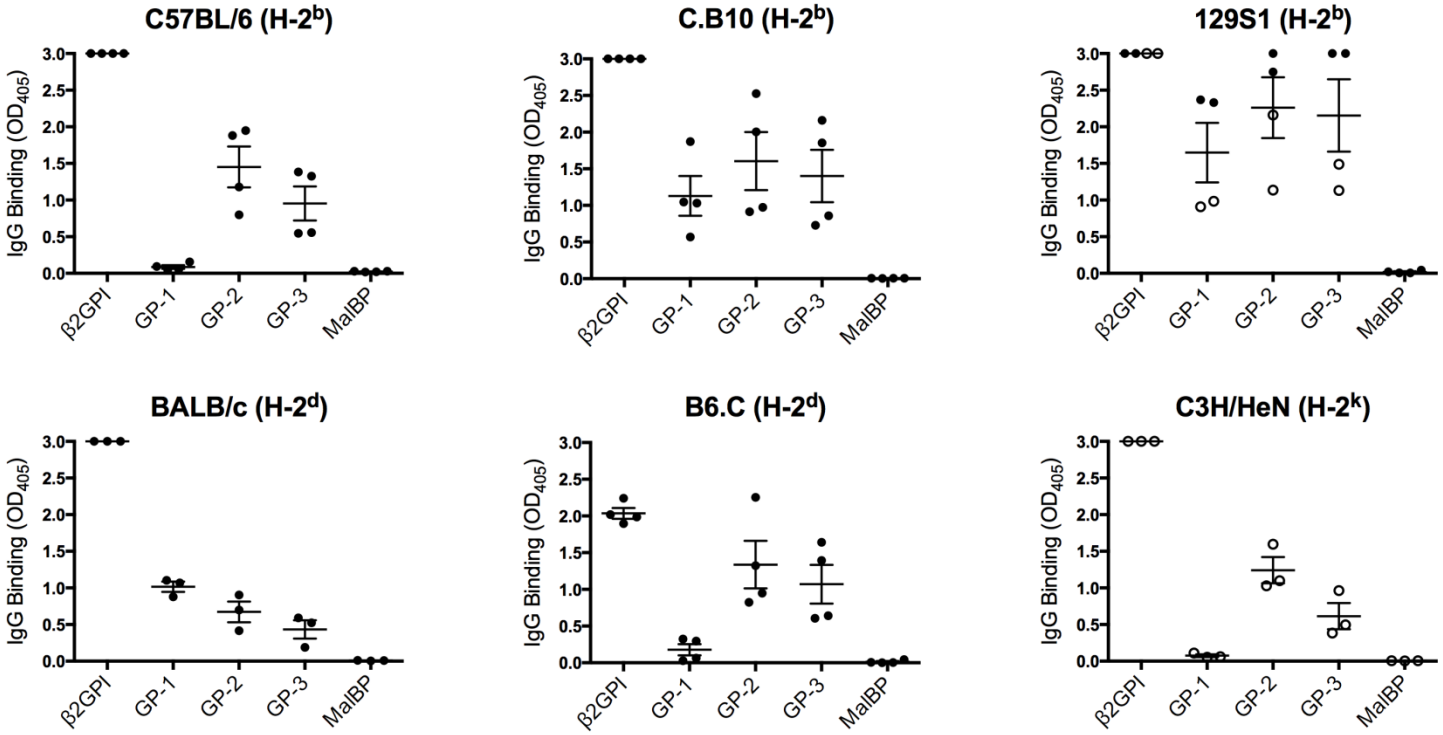

Figure 5. B cell epitope spread to other β2GPI domains occurs in the presence of a β2GPI-specific T cell response. Sera from β2GPI/LPS-immunized mice were tested by ELISA for antibodies to human β2GPI (native protein), or recombinant protein fragments of human β2GPI (GP-1, GP-2, GP-3) or MalBP (control fusion protein). The data shown for C57BL/6, C.B10, BALB/c, and B6.C mice are post-third immunization (black circles), while the data for all C3H/HeN mice and two 129S1 mice are post-second immunization (white circles) (see Methods - Mice and immunization). Each dot represents the mean IgG binding (OD_{405} ± SE) of duplicate samples (1/1000 dilution, except for C57BL/6 [1/100 dilution]) for an individual mouse (n = 3 to 4 mice/group), and the data shown are representative of 3 independent experiments.

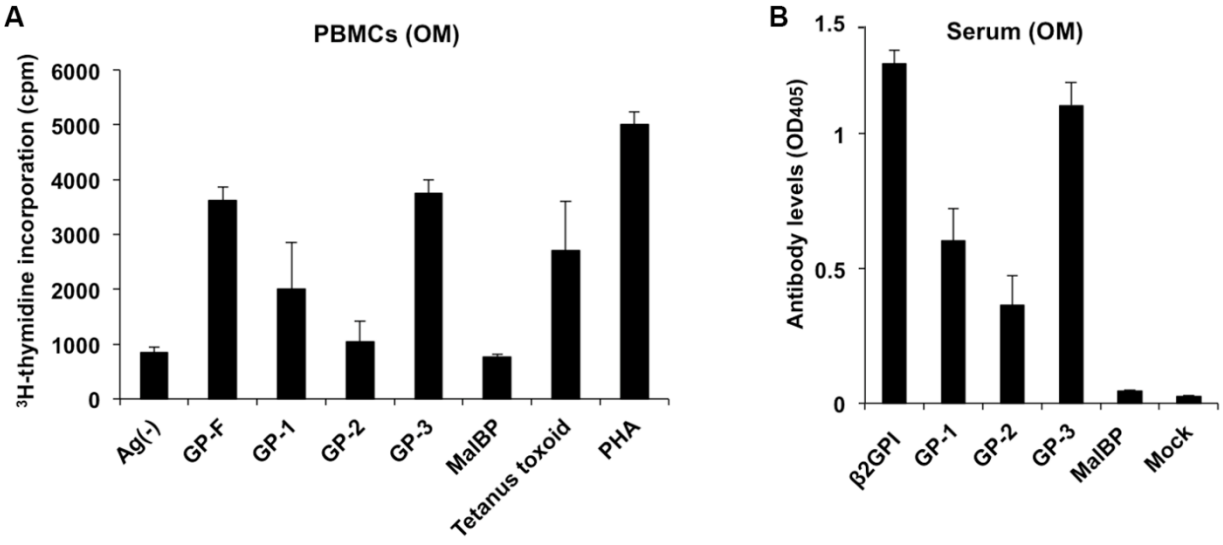

Figure 6. Primary T cells and antibodies from an autoimmune patient react with a recombinant fragment containing Domains I and II. A. An *in vitro* T cell assay for evaluating proliferative responses to GP-1 (Domains I-II), GP-2 (Domains III-IV), GP-3 (Domains IV-V), GP-F (full length β2GPI), or MalBP (control fusion protein) (20 μg/ml), or peptides (10 μg/ml) covering the entire sequence of Domains I-II of β2GPI, was done using peripheral blood mononuclear cells (PBMCs) isolated from an APS patient (OM). Tetanus toxoid and PHA were used for evaluating T cell viability. Values represent the mean ^{3}H-thymidine incorporation (cpm) + SE of triplicate samples. The data shown are representative of 3 independent experiments. **B.** ELISAs were performed to detect antibodies to GP-1, GP-2, GP-3, GP-F, and MalBP in sera from a donor OM (1/100 serum dilution). Values represent the mean IgG binding (OD_{405}) + SD of triplicate samples. The data shown are representative of 3 independent experiments.

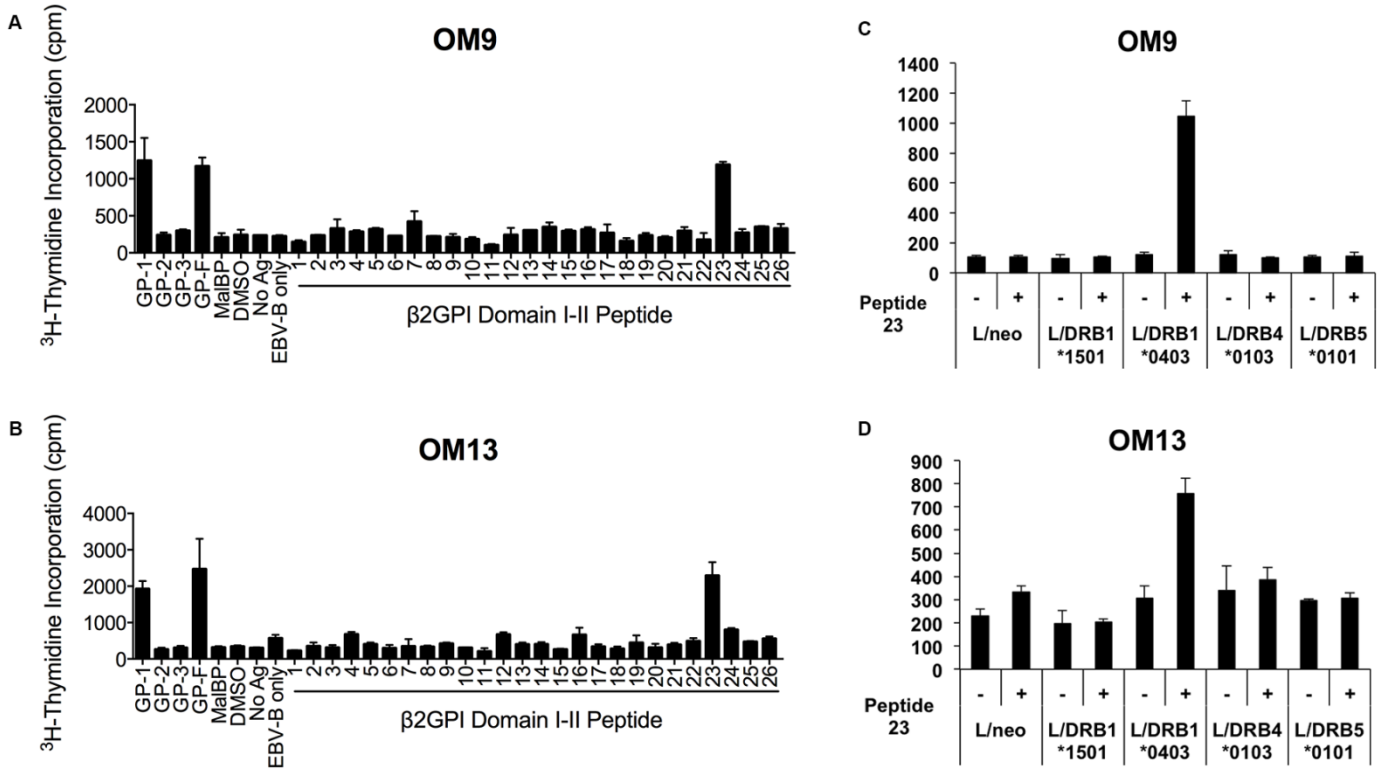

Figure 7. β2GPI-reactive CD4$^+$ T cell clones derived from an autoimmune patient recognize the same peptide as H-2^b-bearing mice. β2GPI-specific T cell clones OM9 (**A**) and OM13 (**B**) were evaluated for proliferative responses to GP-1 (Domains I-II), GP-2 (Domains III-IV), GP-3 (Domains IV-V), GP-F (full length β2GPI), or MalBP (control fusion protein) (20 μg/ml), or peptides (10 μg/ml) covering the entire sequence of Domains I-II of β2GPI, using an *in vitro* T cell assay. MHC class II haplotype restriction of β2GPI-specific T cell clones OM9 (**C**) and OM13 (**D**) was evaluated using a series of L-cell transfectants expressing a single human HLA class II molecule, in the presence or absence of peptide 23. Cells were incubated for 72 hours and cell proliferation was measured using ^{3}H-thymidine incorporation. Values represent the mean ^{3}H-thymidine incorporation (cpm) + SE of triplicate samples, and the data shown are representative of 2 independent experiments.

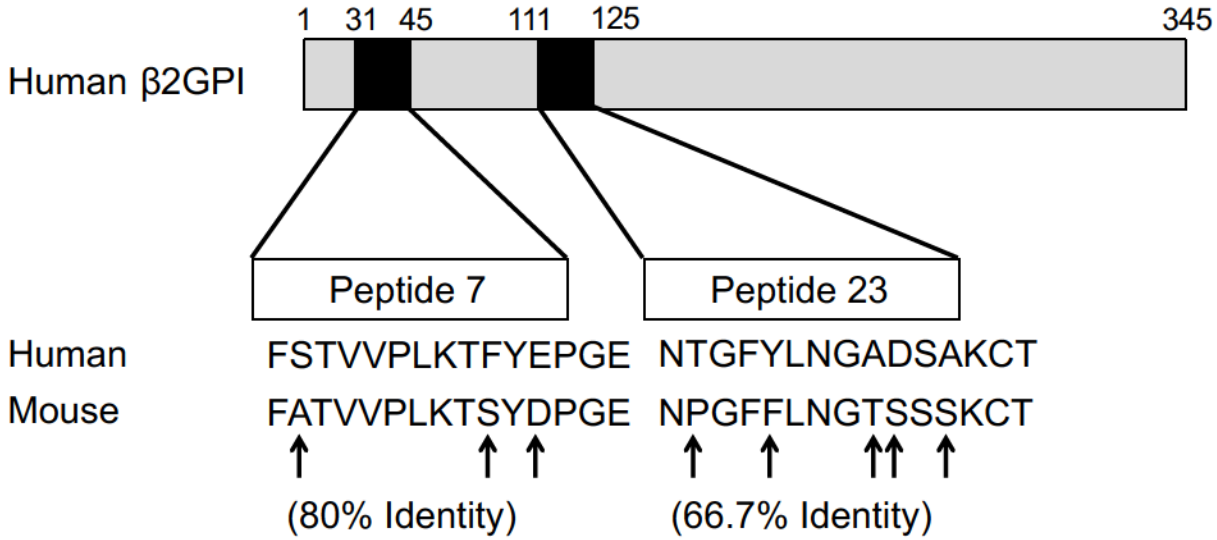

Figure 8. Human β2GPI-derived peptide sequences recognized by β2GPI-reactive T cells share sequence identity with murine β2GPI. This figure shows a schematic representation of the full amino acid sequence of human β2GPI (top), and the peptides (black regions) recognized by T cells from human β2GPI/LPS-immunized mice. The expanded regions show the amino acid sequences of epitopes within Domains I (peptide 7) and II (peptide 23) recognized by T cells from H-2^d- and H-2^b-bearing mice, respectively. Peptide 23 was also recognized by HLA-DRB1*0403-bearing human $CD4^+$ T cell clones. Human β2GPI-derived sequences for peptides 7 and 23 are aligned with the comparable sequences from murine β2GPI to illustrate the percent identity between the sequences.

CHAPTER 3

T CELLS FROM INDUCED AND SPONTANEOUS MODELS OF SLE RECOGNIZE A COMMON T CELL EPITOPE ON β2-GLYCOPROTEIN I

3.1 PREFACE

In Chapter 2, we determined the Domain I-II epitope specificity of β2GPI-reactive T cells in mice with induced SLE. Using that limited library of peptides, we found that epitope specificity was strongly governed by MHC class II haplotype and that mice with different MHC class II haplotypes did not share β2GPI Domain I-II T cell epitopes. However, one β2GPI T cell epitope (peptide 23 [NTGFYLNGADSAKCT]) was recognized by T cells from both B6 mice with induced SLE and an autoimmune patient with APS. In this chapter, we use a peptide library spanning the complete sequence of human β2GPI to identify common and potentially dominant β2GPI T cell epitopes in both mice with induced SLE and MRL/*lpr* mice that develop SLE-like disease spontaneously. We also characterize the phenotype of β2GPI-reactive T cells in mice with induced SLE and mice with spontaneous SLE.

T CELLS FROM INDUCED AND SPONTANEOUS MODELS OF SLE RECOGNIZE A COMMON T CELL EPITOPE ON β2-GLYCOPROTEIN I

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Running title: Common T cell epitope in induced and spontaneous SLE

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Abbreviations: β2-glycoprotein, β2GPI; anti-CL, anti-cardiolipin; anti-β2GPI, anti-β2-glycoprotein I; anti-dsDNA, anti-double stranded DNA; aPL, anti-phospholipid antibodies; LPS, lipopolysaccharide; MHC, major histocompatibility complex; SLE, systemic lupus erythematosus.

Keywords: β2-glycoprotein I; T cells; systemic lupus erythematosus; autoantibodies; MHC class II haplotypes;

3.2 ABSTRACT

Systemic lupus erythematosus is a prototypic model for B cell epitope spread in autoimmunity. Autoantibodies to numerous molecularly distinct self-antigens emerge in a sequential manner over several years, leading to disease manifestations. Among the earliest autoantibodies to appear are those targeting phospholipid-binding proteins, particularly β2-glycoprotein I. Notably, mice immunized with β2-glycoprotein I and lipopolysaccharide develop a strong T cell response to β2-glycoprotein I that is associated with autoantibody production and renal disease similar to that seen in human SLE. Here, we hypothesized that mice with murine systemic lupus erythematosus, whether induced or spontaneous, should have T cells that recognize β2-glycoprotein I. We evaluated the proliferative response of splenic T cells from mice with induced (C57BL/6 and C3H/HeN) and spontaneous (MRL/lpr) systemic lupus

erythematosus to peptides spanning the entire sequence of human β2GPI. We found that mice with induced and spontaneous systemic lupus erythematosus recognize a common T cell epitope (peptide 31; LYRDTAVFECLPQHAMFG) in Domain III in β2-glycoprotein I. β2GPI-reactive $CD4^+$ T cells from the two models differed primarily in cytokine production: T cells from mice with induced SLE expressed IFN-γ, while T cells from MRL/lpr mice expressed both IL-17 and IFN-γ, indicating that IL-17-expressing T cells are not necessary for generating a β2GPI-reactive T cell response. These data suggest that the generation of a β2-glycoprotein I-reactive T cell response is shared by both induced and spontaneous models of systemic lupus erythematosus, and that this T cell response may mediate epitope spread to autoantibodies in both models.

3.3 INTRODUCTION

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease in which individuals develop multiple different autoantibodies, as well as a diversity of organ-related pathologies[1]. Despite this heterogeneity, autoantibodies emerge in a sequential manner with anti-β2 glycoprotein I (β2GPI) being among the first to appear[2]. We have previously shown that immunization of nonautoimmune mice with β2GPI and lipopolysaccharide (LPS) induces a murine model of SLE that closely mimics human disease[3]. These mice develop a strong and persistent T cell response to β2GPI, which is associated with epitope spread to other SLE-related autoantibodies and the development of SLE-like disease[4]. The order of appearance of autoantibodies in our induced model mimics that seen in spontaneous human SLE[2,3]. We have shown that multiple β2GPI T cell epitopes are associated with the production of SLE autoantibodies in this induced model[4]. Here, we hypothesized that mice with spontaneous systemic lupus erythematosus should also have T cells recognizing β2-glycoprotein I, and that a common β2GPI T cell epitope might exist between induced and spontaneous models of SLE. We evaluated the T cell response to peptides spanning the entire sequence of β2GPI in two MHC class II-distinct strains of mice with induced SLE and in the MRL/lpr spontaneous model of SLE, and characterized the differences between β2GPI-reactive T cells in the two models.

$CD4^+$ T cells, particularly T helper type 1 and type 17 cells (Th1/Th17), have been shown to be necessary for disease pathogenesis in murine and human SLE[5-7]. Interferon-γ and

interleukin-17-producing T cells are important for the development of nephritis in MRL/lpr mice, a spontaneous murine model of SLE[8]. However, characterization of the epitopes recognized by these pathogenic autoreactive T cells has been less well-studied and limited to a few autoantigens, primarily in models of spontaneous SLE[9-11]. In the current study, we used an induced model of SLE with a known initiating antigen, β2GPI, and in which the generation of a strong T cell response to that initiating antigen is involved in disease progression[4]. We map the dominant β2GPI-reactive T cell epitopes in our induced model across two MHC haplotypes and evaluate whether T cells from a spontaneous model of SLE share the same epitopes. Furthermore, we characterize the phenotypes of the β2GPI-reactive T cells. We propose that a common β2GPI-reactive T cell epitope may be responsible for SLE autoantibody production in both induced and spontaneous SLE.

3.4 EXPERIMENTAL PROCEDURES

3.4.1 Mice and immunization

Specific pathogen-free female C57BL/6 mice (8-12 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, Maine). Female C3H/HeN were generously provided by Dr. Salman Qureshi and bred in-house. MRL/MpJ-*Tnfrsf6*^{lpr} (MRL/lpr) mice were purchased from The Jackson Laboratory and bred in-house. The MRL/lpr strain was derived originally from crosses among mouse strains LG, AKR, C3H/Di, and C57BL/6[12]. These mice develop systemic autoimmunity and immune complex glomerulonephritis that closely resembles human SLE. Mice were maintained and bred according to Canadian Council on Animal Care (CCAC) guidelines, and maintained on food and water *ad libitum*. Animal experiments were approved by the McGill University Animal Care Committee.

C57BL/6 and C3H/HeN mice were immunized with 20 μg human β2GPI and 10 μg LPS, as described previously[13]. Mice were injected intravenously every two weeks, and bled for serum 10 days following the second and third immunizations. The number of immunizations required was determined by observed levels of anti-β2GPI antibodies. C57BL/6 mice received four immunizations and C3H/HeN mice received two immunizations[4]. MRL/lpr mice (4-8

weeks of age) were injected with a single dose of 20 μg GP-F (full-length recombinant human β2GPI; see below) and 10 μg LPS. Untreated MRL/lpr mice were used at 9-14 weeks of age.

3.4.2 Reagents

Unless stated otherwise, all reagents were obtained commercially from the following sources and used without further purification: human β2GPI (≥95% pure; Crystal Chem, Downers Grove, IL); LPS (*Escherichia coli*-derived, serotype O111:B4) (List Biological Laboratories, Campbell, CA); bovine heart cardiolipin (CL) (Avanti Polar Lipids, Alabaster, AL); *E. coli* DNA (dsDNA) (Worthington Biochemical Corporation, Lakewood, NJ); Anti-Mouse Ig κ / Negative Control Compensation Particles Set, Anti-Rat and Anti-Hamster Ig κ / Negative Control Compensation Particles Set, Fc block (FcgIII/II receptor-CD16/32), mouse interleukin-2 (IL-2) ELISA set (BD OptEIA kit), mouse interferon-γ (IFN-γ) ELISA set (BD OptEIA kit), and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate reagent set (BD OptEIA kit) from BD Biosciences (Mississauga, ON); alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG and AP-conjugated streptavidin from Southern Biotech (Birmingham, AL); p-nitrophenyl phosphate, phorbol 12-myristate 13-acetate (PMA), and ionomycin from Sigma-Aldrich (St. Louis, MO); Alexa fluor 488 anti-mouse IFN-γ (XMG1.2), Alexa fluor 647 anti-mouse forkhead box P3 (FoxP3) (MF-14), Alexa fluor 700 anti-mouse CD8α (53-6.7), brilliant violet 421 anti-mouse IL-4 (11B11), brilliant violet 605 anti-mouse CD185 (CXCR5) (L138D7), brilliant violet 785 anti-mouse CD4 (RM4-5), PE anti-human/mouse B-cell lymphoma 6 protein (Bcl-6) (7D1), PE/Dazzle 594 anti-mouse IL-17α (TC11-18H10.1), monensin solution, True-Nuclear transcription factor buffer set, Zombie NIR fixable viability kit and recombinant human IL-7 from BioLegend (San Diego, CA); recombinant human IL-2 (generously provided by Dr. Piccirillo [gift of the Surgery Branch, National Cancer Institute, National Institutes of Health]).

3.4.3 β2GPI recombinant fragments and synthetic peptides

Recombinant maltose-binding protein (MalBP) fusion proteins encoding different regions of human β2GPI were used as antigens for T cell stimulation, as previously described[4,14]. These fusion proteins, expressed as recombinant proteins in *E. coli* and described in detail

previously[15], included: GP-F, encoding the entire amino acid sequence of β2GPI (amino acid residues 1-326); GP-1, encoding Domains I and II (amino acid residues 1-133); GP-2, encoding Domains III and IV (amino acid residues 119-254); and GP-3, encoding Domains IV and V (amino acid residues 182-326). Recombinant MalBP was used as a control antigen. All recombinant fragments were used at a concentration of 20 μg/ml in 0.01 M phosphate-buffered saline, pH 7.3 (PBS).

Twenty-six 15-mer peptides (10 residue overlap) spanning Domains I and II, and thirty-two 18-mer peptides (12 residue overlap) spanning Domains III-V of human β2GPI were synthesized and their purity determined by high performance liquid chromatography (Sigma-Aldrich). The peptides were dissolved in 200 μl of dimethyl sulfoxide (DMSO), and further diluted in 500 μl PBS. Peptide stock solutions in DMSO and PBS were stored at -70°C. The peptides were added to T cells at a final concentration of 10 μg/ml in PBS, as described below (see "Evaluation of domain and epitope specificity of T cells").

3.4.4 Cell culture

Unless stated otherwise, all cells were cultured in DMEM medium (4.5 g/L glucose, 110 mg/mL sodium pyruvate), containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% L-glutamate, 1% HEPES, 1% non-essential amino acids, and 0.1% 2-mercaptoethanol (medium and supplements were from Life Technologies Inc., Burlington, ON), hereafter referred to as complete DMEM. Splenic T cells from immunized mice were isolated using an EasySep T cell kit (StemCell Technologies, Vancouver, BC) and were cultured in complete DMEM containing β2GPI-depleted FBS. FBS was depleted of β2GPI using a HiTrap Heparin HP column (GE Healthcare, Piscataway, NJ) to eliminate the potential influence of bovine β2GPI. β2GPI depletion was verified by the inability of treated FBS to support the binding of a bovine β2GPI-dependent murine monoclonal antibody to cardiolipin by ELISA (data not shown).

3.4.5 Evaluation of domain and epitope specificity of T cells

Splenocytes from β2GPI/LPS-immunized C57BL/6 and C3H/HeN, or naïve or GP-F/LPS-injected MRL/lpr mice, were plated at 1 x 10^6 cells/well in complete DMEM containing β2GPI-depleted FBS. Commercial human β2GPI, recombinant protein fragments of human β2GPI or MalBP, or Domain I-II and Domain III-V peptides were then added to the T cells and incubated for 48 hours (37°C, 5% CO_2). Cell supernatants were collected, and IFN-γ and IL-2 levels were quantified by ELISA. Results were expressed as the mean IFN-γ or IL-2 concentration (pg/ml) of duplicate samples, as determined from a standard curve using recombinant IFN-γ or IL-2.

3.4.6 Expansion of β2GPI-reactive T cells

Splenocytes from β2GPI/LPS- or PBS/LPS-immunized C57BL/6 or C3H/HeN mice were plated at 1 x 10^7 cells/well (24-well plates) in complete DMEM containing β2GPI-depleted FBS. Splenocytes were cultured for five days in the presence of commercial human β2GPI (15 μg/ml), IL-2 (10 units/ml), and IL-7 (10 ng/ml). For MRL/lpr mice, splenocytes from GP-F/LPS- or PBS/LPS-injected mice (10 weeks of age) were cultured for five days with GP-F (15 μg/ml), IL-2, and IL-7. Cells were then washed, transferred into 6-well plates, and cultured for an additional two days in the presence of only IL-2 and IL-7. Cells were then stimulated with PMA (20 ng/ml) and ionomycin (750 ng/ml) for 3 hours, in the presence of monensin solution, and then harvested and stained for surface and intracellular markers. Cells were analyzed using a BD LSRFortessa flow cytometer and FACSDiva software (BD Bioscience), and plots, gated on viable cells, were generated using FlowJo X software.

3.4.7 Detection of autoantibodies

Serum antibodies to β2GPI, recombinant β2GPI domain fragments, CL, and dsDNA were determined by ELISA, as previously described[3,4]. The anti-β2GPI (direct binding) assay detects antibodies that bind to human β2GPI, while the anti-CL assay detects antibodies that recognize CL-bound β2GPI (bovine β2GPI from FBS in the assay buffer and, potentially, murine β2GPI within the serum sample). Reactivity to bound β2GPI was confirmed by a similar anti-CL ELISA where plates were incubated with either bovine or human serum, or purified human β2GPI, for 16 hr at 37°C prior to the addition of murine serum samples, as previously described[3].

Sera from β2GPI/LPS-immunized C3H/HeN were collected 10-14 days after the first, second and third immunizations, respectively. Sera from untreated MRL/lpr female mice were collected every 2 weeks, starting at 4 weeks of age.

3.4.8 Statistical analysis

Statistical significance was determined by a two-tailed unpaired non-parametric T-test using Prism 7.0 (GraphPad Software Inc., San Diego, CA). The minimal threshold for significance was $p < 0.05$.

3.5 RESULTS

3.5.1 T cells from mice with induced SLE recognize common β2GPI epitopes

We have previously evaluated the T cell response to epitopes within Domains I and II of β2GPI in mice immunized with β2GPI and LPS, and found no common epitopes between mice of different haplotypes[4,13]. Here, we extend our analysis to determine whether T cells from β2GPI/LPS-immunized mice also recognize epitopes within Domains III-V of β2GPI, and ask whether these epitopes are shared between haplotypes. Furthermore, we evaluate whether mice with induced and spontaneous SLE recognize shared T cells epitopes on β2GPI.

Splenic T cells from β2GPI/LPS-immunized mice of two different MHC class II haplotypes (C57BL/6 [H-2^b] and C3H/HeN [H-2^k]) were evaluated for their response to native human β2GPI; recombinant proteins encoding Domains I-V of human β2GPI (or MalBP as control); and synthetic peptides covering the entire human β2GPI sequence. T cells from both C57BL/6 (Figure 1A) and C3H/HeN (Figure 1B) mice recognized native human β2GPI and full-length recombinant β2GPI (GP-F), but domain recognition differed between the two strains. While C57BL/6 T cells recognized all recombinant fragments (GP-1, 2, and 3) of human β2GPI, C3H/HeN T cells were most responsive to recombinant fragment GP-2 (Domains III-IV). T cell recognition of peptides revealed both distinct and common epitopes. C57BL/6 T cells recognized peptides 23 (Domain II) and 30/31 (Domain III), while C3H/HeN T cells recognized

peptides 30/31, 38, and 46 (Domains III and IV). T cell responses were specific to human β2GPI, as shown by the lack of recognition of the control recombinant protein MalBP. These data demonstrate that, despite different MHC class II haplotypes, T cells from mice with induced SLE respond to a common β2GPI epitope (peptides 30/31 [SAGNNSLYRDTAVFECLP/ LYRDTAVFECLPQHAMFG]).

3.5.2 T cells from mice with induced SLE are Th1-biased and produce IFN-γ

We next evaluated the characteristics of β2GPI-reactive T cells from C57BL/6 or C3H/HeN mice that had been expanded *in vitro* with β2GPI. β2GPI-expanded T cells from β2GPI/LPS-immunized C57BL/6 and C3H/HeN mice both had a much higher proportion of $CD4^+$ (24-28%) than CD8 (<10%) T cells, compared with approximately equal proportions (18-25% $CD4^+$; 22-23% $CD8^+$) in similarly expanded T cells from PBS/LPS-immunized mice (Figure 2). These findings suggest expansion of $CD4^+$, but not $CD8^+$, cells in response to β2GPI. This is also reflected by the ratio of CD4 to CD8 T cells, which was 3-4-fold higher in β2GPI/LPS-immunized mice than control mice. Approximately 50% of the β2GPI-expanded T cells were IFN-γ^+, with minimal IL-4^+ T cells seen in this population, suggesting a clear Th1 orientation. The percentage of IFN-γ^+ cells in the β2GPI-expanded population from β2GPI/LPS-immunized mice was 2-5-fold greater than that in expanded T cells from PBS/LPS-immunized mice. In contrast, IL-17^+ cells were minimally changed in β2GPI-expanded T cells, relative to the PBS control.

3.5.3 T cells from mice with spontaneous SLE share β2GPI epitopes with induced SLE

We were interested in determining whether T cells from a spontaneous model of SLE also respond to β2GPI. We found that T cells from MRL/lpr mice did not recognize native serum-derived human β2GPI, but reacted very strongly to GP-F, a recombinant full-length β2GPI. The lack of recognition of native β2GPI by MRL/lpr T cells is similar to findings for human β2GPI-reactive T cells from patients with anti-phospholipid syndrome, which recognize recombinant but not native β2GPI[15,16]. For this reason, recombinant GP-F (rather than native β2GPI) was used to expand the numbers of β2GPI-reactive T cells in MRL/lpr mice. Splenic T cells isolated

from naïve 10-week old MRL/lpr (H-2^k) mice were evaluated for their response to native human β2GPI; recombinant proteins encoding Domains I-V of human β2GPI (or MalBP as control); and synthetic peptides covering the human β2GPI sequence (Figure 3A). T cells responded to all of the recombinant fragments of human β2GPI, but poorly to native β2GPI. T cells also responded to multiple peptides over the sequence of β2GPI, but IFN-γ levels were low and variable. These data from naive MRL/lpr mice suggest that β2GPI-reactive T cells are present, but in very low numbers.

To increase the number of β2GPI-reactive T cells, MRL/lpr mice were injected once with recombinant full-length β2GPI (GP-F) and LPS. Splenic T cells from the GP-F-injected mice demonstrated a strong IFN-γ response to all recombinant fragments of β2GPI (Figure 3B), as well as a clear pattern of peptide recognition, particularly for peptides 30/31 and 38. The IL-2 response of these T cells was similar to that of IFN-γ (data not shown). Of note, the pattern of peptide recognition for MRL/lpr T cells was virtually identical to that of T cells from β2GPI/LPS-immunized C3H/HeN mice, which share the same MHC class II haplotype (H-2^k). Importantly, MRL/lpr mice responded to peptides 30/31, which both C3H/HeN and C57BL/6 mice recognize. These data demonstrate that T cells from both spontaneous (MRL/lpr) and induced (β2GPI/LPS-immunized C57BL/6 and C3H/HeN) mice recognize a shared epitope (peptides 30/31) on human β2GPI.

3.5.4 T cells from mice with spontaneous SLE are Th1/Th17-biased

To determine the phenotype of β2GPI-reactive T cells from mice with spontaneous SLE, T cells from MRL/lpr mice (10 weeks) that had received a single injection of either GP-F/LPS or PBS/LPS were expanded *in vitro* with GP-F. Similar to T cells from mice with induced SLE (Figure 2), GP-F-expanded T cells from GP-F/LPS-boosted MRL/lpr mice were predominantly $CD4^+$. However, unlike mice with induced SLE, MRL/lpr-derived T cells expressed moderate levels of IFN-γ but 2-3-fold higher levels of IL-17a, compared to PBS/LPS-boosted mice (Figure 4). These data are consistent with a Th17 phenotype in β2GPI-reactive MRL/lpr T cells[17].

3.5.5 β2GPI-reactive T cells precede the production of SLE autoantibodies

We wanted to ensure that the single injection of β2GPI and LPS used to expand the number of β2GPI-reactive T cells in the spontaneous SLE model (MRL/lpr mice) was not producing an "induced" autoantibody response, similar to that seen in our induced SLE model. In the induced model, we immunize mice repeatedly (3-5 injections) with β2GPI and LPS, whereas MRL/lpr mice received a single injection of recombinant β2GPI (GP-F) and LPS. Moreover, we injected young (5-week-old) MRL/lpr mice, an age defined as being pre-diseased[17] in this model. We evaluated SLE autoantibodies in both GP-F-immunized mice and unimmunized MRL/lpr mice of different ages. At 4-6 weeks, levels of anti-β2GPI and anti-dsDNA were very low or undetectable in unimmunized MRL/lpr mice (Figure 5). Anti-CL levels were low at 4 weeks, but were higher than anti-β2GPI and anti-dsDNA autoantibodies and rose quickly between 4 and 8 weeks. Importantly, in GP-F-injected mice that were used for T cell isolation (i.e., at 7 weeks of age), the levels of anti-β2GPI, anti-CL, and anti-dsDNA were comparable to those of unimmunized MRL/lpr mice of the same age. Further characterization of the anti-CL antibodies in immunized C3H/HeN and unimmunized MRL/lpr mice indicated that C3H/HeN antibodies recognized CL-bound bovine and human β2GPI, while those in MRL/lpr sera had the characteristics of β2GPI-independent antibodies and were inhibited by FBS and human serum (as described by others[18,19]) (Supplemental Figure 1 [top]). In contrast, in direct binding assays on native or recombinant fragments of β2GPI, both C3H/HeN and MRL/lpr sera reacted with native human β2GPI, GP-2 (Domains III and IV), and GP-3 (Domains IV and V). (Supplemental Figure 1 [bottom]). These data indicate that the single injection of GP-F used to expand β2GPI-reactive T cells did not induce SLE autoantibodies in MRL/lpr mice, which had autoantibody levels similar to those of unimmunized mice. These findings suggest that β2GPI-reactive autoantibodies (anti-CL) also are among the earliest to develop in MRL/lpr mice, and that a β2GPI-reactive T cell response precedes the development of SLE autoantibodies in these mice.

3.6 DISCUSSION

In this study, we show that mice with induced and spontaneous SLE share a common T cell epitope on human β2GPI. Both murine models develop the full spectrum of autoantibodies and renal disease seen in human SLE[2,3,20]. T cells from β2GPI/LPS-immunized mice (induced SLE)

and from MRL/lpr mice (spontaneous SLE) recognized peptide 31 (^{165}LYRDTAVFECLPQHAMFG^{182}) in Domain III of β2GPI, as well as adjacent peptide 30 (^{159}SAGNNSLYRDTAVFECLP^{176}) (Figure 6). Interestingly, this T cell epitope was shared across two different MHC class II haplotypes. T cells from mice with induced and spontaneous SLE also shared MHC class II-restricted T cell epitopes; peptide 38 (^{208}PSRPDNGFVNYPAKPTLY^{225}) in Domain IV was recognized by T cells from C3H/HeN and MRL/lpr mice, which both bear the H-2^{k} haplotype. In contrast, there were other MHC class II-restricted peptides, such as peptide 23 (^{111}NTGFYLNGADSAKCT^{125}) and peptide 46 (^{256}AMPSCKASCKVPVKKATV^{273}), that were recognized solely by T cells from mice with induced SLE. The finding of shared T cell epitopes between mice with induced and spontaneous SLE suggests a potential common autoantigenic trigger and T cell potentiation of autoantibodies.

Autoantibodies reactive with β2GPI are among the earliest to occur[2] in human SLE. Moreover, individuals positive for β2GPI-reactive antibodies develop other SLE-related autoantibodies earlier and appear to have a more severe clinical outcome than individuals negative for these antibodies[21], suggesting that β2GPI may be one of the first autoantigens to which immune tolerance is broken. In our induced model, we use a known initiating antigen (β2GPI) to induce a strong β2GPI-reactive T cell response that drives epitope spread to other SLE autoantibodies and ultimately renal disease[3,4]. Here, our data suggest that a β2GPI-reactive T cell response may be important in driving autoantibody production in both induced and spontaneous murine SLE.

Our rationale for comparing the T cell response in this induced model to that of a spontaneous SLE model (MRL/lpr) that mimics human SLE is to provide evidence for common T cell mechanisms in murine and human SLE. Our induced murine model of SLE is generated by immunization of mice with human β2GPI with LPS[3] and we have recently shown that the T cell response associated with epitope spread to SLE autoantibodies is directed to T cell epitopes derived from human β2GPI[4]. For this reason, human β2GPI was used throughout the study. The finding that T cells from MRL/lpr mice with spontaneous SLE recognize peptides derived from human β2GPI may at first seem surprising, but becomes understandable when considering the high degree of homology between human and murine β2GPI (76.5% sequence identity).

Notably, the human β2GPI epitopes recognized by T cells from MRL/lpr and human β2GPI/LPS-immunized mice are closely related to comparable amino acid sequences within murine β2GPI (83.3% and 66.7% identity for peptides 30 and 31, and 77.8% and 72.2% identity for peptides 37 and 38, respectively). The recognition of human β2GPI-derived peptides by T cells from mice with spontaneous and induced SLE indicates that both models likely have T cells that recognize autologous murine β2GPI. Future studies will use murine β2GPI-derived peptides to define the precise autoreactive T cell epitopes within the autologous protein.

The finding that MRL/lpr T cells recognize recombinant human β2-glycoprotein I (β2GPI) (GP-F), but not native human β2GPI, is also consistent with published data for human T cells from healthy controls and from patients with anti-phospholipid syndrome (APS) Human β2GPI-reactive T cells respond to bacterially expressed recombinant β2GPI fragments and chemically reduced β2GPI, but fail to respond to native β2GPI, suggesting that the generation of epitopes recognized by β2GPI-reactive T cells requires unfolding or structural modification of β2GPI. Kuwana et al.[22] provided direct evidence that anionic phospholipid may be involved in the generation of these cryptic T cell epitopes. Dendritic cells or macrophages pulsed with vesicles containing anionic phospholipid and β2GPI, but not β2GPI or phospholipid alone, induced a response in Domain V epitope (p276-290)-specific T cell lines generated from patients in an HLA-DR-restricted manner. These data suggest disease-relevant T cell epitopes in β2GPI may arise as a consequence of antigen processing of anionic phospholipid-bound β2GPI.

The sharing of β2GPI T cell epitopes in both spontaneous and induced SLE models also may speak to common mechanisms for generating these peptides. In particular, the induced model in C3H/HeN mice and spontaneous model in MRL/lpr mice can be compared, as they share the same MHC class II haplotype (H-2^k). That being said, there are major differences between MRL/lpr and C3H/HeN mice. Both the autoimmune predisposition from the MRL/+ background and the *lpr* mutation in Fas impact immune cell composition and function in these mice[20]. The finding that the T cell epitope response to β2GPI is so similar between nonautoimmune C3H/HeN mice and MRL/lpr mice suggests that abnormalities due to the MRL/+ background and the Fas mutation do not impact the antigenic processing and

presentation of β2GPI-derived T cells epitopes. Divergence in T cell responses between these two strains therefore likely lies at a level distinct from antigenic processing and presentation.

One of the differences that we observed was the Th17-orientation of the β2GPI-reactive T cell response in MRL/lpr mice, compared to the Th1-orientation of the response in C3H/HeN and C57BL/6 mice. β2GPI-reactive $CD4^+$ T cells from MRL/lpr mice expressed IL-17 and IFN-γ, while T cells from mice with induced SLE expressed IFN-γ but no IL-17. Th17 cells play a central role in the pathogenesis of human and murine SLE[7,23]. SLE patients have increased numbers of Th17 cells, as well as high serum levels of IL-17[23]. Th17 cells, as well as TCRαβ double negative ($CD3^+CD4^-CD8$) T cells and γδ T cells, produce increased amounts of IL-17 in the kidneys of SLE patients and SLE-prone mice[23]. Of relevance to our findings, Yang et al.[24] found an increased proportion of IL-17^+ $CD4^+$ T cells and a higher expression of IL-17A mRNA in splenocytes from MRL/lpr mice, as compared to splenocytes from C57BL/6. Dai et al.[25] have shown that increased numbers of IL-17-expressing T cells are found in the spleens and lymph nodes of both MRL/lpr and C57BL/6-lpr mice, relative to their control strains (MRL/MpJ and C57BL/6, respectively). In both *lpr*-bearing strains, the major population of IL-17-expressing cells was double negative T cells. These data suggest that IL-17-expressing cells (particularly double negative T cells) are increased in the presence of the *lpr* mutation. The genetic background, however, sets the baseline levels of these cells, which appear to be higher in MRL/MpJ compared with C57BL/6 mice[25]. Dai at al.[25] further showed that lymphocytes from SLE-prone animals expressed progressively higher levels of IL-23 receptor mRNA as their disease developed, and that *in vitro* treatment of lymphocytes with IL-23 resulted in increased levels of IL-23 receptor mRNA and IL-17A mRNA, as well as increased numbers of IL-17A cells and double negative cells. They suggest that IL-23 acts as a "trophic/inducing" cytokine in SLE-prone mice, particularly on double negative T cells, and induces the production of IL-17 instead of IL-2 and IFN-γ. These findings fit well with our findings of IL-17-expressing cells in the spontaneous SLE of MRL/lpr, but not in an induced model of SLE, and suggest that either the MRL/+ or the *lpr* mutation would be needed to observe IL-17-producing cells in our induced model. The data from our induced model further indicate that IL-17-expressing T cells are not necessary for generating a β2GPI-reactive T cell response or for epitope spread to multiple SLE autoantibodies.

The mechanism responsible for generating a β2GPI T cell epitope that is shared not only between spontaneous and induced SLE, but also across MHC class II haplotypes, remains to be determined. In APS, de Moerloose and coworkers[26] demonstrated an immunodominant T cell epitope in Domain I of β2GPI, and related it to anti-β2GPI antibody-binding motifs that could be shared among individuals with different MHC class II haplotypes. To date, there is a paucity of knowledge about common T cell epitopes shared across MHC class II haplotypes in SLE. However, in the more general T cell literature, it is clear that the same peptide can be presented by different MHC class II haplotypes. Greenbaum et al.[27] classified MHC class II molecules into groups (called "supertypes") based on binding to shared peptide motifs or structures, and found a surprising degree of sharing across MHC class II supertypes. They attribute this shared peptide binding to MHC class II backbone interactions rather than peptide anchor residues. There is also evidence for recognition of self-antigen-derived peptides across different MHC class II haplotypes. Individuals with different MHC class II haplotypes present identical endogenous peptides, suggesting that self-peptide expression can be quite promiscuous[28]. Tsai and Santamaria[29] propose that this MHC promiscuity may allow for both protective and pathogenic T cells to interact with the same peptide, but with very different outcomes. They hypothesize that the same peptide may be recognized with higher affinity/avidity when bound to a protective MHC class II molecule, possibly resulting in negative selection or T regulatory cell development, whereas a lower affinity/avidity interaction with a disease-promoting MHC class II molecule would lead to positive selection of autoreactive T cells[29].

The lack of consistent MHC class II associations in SLE, and the multitude of autoantigens targeted, make identification of critical T cell antigen(s) in this disease a major challenge. Multiple HLA alleles, including HLA-DR2 and HLA-DR3, are associated with SLE, but the strength of this association and the specific allele(s) depend on the ethnic group and clinical presentation studied[30]. Identifying a dominant and shared T cell epitope across MHC class II haplotypes suggests a common initiating antigen and should help to identify the mechanisms involved in the initiation and progression of SLE. Previous T cell studies in the MRL/lpr model of SLE have focused mainly on $CD4^+$ T cells reactive to an epitope on the 70K small nuclear ribonucleoprotein (snRNP)[9-11]. However, it is unclear how these snRNP-reactive T cell subsets

fit into the temporal sequence of events leading to SLE. Autoantibodies to snRNP are produced relatively late in the sequential emergence of SLE autoantibodies, while autoantibodies to β2GPI are among the earliest autoantibodies detected in SLE patients[2]. In our induced model, anti-β2GPI antibodies are produced first, but followed soon after by the emergence of multiple SLE autoantibodies in a progression mimicking human SLE[2-4]. Of note, in MRL/lpr mice, anti-CL autoantibodies (which react with bovine β2GPI) appeared early on (4 weeks), while significant levels of anti-dsDNA autoantibodies appeared later (between 6 and 8 weeks) (Figure 5). This finding suggests that recognition of β2GPI epitopes occurs very early in the immune response in both spontaneous (murine and human) and induced SLE. We have previously proposed a model of epitope spread in SLE in which β2GPI-reactive T cells provide help to B cells of different specificities, as long as the B cell presents MHC class II-bound β2GPI epitopes on its surface[3].

In summary, our current data suggest that a common β2GPI-reactive T cell epitope may promote SLE autoantibody production in both induced and spontaneous SLE. Identification of a shared T cell autoantigen in both murine models opens up the possibility that similar findings in human SLE could lead to T cell-based prognostic and therapeutic interventions.

3.7 ACKNOWLEDGEMENTS

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3.8 REFERENCES

1. Tsokos GC, Lo MS, Costa Reis P & Sullivan KE. New insights into the immunopathogenesis of systemic lupus erythematosus. *Nat Rev Rheumatol* 2016; **12**: 716-730.

2. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA *et al.* Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* 2003; **349**: 1526-1533.

3. Levine JS, Subang R, Nasr SH, Fournier S, Lajoie G, Wither J *et al.* Immunization with an apoptotic cell-binding protein recapitulates the nephritis and sequential autoantibody emergence of systemic lupus erythematosus. *J Immunol* 2006; **177**: 6504-6516.

4. Salem D, Subang R, Okazaki Y, Laplante P, Levine JS, Kuwana M *et al.* beta2-Glycoprotein I-specific T cells are associated with epitope spread to lupus-related autoantibodies. *J Biol Chem* 2015; **290**: 5543-5555.

5. Masutani K, Akahoshi M, Tsuruya K, Tokumoto M, Ninomiya T, Kohsaka T *et al.* Predominance of Th1 immune response in diffuse proliferative lupus nephritis. *Arthritis Rheum* 2001; **44**: 2097-2106.

6. Shin MS, Lee N & Kang I. Effector T-cell subsets in systemic lupus erythematosus: update focusing on Th17 cells. *Curr Opin Rheumatol* 2011; **23**: 444-448.

7. Suarez-Fueyo A, Bradley SJ, Klatzmann D & Tsokos GC. T cells and autoimmune kidney disease. *Nat Rev Nephrol* 2017; **13**: 329-343.

8. Hunemorder S, Treder J, Ahrens S, Schumacher V, Paust HJ, Menter T *et al.* TH1 and TH17 cells promote crescent formation in experimental autoimmune glomerulonephritis. *J Pathol* 2015; **237**: 62-71.

9. Monneaux F, Briand JP & Muller S. B and T cell immune response to small nuclear ribonucleoprotein particles in lupus mice: autoreactive CD4(+) T cells recognize a T cell epitope located within the RNP80 motif of the 70K protein. *Eur J Immunol* 2000; **30**: 2191-2200.

10. Monneaux F, Lozano JM, Patarroyo ME, Briand JP & Muller S. T cell recognition and therapeutic effect of a phosphorylated synthetic peptide of the 70K snRNP protein administered in MR/lpr mice. *Eur J Immunol* 2003; **33**: 287-296.

11. Kattah NH, Newell EW, Jarrell JA, Chu AD, Xie J, Kattah MG *et al.* Tetramers reveal IL-17-secreting CD4+ T cells that are specific for U1-70 in lupus and mixed connective tissue disease. *Proc Natl Acad Sci U S A* 2015; **112**: 3044-3049.

12. Andrews BS, Eisenberg RA, Theofilopoulos AN, Izui S, Wilson CB, McConahey PJ *et al.* Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *J Exp Med* 1978; **148**: 1198-1215.

13. Tolomeo T, Rico De Souza A, Roter E, Dieude M, Amireault P, Subang R *et al.* T cells demonstrate a Th1-biased response to native beta2-glycoprotein I in a murine model of anti-phospholipid antibody induction. *Autoimmunity* 2009; **42**: 292-295.

14. Arai T, Yoshida K, Kaburaki J, Inoko H, Ikeda Y, Kawakami Y *et al.* Autoreactive CD4(+) T-cell clones to beta2-glycoprotein I in patients with antiphospholipid syndrome: preferential recognition of the major phospholipid-binding site. *Blood* 2001; **98**: 1889-1896.

15. Hattori N, Kuwana M, Kaburaki J, Mimori T, Ikeda Y & Kawakami Y. T cells that are autoreactive to beta2-glycoprotein I in patients with antiphospholipid syndrome and healthy individuals. *Arthritis Rheum* 2000; **43**: 65-75.

16. Kuwana M. Beta2-glycoprotein I: antiphospholipid syndrome and T-cell reactivity. *Thromb Res* 2004; **114**: 347-355.

17. Perry D, Sang A, Yin Y, Zheng YY & Morel L. Murine models of systemic lupus erythematosus. *J Biomed Biotechnol* 2011; **2011**: 271694.

18. Hashimoto Y, Kawamura M, Ichikawa K, Suzuki T, Sumida T, Yoshida S *et al.* Anticardiolipin antibodies in NZW x BXSB F1 mice. A model of antiphospholipid syndrome. *J Immunol* 1992; **149**: 1063-1068.

19. Verthelyi D & Ansar Ahmed S. Characterization of estrogen-induced autoantibodies to cardiolipin in non-autoimmune mice. *J Autoimmun* 1997; **10**: 115-125.

20. Peng SL. Experimental use of mouse models of systemic lupus erythematosus. *Methods Mol Biol* 2012; **900**: 135-168.

21. McClain MT, Arbuckle MR, Heinlen LD, Dennis GJ, Roebuck J, Rubertone MV *et al.* The prevalence, onset, and clinical significance of antiphospholipid antibodies prior to diagnosis of systemic lupus erythematosus. *Arthritis Rheum* 2004; **50**: 1226-1232.

22. Kuwana M, Matsuura E, Kobayashi K, Okazaki Y, Kaburaki J, Ikeda Y *et al.* Binding of beta 2-glycoprotein I to anionic phospholipids facilitates processing and presentation of a cryptic epitope that activates pathogenic autoreactive T cells. *Blood* 2005; **105**: 1552-1557.

23. Koga T, Ichinose K & Tsokos GC. T cells and IL-17 in lupus nephritis. *Clin Immunol* 2017; **185**: 95-99.

24. Yang J, Chu Y, Yang X, Gao D, Zhu L, Yang X *et al.* Th17 and natural Treg cell population dynamics in systemic lupus erythematosus. *Arthritis Rheum* 2009; **60**: 1472-1483.

25. Dai H, He F, Tsokos GC & Kyttaris VC. IL-23 Limits the Production of IL-2 and Promotes Autoimmunity in Lupus. *J Immunol* 2017; **199**: 903-910.

26. de Moerloose P, Fickentscher C, Boehlen F, Tiercy JM, Kruithof EKO & Brandt KJ. Patient-derived anti-beta2GP1 antibodies recognize a peptide motif pattern and not a specific sequence of residues. *Haematologica* 2017; **102**: 1324-1332.

27. Greenbaum J, Sidney J, Chung J, Brander C, Peters B & Sette A. Functional classification of class II human leukocyte antigen (HLA) molecules reveals seven different supertypes and a surprising degree of repertoire sharing across supertypes. *Immunogenetics* 2011; **63**: 325-335.

28. Costantino CM, Spooner E, Ploegh HL & Hafler DA. Class II MHC self-antigen presentation in human B and T lymphocytes. *PLoS One* 2012; **7**: e29805.

29. Tsai S & Santamaria P. MHC Class II Polymorphisms, Autoreactive T-Cells, and Autoimmunity. *Front Immunol* 2013; **4**: 321.

30. Mohan C & Putterman C. Genetics and pathogenesis of systemic lupus erythematosus and lupus nephritis. *Nat Rev Nephrol* 2015; **11**: 329-341.

3.9 FIGURES & LEGENDS

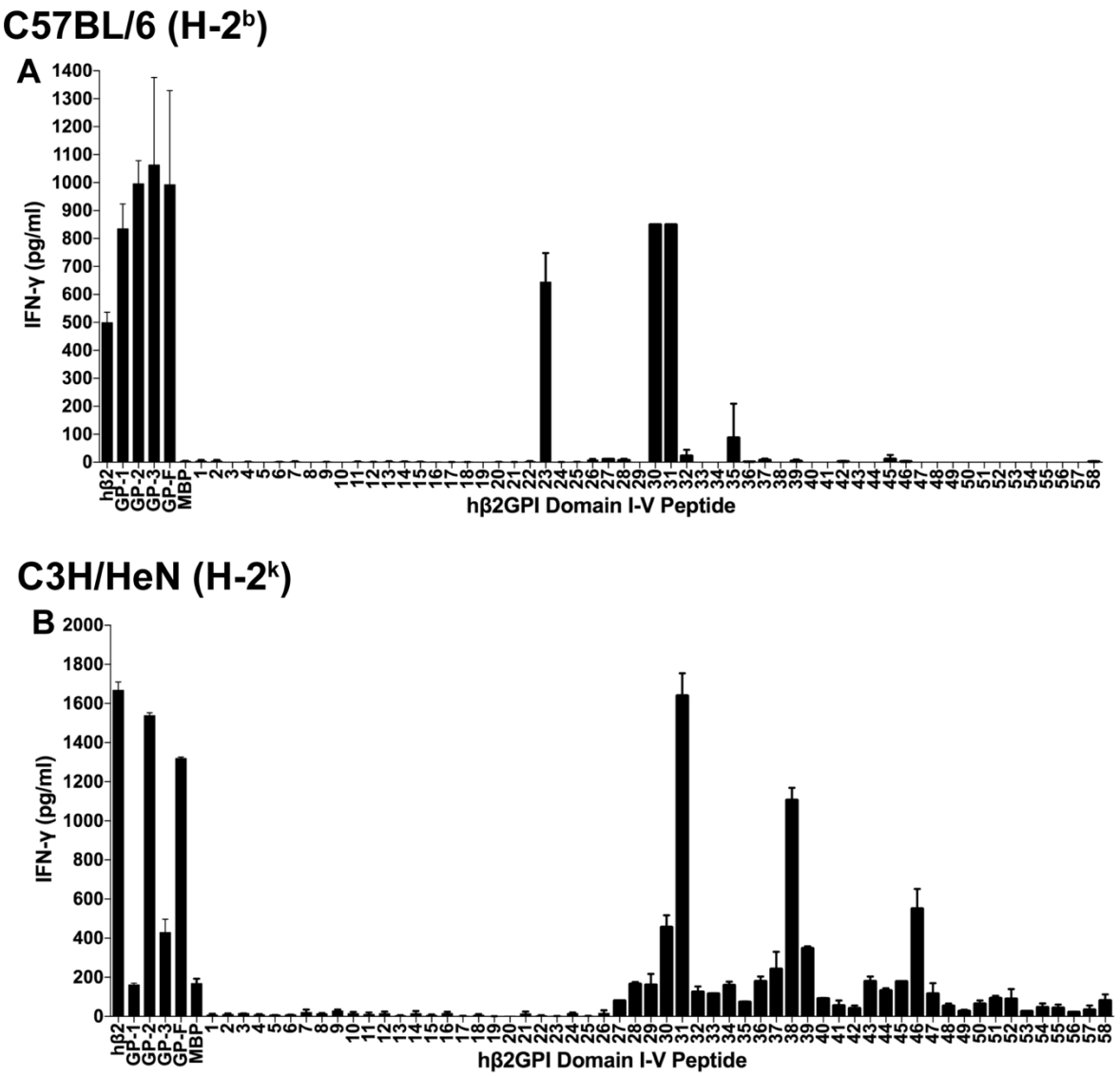


Figure 1. Mice with induced SLE recognize a common β2GPI-T cell epitope. Splenocytes from β2GPI/LPS-immunized mice (C57BL/6 [H-2^b] or C3H/HeN [H-2^k]) were cultured with either human β2GPI (hβ2), GP-1 (Domains I-II), GP-2 (Domains III-IV), GP-3 (Domains IV-V), GP-F (full length β2GPI), or MalBP (MBP; control fusion protein) at a concentration of 20 μg/ml, and individual peptides from Domains I-II (peptides 1-26) or Domains III-V (peptides 27-58) were added at 10 μg/ml. Cells were incubated for 48 hours and IFN-γ production in the supernatant was measured by ELISA. Values represent the mean IFN-γ concentration (pg/ml) ± SE of duplicate samples, and the data shown are pooled from 3 independent experiments.

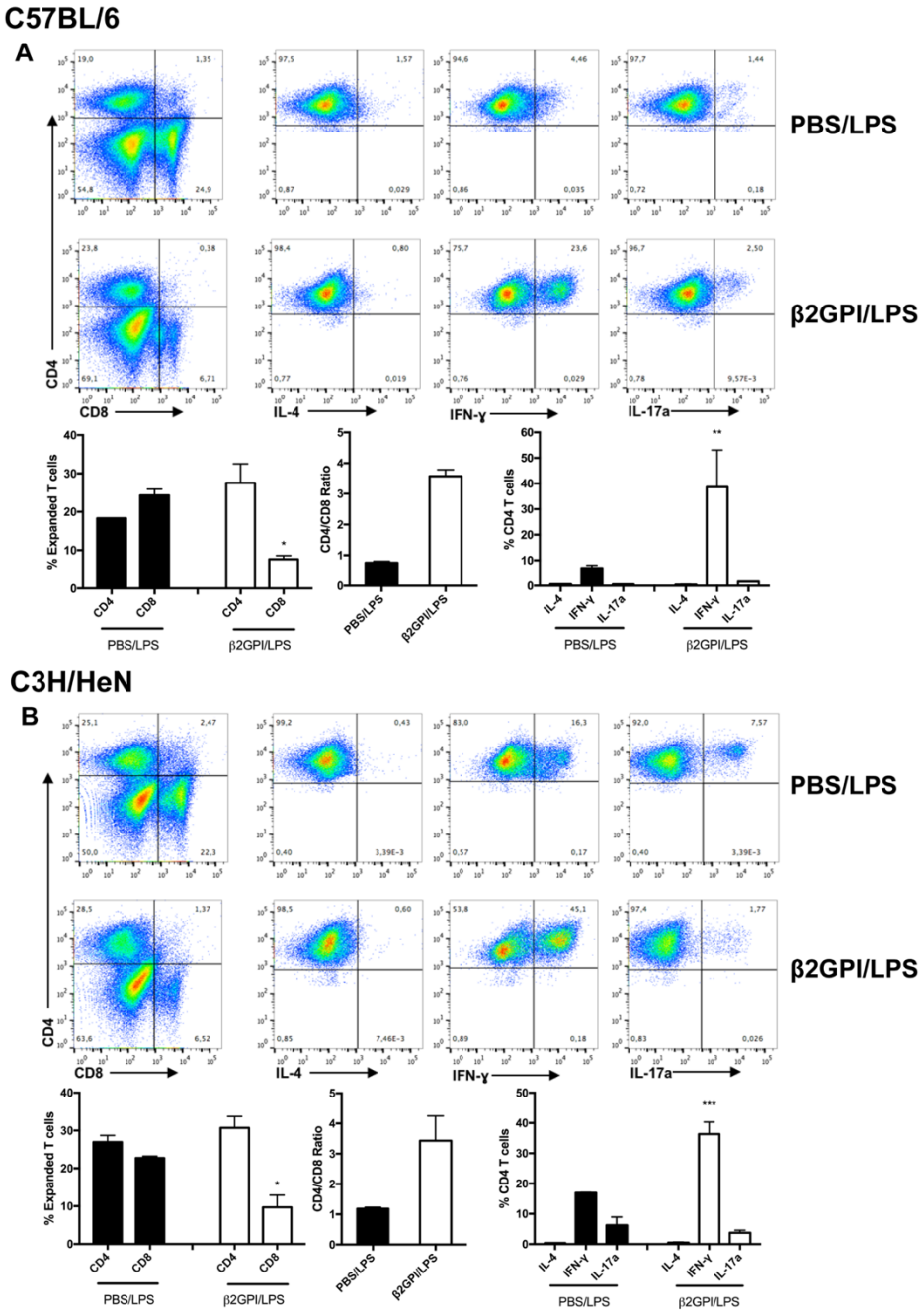


Figure 2. T cells from mice with induced SLE are Th1-biased and produce IFN-γ. Splenocytes from β2GPI/LPS-immunized mice **(**C57BL/6 **[A])** or C3H/HeN **[B]**) were cultured with human β2GPI (15 μg/ml), IL-2 (10 units/ml), and IL-7 (10 ng/ml) for 7 days. Cells were then stimulated with PMA/ionomycin for 3 hours in the presence of monensin and stained for surface and intracellular markers. Flow cytometry plots depict CD4 versus CD8 populations, followed by IL-4, IFN-γ, and IL-17a staining for live $CD4^+$ cells. All cytokine data shown were gated on 3 x 10^4 live $CD4^+$ T cells. Bar graphs summarizing the flow cytometry data show pooled data ± SE of two independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

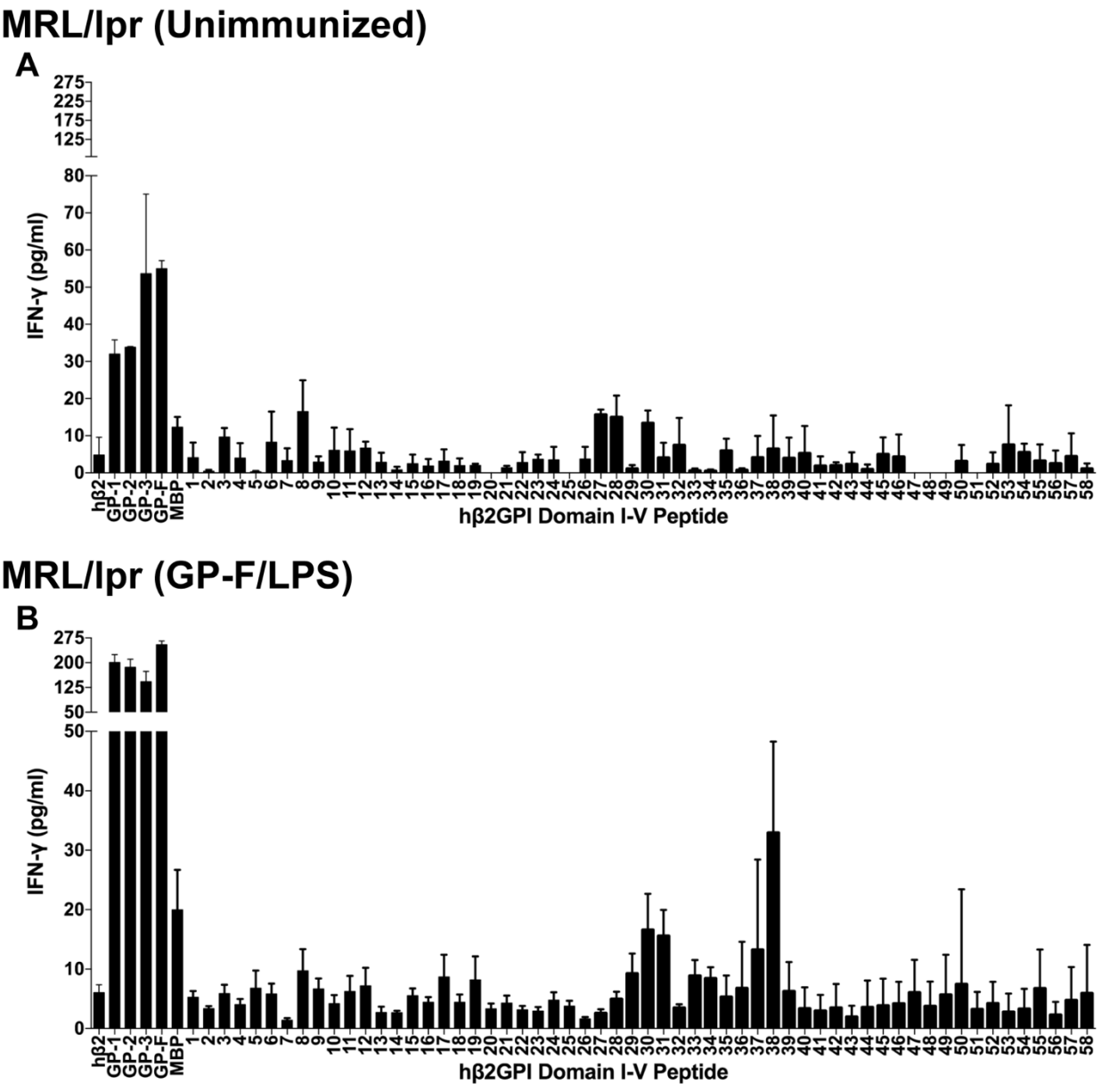


Figure 3. Mice with spontaneous SLE recognize the common β2GPI T cell epitope found in induced SLE. MRL/lpr mice (5 weeks of age) were either left unimmunized **(A)** or boosted once with GP-F and LPS **(B)**, and splenocytes were harvested 10-14 days later. Splenocytes were cultured with either human β2GPI (hβ2), GP-1 (Domains I-II), GP-2 (Domains III-IV), GP-3 (Domains IV-V), GP-F (full length β2GPI), or MalBP (MBP; control fusion protein) at a concentration of 20 μg/ml, and individual peptides from Domains I-II (peptides 1-26) or Domains III-V (peptides 27-58) were added at 10 μg/ml. Cells were incubated for 48 hours and IFN-γ production in the supernatant was measured by ELISA. Values represent the mean IFN-γ concentration (pg/ml) ± SE of duplicate samples, and the data shown are pooled from 3 independent experiments.

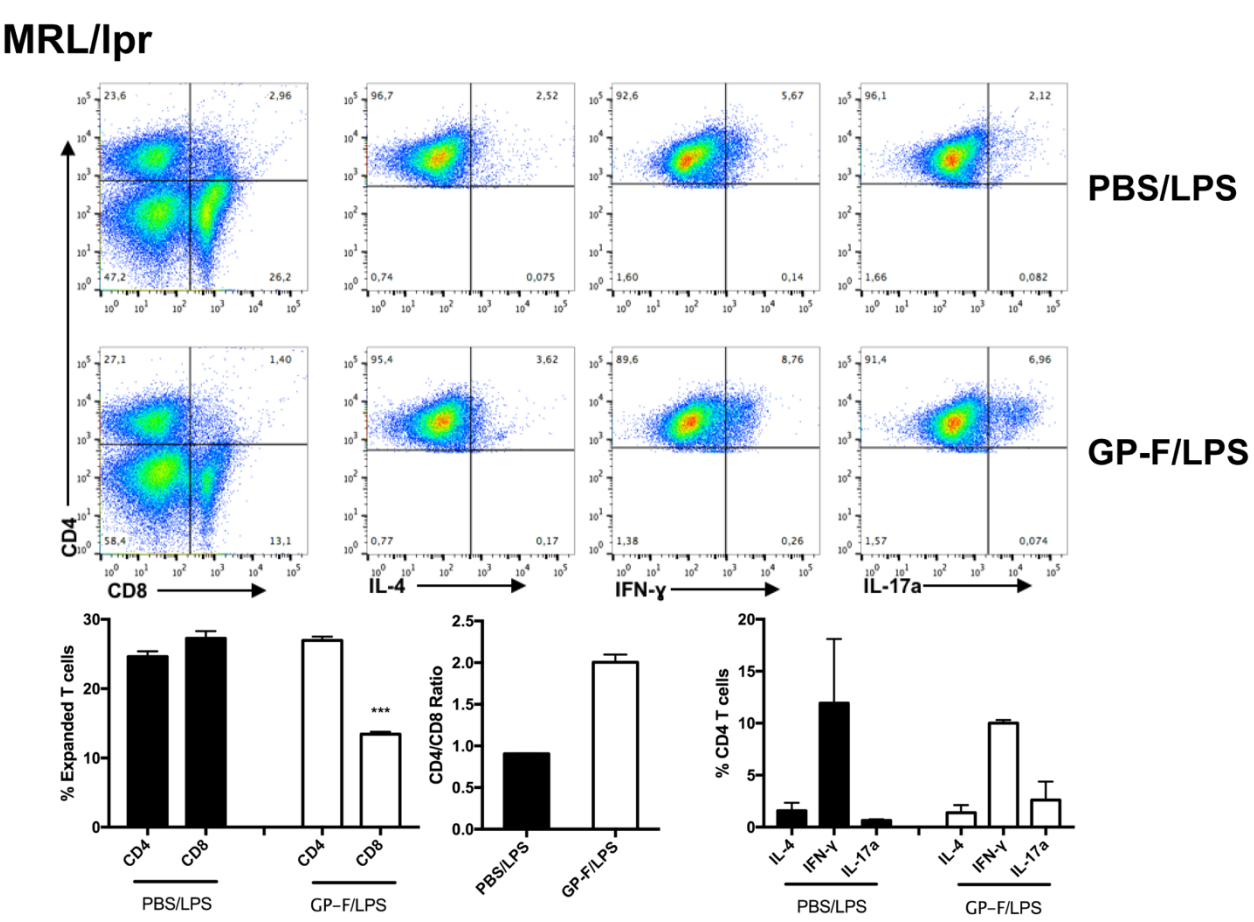


Figure 4. T cells from mice with spontaneous SLE are Th17-biased. Splenocytes from GP-F/LPS or PBS/LPS-boosted MRL/lpr mice (10 weeks old) were cultured with GP-F (15 μg/ml), IL-2 (10 units/ml), and IL-7 (10 ng/ml) for 7 days. Cells were then stimulated with PMA/ionomycin for 3 hours in the presence of monensin and stained for surface and intracellular markers. Flow cytometry plots depict CD4 versus CD8 populations, followed by IL-4, IFN-γ, and IL-17a staining for live $CD4^+$ cells. All cytokine data shown were gated on 3 x 10^4 live $CD4^+$ T cells. Bar graphs graphs summarizing the flow cytometry data show pooled data ± SE of two independent experiments (***, $P < 0.001$).

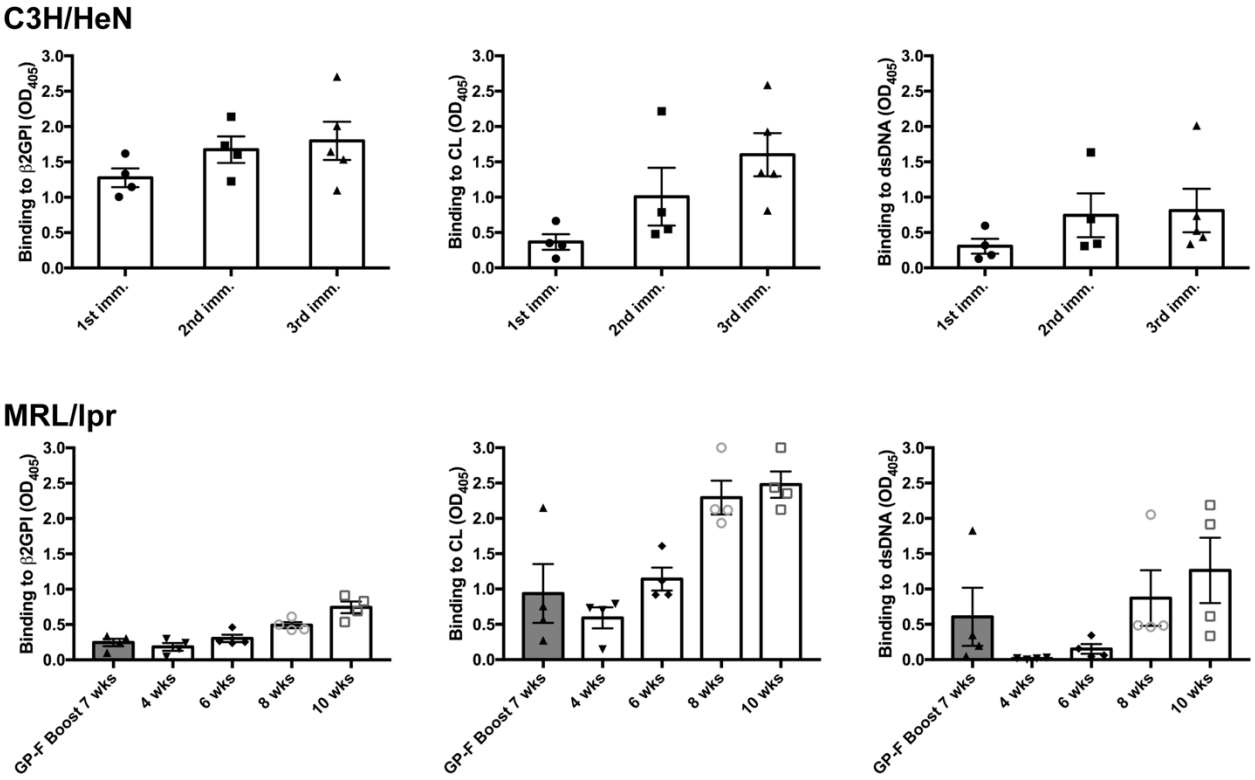


Figure 5. Progression of SLE autoantibody production in induced and spontaneous SLE. Sera from β2GPI/LPS-immunized C3H/HeN mice were tested for IgG anti-β2GPI, anti-CL, and anti-dsDNA autoantibodies by ELISA 14 days after each immunization. As autoantibody titers increased over time, sera were evaluated at multiple dilutions. The dilution shown for each autoantibody and bleed is indicated in square brackets: anti-β2GPI (1st immunization [1/100], 2nd immunization [1/3000], and 3rd immunization [1/5000]); anti-CL (1st immunization [1/100], 2nd and 3rd immunizations [1/5000]); and anti-dsDNA (all immunizations [1/100]). Sera from MRL/lpr mice were obtained every 2 weeks, starting from 4 weeks until 10 weeks of age, and were tested for anti-β2GPI (1/100 dilution), anti-CL (1/1000 dilution) and anti-dsDNA (1/1000 dilution). Each dot represents the mean IgG antibody binding of duplicate samples for an individual mouse (n = 4 - 6 mice/group), and bars indicate the mean blanked OD_{405} ± SE for each group.

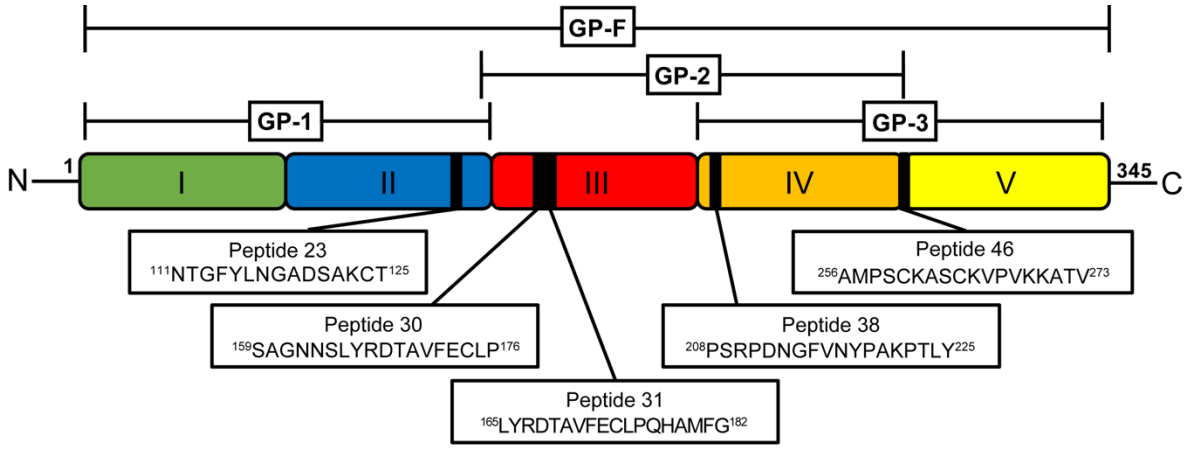
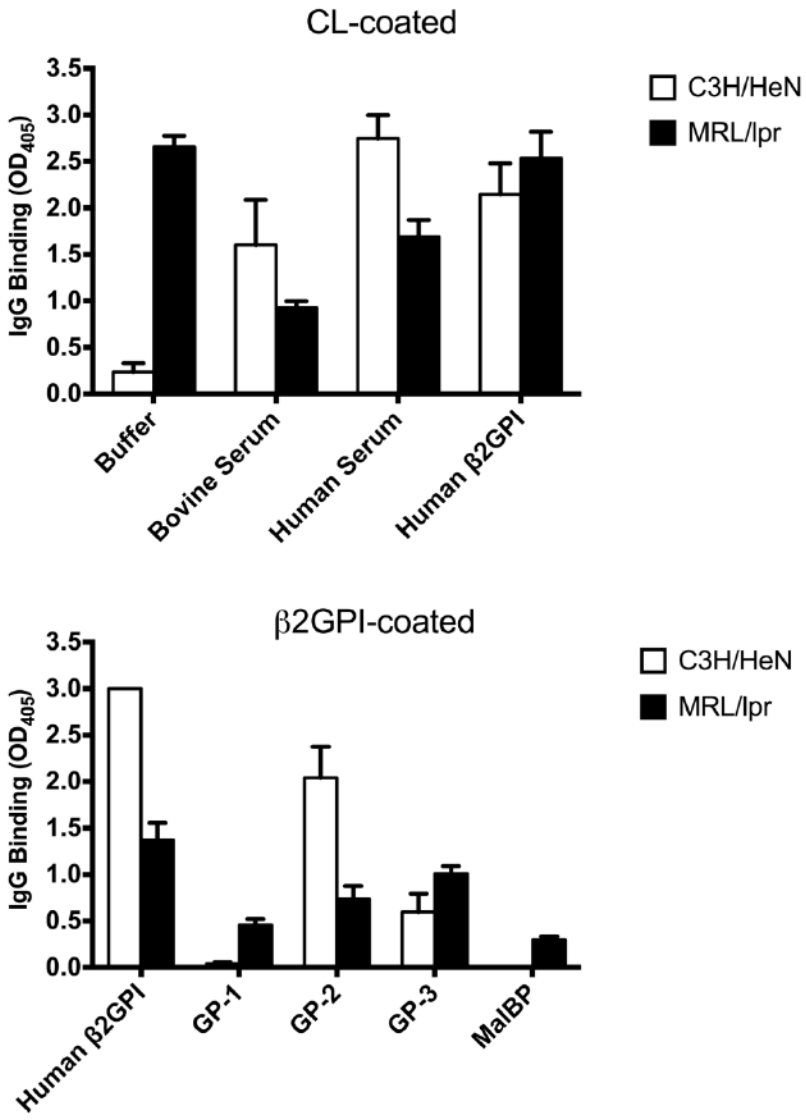


Figure 6. Human β2GPI-derived peptide sequences recognized by mice with induced and spontaneous SLE. This is a schematic representation of the full amino acid sequence of human β2GPI **(center)** and its 5 domains **(shown in color)**. Recombinant fusion proteins GP-1, GP-2, GP-3, and GP-F, and the domains that each fusion protein comprises, are shown above the coloured image of β2GPI. The epitopes (peptides) recognized by T cells from mice with induced (C57BL/6 [H-2^b] and C3H/HeN [H-2^k]) and spontaneous (MRL/lpr [H-2^k]) SLE are shown by **black bars** within the coloured image of β2GPI, with boxes below the image showing the amino acid sequences of each peptide. Notably, peptide 31 in Domain III is recognized by T cells from both induced and spontaneous SLE.



Supplemental Figure 1. Specificity of anti-β2GPI antibodies in induced and spontaneous SLE. Sera from β2GPI/LPS-immunized C3H/HeN (post-second immunization) and MRL/lpr (unimmunized; 10 weeks of age) mice were evaluated for IgG anti-β2GPI antibodies. Top. Cardiolipin (CL)-coated plates were incubated with buffer, 10% fetal bovine serum (FBS), 10% human serum (healthy control), or purified human β2GPI (10 μg/mL) prior to addition of murine serum samples to assess reactivity to CL-bound bovine or human β2GPI. Sera were tested at 1/1000 dilution. Bottom. C3H/HeN (1/1000 dilution) and MRL/lpr (1/100 dilution) sera were evaluated for reactivity to purified native human β2GPI, recombinant protein fragments of human β2GPI (GP-1, GP-2, or GP-3), or MalBP (control fusion protein) coated directly on ELISA plates. For panels A and B, each bar represents the mean IgG antibody binding of duplicate samples for an individual mouse (n = 4 mice/group), and bars indicate the mean blanked OD405 ± SE for each group.

CHAPTER 4

NECROPTOTIC CELL BINDING OF B2-GLYCOPROTEIN I PROVIDES A POTENTIAL AUTOANTIGENIC STIMULUS IN SYSTEMIC LUPUS ERYTHEMATOSUS

4.1 PREFACE

In Chapter 3, we identified a T cell epitope (peptide 31 [LYRDTAVFECLPQHAMFG]) in Domain III of β2GPI that is recognized in induced and spontaneous SLE across different MHC class II haplotypes. Expanded β2GPI-reactive T cells from mice with induced SLE showed a bias towards a Th1 phenotype, whereas T cells from spontaneous SLE mice were skewed more towards a Th17 phenotype. The β2GPI-reactive T cell response in both induced and spontaneous SLE appeared before B cell epitope spread to multiple SLE autoantibodies. In this chapter, we determined the origin of this pro-inflammatory β2GPI-reactive T cell response. We show that both apoptotic and necroptotic cells bind β2GPI, but only necroptotic cells activate antigen presenting cells, and thus activate β2GPI-reactive T cells. We provide evidence for a potential mechanism in which necroptotic cells could promote loss of tolerance of β2GPI, thereby initiating a β2GPI-reactive T cell response and subsequent B cell epitope spread.

NECROPTOTIC CELL BINDING OF β2-GLYCOPROTEIN I PROVIDES A POTENTIAL AUTOANTIGENIC STIMULUS IN SYSTEMIC LUPUS ERYTHEMATOSUS

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Running title: *Necroptotic cell binding of β2-glycoprotein I*

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4.2 ABSTRACT

Systemic lupus erythematosus (SLE) is characterized by the development of autoantibodies against diverse self-antigens with damage to multiple organs. Immunization with β2-glycoprotein I (β2GPI), an SLE autoantigen, induces a murine model of SLE. We hypothesize that binding of β2GPI to apoptotic and necroptotic cells presents the immune system with a "scaffold" of cellular self-antigens, but only necroptotic cells do so in a pro-inflammatory and immunogenic context. Here, we show that β2GPI binds to necroptotic L929 cells, providing a potential target for SLE autoantibodies. We demonstrate that necroptotic, but not apoptotic, cells enhance pro-inflammatory cytokine (TNF-α) secretion by activated macrophages and dendritic cells *in vitro*. Moreover, necroptotic cells promote MHC class II and costimulatory molecule

expression in immature dendritic cells, leading to an enhanced CD4 T cell response to β2GPI *in vitro*. Finally, we show that mice deficient in *Ripk3* (receptor-interacting serine/threonine-protein kinase 3), and hence necroptosis, show poor induction of SLE. Our data suggest that necroptotic cells provide both self-antigens and pro-inflammatory signals that may be sufficient to overcome immune tolerance and induce SLE.

4.3 INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that can result in life-long suffering and premature death. It is characterized by the development of autoantibodies against diverse self-antigens, resulting in damage to multiple organs. Dying cells represent an abundant source of self-antigens[1,2], and apoptosis has long been thought to play a key role in the pathogenesis of SLE[3,4]. Apoptotic cells are typically anti-inflammatory and tolerogenic[5-7], making it unclear how these cells promote autoimmunity. In fact, association of antigen with apoptotic cells generally suppresses the adaptive response to that antigen[5-7].

Necroptosis is a caspase-independent form of cell death with features of both apoptosis and necrosis[8-13]. Like apoptosis, necroptosis is regulated by a defined molecular cascade. However, unlike apoptosis, necroptotic cells rupture and release damage-associated molecular patterns that promote inflammation[10-12,14]. Thus, necroptotic cells have the capacity to be pro-inflammatory[10,12,15-17] and immunogenic[12,18-20]. Necroptosis has garnered much attention recently because of its apparent role in inflammatory pathologies[10,12,15-17]. Pharmacologic or genetic inhibition of necroptosis is protective in numerous animal models of inflammatory pathology[10,12,16,21]. However, there is a paucity of published data on necroptosis in SLE[22,23].

We have previously shown that β2-glycoprotein I (β2GPI), a circulating plasma phospholipid-binding protein and known SLE autoantigen, binds to apoptotic cells[24]. We have also demonstrated that immunization with β2GPI, in the presence of a pro-inflammatory stimulus like lipopolysaccharide (LPS), elicits a murine model of SLE, complete with the production of SLE autoantibodies and glomerulonephritis[2]. The emergence of distinct SLE autoantibodies occurs in a sequential manner exactly paralleling that seen in human SLE[25]. Moreover,

autoantibody production is associated with a strong β2GPI-reactive T cell response[26].

Here, we determined whether the paradigm for autoimmune recognition of β2GPI bound to apoptotic cells can be extended to necroptotic cells. We hypothesized that necroptotic cells, like apoptotic cells, provide a "scaffold" of cellular self-antigens, but, unlike apoptotic cells, necroptotic cells do so in a pro-inflammatory and immunogenic context. We demonstrate that β2GPI indeed binds to necroptotic cells and serves as a target for anti-β2GPI autoantibodies. We further demonstrate that necroptotic cells promote antigenic presentation of β2GPI to CD4 T cells by dendritic cells. Finally, we show that mice deficient in *Ripk3* (receptor-interacting serine/threonine-protein kinase 3), and consequently unable to undergo necroptosis, show poor induction of SLE autoantibodies. Together, these data suggest that necroptotic cells provide an immunogenic "scaffold" of cellular self-antigens that may trigger the initiation of SLE.

4.4 EXPERIMENTAL PROCEDURES

4.4.1 Mice

Specific pathogen-free female C57BL/6 mice (8-12 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME). Female RIPK3$^{-/-}$ C57BL/6 mice were generously provided by Vishva Dixit (Genentech, San Francisco) (34,43). Mice were maintained and bred according to Canadian Council on Animal Care (CCAC) guidelines (consistent with the National Institutes of Health guide for the care and use of laboratory animals [NIH Publications No. 8023, revised 1978]), and maintained on food and water *ad libitum*. Animal experiments were approved by the McGill University Animal Care Committee.

4.4.2 Reagents

Unless stated otherwise, all reagents were obtained commercially from the following sources and used without further purification: qualified fetal bovine serum (FBS; Invitrogen, Thermo Fisher Scientific, Mississauga, ON); human β2 glycoprotein I (β2GPI) (Crystal Chem, Downers Grove, IL); bovine heart cardiolipin (CL) (Avanti Polar Lipids, Alabaster, AL);

lipopolysaccharide (LPS; *Escherichia coli*-derived, serotype O111:B4; List Biological Laboratories, Campbell, CA); recombinant mouse tumor necrosis factor-α (TNF-α), Zombie NIR fixable viability kit, PerCP Cy5.5-conjugated anti-mouse CD11c, PE-Cy7-conjugated anti-mouse CD86, APC-conjugated anti-mouse CD40, Alex-Fluor 488-conjugated anti-mouse MHC-II (I-A^b), and LEGENDplex Multi-Analyte Flow Assay Kit for IL-1β, IL-10, IFN-α, and IFN-β (BioLegend, San Diego, CA); z-VAD-fmk (Enzo Life Sciences, Farmingdale, NY); actinomycin-D, staurosporine, and p-nitrophenyl phosphate (Sigma Aldrich Canada, Oakville, ON); 7-Cl-O-Necrostatin-1 (Nec-1s) (EMD Millipore, Etobicoke, ON); GSK'872 (EMD Millipore); PE-conjugated annexin V, Negative Control Compensation Particles Set (Anti-Mouse Ig κ; and Anti-Rat and Anti-Hamster Ig κ), F_C block (FcgIII/II receptor-CD16/32), Alexa-Fluor 700-conjugated anti-mouse CD11b, PE anti-mouse CD80, FITC-conjugated Active Caspase-3 Apoptosis Kit, mouse TNF-α enzyme-linked immunoassay (ELISA) set II, mouse interleukin-2 (IL-2) ELISA set, mouse interferon-γ (IFN-γ) ELISA set (AN-18), and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate reagent set (BD OptEIA kit) (BD Biosciences, Mississauga, ON); 7-aminoactinomycin D (7-AAD) (eBioscience, San Diego, CA); Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-human IgG (Southern Biotech, Birmingham, AL); *E. coli* DNA (dsDNA) (Immunovision, Springdale, AR); alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Southern Biotech); and nProtein A Sepharose 4 Fast Flow column (GE Healthcare Life Sciences, Chicago, IL). Murine monoclonal anti-human β2GPI antibody (21C5-48.2) and isotype control monoclonal antibody 29J3-119 were produced in our laboratory, and have been described previously[27].

4.4.3 Cell culture

The L929 mouse fibroblast cell line was generously provided by Dr. Samuel David (McGill University), but was originally from American Type Culture Collection (ATCC) (ATCC #CCL-1; ATCC, Manassas, VA). L929 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) medium (4.5 g/L glucose, 110 mg/mL sodium pyruvate) containing 10% heat-inactivated FBS, 1% penicillin-streptomycin, and 1% Minimal Essential Medium (MEM) vitamin (medium and supplements from Life Technologies Inc., Burlington, ON) (hereafter referred to as "L929 medium"). The B10R mouse macrophage cell line (henceforth referred to

as “B10R macrophages”) was generously provided by Dr. Danuta Radzioch (McGill University). B10R macrophages were grown in DMEM medium (4.5 g/L glucose, 110 mg/mL sodium pyruvate), containing 7% heat-inactivated FBS and 1% penicillin-streptomycin (B10R medium), and used at passage 2-6. All cells were grown at 37°C (5% CO_2), unless stated otherwise.

Bone marrow derived macrophages (BMDMs) and dendritic cells (BMDCs) were obtained from C57BL/6 mice. Briefly, bone marrow cells were cultured with either L929 cell-derived macrophage colony-stimulating factor (M-CSF; 40% for the first 5 days, and 10% for the final 2 days) for BMDMs, or 20% granulocyte macrophage colony-stimulating factor (GM-CSF) derived from X-63 GM-CSF cells[44] (a kind gift of Dr. Brigitta Stockinger [The Francis Crick Institute, London, UK]) for 7 days for BMDCs. In some experiments, GM-CSF from PeproTech (Rocky Hill, NJ) was used at 20 ng/mL (final concentration). BMDM medium consisted of Roswell Park Memorial Institute (RPMI) containing 10% heat-inactivated FBS, 1% penicillin-streptomycin, 2% HEPES, 1% L-glutamine, 1% sodium pyruvate, and 1% non-essential amino acids. BMDC medium consisted of RPMI containing 10% heat-inactivated FBS, 1% penicillin-streptomycin, 1% HEPES, 1% L-glutamine, 1% non-essential amino acids, and 0.1% 2-mercaptoethanol.

4.4.4 Induction and detection of cell death

L929 cells were plated ($2x10^5$ cells/well) in 700 µl of L929 medium in 24-well plates, and incubated for 16 hr. Apoptosis was induced by treatment of plated L929 cells with actinomycin D (4 µg/mL final concentration) and TNF-α (100 ng/mL final concentration), or vehicle (0.01% DMSO) as control, in a total volume of 1 mL, and incubated for 16 hr. Where indicated, apoptosis was also induced by treatment of plated L929 cells with staurosporine (1 µg/mL final concentration), or vehicle (0.01% DMSO) as control, in a total volume of 1 mL, and incubated for 4 hr. Necroptosis was induced by treatment of plated L929 cells with zVAD-fmk (25 µM final concentration) and TNF-α (100 ng/mL final concentration), or vehicle (0.01% DMSO), in a total volume of 1 mL for 6 hr. When used, necroptosis inhibitor Nec-1s (50 µM) or GSK’872 (50 µM) was added immediately following the addition of zVAD-fmk and TNF-α. After induction of apoptosis or necroptosis, non-adherent cells were removed and kept, and adherent

cells were detached with 0.25% Trypsin-EDTA (10 minutes at 23ºC). Non-adherent and detached cells were pooled and washed twice with cold 0.01 M phosphate-buffered saline, pH 7.3 (PBS), resuspended in binding buffer (0.01 M HEPES, 0.14 M NaCl, and 2.5 mM $CaCl_2$, pH 7.4), and stained with 7-AAD and PE-annexin V for 15 minutes in the dark at 23ºC. Anti-active caspase-3-detection was done separately on fixed/permeabilized cells according to the kit protocol. Cells were analyzed using a FACSCanto II flow cytometer and FACSDiva software (BD Bioscience), and plots, gated on viable cells, were generated using FlowJo software (Tree Star, Ashland, OR).

4.4.5 Detection of β2GPI/anti-β2GPI binding

After induction of apoptosis or necroptosis, cells were washed with cold FACS Buffer (10 mM HEPES, 140 mM NaCl, 1% bovine serum albumin, 0.02% NaN_3, pH 7.4) and incubated with 100 µl of human β2GPI (10 µg/mL in FACS Buffer) for 20 minutes on ice. Purified murine monoclonal anti-human β2GPI antibody (21C5-48.2), or isotype control antibody 29J3-119, was then added (100 µl; 20 µg/mL final concentration, in FACS Buffer) to the cells and incubated for 30 minutes on ice. Cells were washed with PBS and stained with Alexa Fluor 488-conjugated goat anti-mouse IgG (1/1000 dilution) for 20 minutes on ice, followed by washing and staining with 7-AAD and annexin V as described above. Cells were then either analyzed by flow cytometry or by confocal microscopy (LSM-780; ZEN imaging software [ZEISS, Germany]).

Human sera were obtained with written informed consent from patients in the McGill University Lupus Clinic SLE registry or healthy controls. Patients were classified as having SLE according to the American College of Rheumatology (ACR) criteria[45]. IgG was purified from sera using nProtein A Sepharose 4 beads. Cells were incubated with 100 µl human β2GPI (10 µg/mL in FACS Buffer) for 20 minutes on ice, followed by 100 µl of serum (1/10 dilution in FACS Buffer) or purified serum-derived IgG (100 µg/mL in FACS Buffer) for 30 minutes on ice. Cells were washed and stained with Alexa Fluor 488-conjugated goat anti-human IgG (1:1000), washed twice with PBS, and stained with 7-AAD and annexin V as described above.

4.4.6 Effect of apoptotic versus necroptotic cells on cytokine production

B10R macrophages were plated in 24-well plates ($1x10^5$ cells/well) in DMEM medium containing 7% FBS, and incubated for 16-20 hr. Following aspiration of the culture medium and addition of DMEM medium containing 1% heat-inactivated FBS, the cells were allowed to rest for 16-20 hr. The culture medium was then aspirated and 500 μL of either apoptotic or necroptotic L929 cells in X-VIVO 10 medium (Lonza Inc., Allendale, NJ) were added at varying concentrations (expressed as "apoptotic or necroptotic target: macrophage ratio"; e.g., 1:1), and incubated for 90 minutes. The range of concentrations of target cells was 0.0625 to 1.0. The medium containing the apoptotic or necroptotic cells was then removed, and the B10R cells were washed gently with X-VIVO 10 medium. LPS (1 ng/mL final concentration), or medium (control), was added to the B10R cells, and the cells were cultured for 16 hr. Cell culture medium was collected from each well, and TNF-α levels quantified by ELISA according to the manufacturer's protocol. Results were expressed as the mean TNF-α concentration (pg/mL) of duplicate samples, as determined from a standard curve using recombinant TNF-α.

4.4.7 Effect of apoptotic versus necroptotic cells on MHC class II and costimulatory molecule expression

BMDMs or BMDCs were plated in 6-well plates ($1x10^6$ cells/well) in their respective media and incubated for 16-20 hr. One mL of either apoptotic or necroptotic L929 cells was added to the plated BMDMs or BMDCs at different apoptotic or necroptotic target: BMDM or BMDC ratios (e.g., 1:1), and incubated for 90 minutes. The culture medium containing the apoptotic or necroptotic cells was then removed, and the cells were washed gently with complete RPMI medium containing 10% heat-inactivated FBS, 1% penicillin-streptomycin, 1% HEPES, 1% L-glutamine, 1% non-essential amino acids, and 0.1% 2-mercaptoethanol. LPS (1 ng/mL final concentration), or medium (control), was then added, and the cells were cultured for 16 hr. Cell culture medium was collected from each well, and TNF-α levels quantified by ELISA. Adherent cells were then detached using a cell scraper and washed twice with 1% FBS-PBS.

For flow cytometry analysis, the BMDMs or BMDCs were incubated with 10 μg/mL Fc-Block (15 minutes; 4°C), washed twice with PBS, and stained with viability dye (30 minutes;

23°C). The cells were then washed twice with 1% FBS-PBS, and stained with antibodies to CD11b, CD11c, MHC class II, CD80, CD86, and CD40 (30 minutes; 4°C). Cells were analyzed using a BD LSRFortessa X-20 flow cytometer and FACSDiva software (BD Bioscience), and plots, gated on viable cells, were generated using FlowJo software.

4.4.8 Effect of apoptotic versus necroptotic cells on T cell response to β2GPI

BMDCs were plated in 24-well plates ($1x10^5$ cells/well) in their respective medium and incubated for 16-20 hr. One mL of either apoptotic or necroptotic L929 cells was added to the cells at different concentrations (expressed as apoptotic or necroptotic target: BMDC ratios; e.g., 1:1), and incubated for 90 minutes. The culture medium containing the apoptotic or necroptotic cells was then removed, and the cells were washed gently with BMDC medium. Human β2GPI (15 μg/well) was added to cells and incubated for 2 hr. Then, LPS (1 ng/mL final concentration), or medium (control), was added, and the cells were further incubated for 16 hr. Splenic T cells were isolated from C57BL/6 mice immunized four times with β2GPI and LPS[26] using an EasySep negative isolation T cell kit (StemCell Technologies, Vancouver, BC). The β2GPI-reactive T cells ($3x10^5$ cells/well) were then added to BMDCs and incubated for 48 hr. Cell culture medium was collected, and IL-2 levels quantified by ELISA, according to the manufacturer's protocol. Results were expressed as the mean IL-2 concentration (pg/mL) of duplicate samples, as determined from a standard curve using recombinant IL-2.

4.4.9 Immunization and SLE autoantibody detection

C57BL/6 wild type (WT) and $RIPK3^{-/-}$ mice were immunized intravenously with human β2GPI (20 μg) on Day 1 and LPS (15 μg for first and second immunizations, and 10 μg for third and fourth immunizations) on Day 2 every two weeks, and mice were bled after four immunizations, as previously described[2,26]. Serum levels of anti-β2GPI, anti-cardiolipin (anti-CL), anti-double stranded DNA (anti-dsDNA), anti-Ro (SS-A), anti-La (SS-B), anti-Sm, and anti-nRNP autoantibodies were determined by ELISA, as previously described[2,26].

4.4.10 Histology

Two days prior to sacrifice, β2GPI/LPS-immunized mice received one intravenous injection of 10 μg of β2GPI (without a subsequent LPS injection). Mice were sacrificed, and the kidneys were harvested and fixed in 10% formalin. Following dehydration through a graded series of ethanol, tissue slices were embedded in paraffin, sectioned at 3 microns, and stained with hematoxylin-eosin (H&E) or periodic acid-Schiff (PAS). All slides were read by a pathologist who was blinded to the immunogen used.

4.4.11 Data and statistical analysis

Data were plotted using Prism 7.0 (GraphPad Software Inc., San Diego, CA). Statistical significance was determined by a two-tailed unpaired Student's *t*-test using Microsoft Excel 2013, or a two-way Anova (Prism 7.0). The minimal threshold for significance was $p < 0.05$.

4.5 RESULTS

4.5.1 β2GPI binds to necroptotic cells

We have previously shown that β2GPI binds to apoptotic cells[24]. To determine whether β2GPI also binds to necroptotic cells, we induced apoptosis or necroptosis in L929 cells using well-established protocols for this murine cell line[13]. We established optimal conditions for induction, and observed ~70% early apoptosis (annexin V^+, 7-AAD^-) and ~90% necroptosis (annexin V^+, 7-AAD^+) **(Figure 1A)**. Vehicle-treated viable cells (89% annexin V^-, 7-AAD^-) served as our negative control, and are referred to as "viable cells" throughout our experiments. Cells induced to undergo apoptosis stained positive for active caspase-3, while viable cells and necroptotic cells were negative for active caspase-3. Necroptotic cell death was inhibited by either Nec-1s (RIPK-1 inhibitor) or GSK'872 (RIPK-3 inhibitor), both of which reduced the percentage of annexin V^+, 7-AAD^+ cells to levels similar to that of vehicle-treated viable cells. These data show that we have distinct preparations of viable, apoptotic, and necroptotic L929 cells.

To determine whether human β2GPI binds to necroptotic cells, we used a murine monoclonal anti-human β2GPI antibody (21C5-48.2)[27]. We evaluated binding of β2GPI to apoptotic and viable cells in parallel; viable cells served as an internal negative control. Consistent with previous findings from our[24] and other[28-30] studies, we found that β2GPI bound to apoptotic, but not to viable, cells (mean fluorescence intensity [MFI] ± SE of pooled data from three independent experiments: 1370 ± 347 versus 114 ± 15, $p<0.005$) **(Figure 1B)**. Notably, β2GPI bound equally well to necroptotic cells (MFI ± SE: 1069 ± 362, $p<0.05$). Anti-β2GPI binding was specific for β2GPI, as shown by the lack of antibody binding in the presence of an irrelevant protein (human serum albumin [HSA]). An isotype control antibody was negative for binding to viable, apoptotic, and necroptotic cells. These data demonstrate that, like apoptotic cells, necroptotic cells bind β2GPI and are recognized by monoclonal anti-β2GPI antibodies.

To identify whether β2GPI binds to phosphatidylserine on the surface of dead cells, we co-stained cells with anti-β2GPI, PE-annexin V, and 7-AAD, and analyzed them by confocal microscopy **(Figure 1C)**. Apoptotic cells showed surface staining for anti-β2GPI (green) and annexin V (red), but no co-localization of these stains. As expected, apoptotic cells were negative for staining with 7-AAD (white). Necroptotic cells similarly showed surface staining for anti-β2GPI, but the annexin V staining localized in the cytoplasm and primarily in the nucleus, possibly suggesting movement of annexin V into the cytoplasm and then the nucleus. In addition, the nuclei of the necroptotic cells stained positive for 7-AAD (white), and showed co-localization of PE-annexin V and 7-AAD staining (pink). Viable cells were negative for all markers and visible only using differential interference contrast (DIC) imaging. The specificity of anti-β2GPI binding was confirmed using an isotype control antibody on viable, apoptotic, and necroptotic cells. No IgG binding was observed, but the other markers on these cells were present (i.e., apoptotic cells were annexin V^+, 7-AAD^-, and necroptotic cells were annexin V^+, 7-AAD^+). These data confirm the surface binding of β2GPI to both apoptotic and necroptotic cells. In both forms of cell death, β2GPI binds to the cell surface, but independently of annexin V binding.

4.5.2 Necroptotic cells promote pro-inflammatory cytokine production in antigen-presenting cells

Given the ability of β2GPI to bind to both necroptotic and apoptotic cells[31], and the known role of β2GPI as an antigenic target for SLE autoantibodies[32], we examined the functional effects of these two types of cell death on antigen-presenting cells (macrophages and dendritic cells). First, we evaluated the effect of apoptotic versus necroptotic cells on a macrophage cell line (B10R). As the ratio of apoptotic cells to responder cells (B10R cells) increased from 0.0156 to 4, there was a clear dose-dependent suppression of TNF-α secretion when cells were stimulated with LPS **(Figure 2A)**. In contrast, the same ratios of necroptotic cells resulted in enhanced TNF-α secretion by B10R cells. Maximal effects on TNF-α secretion were observed at a ratio of 1:1 for apoptotic cells (~55% decrease) and 4:1 for necroptotic cells (~160% increase). In contrast to TNF-α, production of the anti-inflammatory cytokine IL-10 was unaffected by apoptotic cells, but suppressed by necroptotic cells (data not shown). Similar effects were observed with primary bone marrow-derived macrophages (BMDMs) **(Figure 2B [top])** and primary bone marrow-derived dendritic cells (BMDCs) **(Figure 2B [bottom])**.

4.5.3 Necroptotic cells promote MHC class II and co-stimulatory molecule expression in dendritic cells

Necroptotic, but not apoptotic, cells enhanced pro-inflammatory cytokine production by antigen-presenting cells, suggesting activation of these cells. We therefore evaluated whether treatment with necroptotic versus apoptotic cells also affected BMDC expression of MHC class II (I-A^b) and costimulatory molecules (CD80, CD86, and CD40) **(Supplemental Figure 1)**. In the presence of apoptotic cells, the percentage of BMDCs (live $CD11c^+CD11b^+$ cells) expressing MHC class II and costimulatory molecules (CD80, CD86, and CD40) was minimally affected **(Figure 3 [left panels])**. In contrast, the presence of necroptotic cells resulted in a dose-dependent increase in BMDCs expressing MHC class II, CD86, and CD40. The increased frequency was ~2.5 fold for MHC class II, CD86, and CD40. There was no change in the frequency of CD80-expressing cells, but this is not surprising given the constitutive expression of CD80. The intensity of expression of these activation markers on BMDCs showed the same trend as their expression frequency **(Figure 3 [right panels])**. These data indicate that

necroptotic cells promote MHC class II and costimulatory molecule expression in dendritic cells, while apoptotic cells have little effect on this expression.

4.5.4 Necroptotic cells promote antigen presentation of β2GPI to T cells

Given the ability of necroptotic cells to enhance MHC class II and costimulatory expression in BMDCs, we next determined whether necroptotic cells promote the presentation of β2GPI to T cells, resulting in a stronger T cell proliferative response to this antigen. For these experiments, BMDCs were derived from a naïve C57BL/6 mouse, and T cells were derived from a β2GPI/LPS-immunized mouse. T cell responses are shown for LPS-stimulated BMDCs, as responses with unstimulated BMDCs were very low (data not shown). While actinomycin D-induced apoptotic cells suppressed the T cell response to β2GPI, as measured by IFN-γ production, necroptotic cells increased the response in a dose-dependent manner (0.0625, 0.25, and 1 dead cell: BMDC ratio) **(Figure 4A)**. Similar findings were observed for IL-2 production by T cells treated with staurosporine-induced apoptotic cells **(Figure 4B)**. These data demonstrate that necroptotic cells promote the response of primary T cells to β2GPI, while apoptotic cells suppress this response.

4.5.5 RIPK3, a regulator of necroptosis, is required for the induction of SLE autoantibodies by β2GPI

Our findings suggest that necroptotic cells can serve both as a target for anti-β2GPI antibody binding and as a promoter of a β2GPI-reactive T cell response. To directly evaluate whether necroptotic cells play a role in the production of autoantibodies in SLE, we used a murine model of SLE in which β2GPI is the primary antigenic stimulus. We have previously shown that induction of SLE in this model[2] is associated with a strong T cell response to β2GPI[26]. We immunized mice deficient in *Ripk3* (RIPK3$^{-/-}$), a critical regulator of the necroptosis pathway[33] or WT C57BL/6 mice with β2GPI and LPS to induce the SLE model. Importantly for our model, RIPK3$^{-/-}$ mice develop adaptive immune responses to T cell-dependent antigens and respond to LPS similarly to WT mice[34]. We found that the RIPK3$^{-/-}$ mice produced significantly lower levels of SLE autoantibodies than WT mice. All SLE

autoantibodies, including ones highly specific to SLE (anti-dsDNA and anti-Sm), were dramatically reduced in the RIPK3$^{-/-}$ mice **(Figure 5A)**. SLE-related renal pathology was reduced in RIPK3$^{-/-}$ mice, compared to the WT strain (**Figure 5B**). Histologic analysis of kidney sections from WT and RIPK3$^{-/-}$ mice stained with H&E and PAS revealed a dense inflammatory infiltrate in the interstitium of WT, but not RIPK3$^{-/-}$, mice that had been immunized with β2GPI and LPS. In the WT kidneys, the inflammatory infiltrate ranged from absent to mild and moderate, while in the RIPK3$^{-/-}$ kidneys, no inflammatory cells were identified. The inflammatory infiltrate was predominantly composed of perivascular lymphocytes and plasmocytes with focal interstitial extension. No other consistent vascular, tubular, or glomerular pathological changes were identified. These data demonstrate that RIPK3 is required for induction of this β2GPI-driven model of murine SLE, suggesting a possible role for necroptosis in this process.

4.6 DISCUSSION

In this study, we demonstrate that, like apoptotic cells, necroptotic cells bind β2GPI and serve as a target for anti-β2GPI autoantibodies. Importantly, we also show that these two forms of cell deaths differ dramatically in their effects on antigen-presenting cells and, consequently, on T cell activation. Necroptotic cells promote pro-inflammatory cytokine production by macrophages and dendritic cells, while apoptotic cells suppress this response. Similarly, necroptotic, but not apoptotic, cells promote MHC class II and co-stimulatory molecule expression in dendritic cells, resulting in an enhanced T cell response to β2GPI. Finally, RIPK3$^{-/-}$ mice, which are deficient in necroptosis, had decreased autoantibody production and pathology in a β2GPI-induced model of SLE. These data suggest that RIPK3-dependent necroptosis may play a role in SLE, either as an inducer or as a target of autoantibodies, or both.

We demonstrate that β2GPI binds to the surface of necroptotic cells, as it does to apoptotic cells. Using a high affinity murine monoclonal antibody to β2GPI antibody to detect β2GPI binding, our data suggest that β2GPI is equivalently displayed on both necroptotic and apoptotic cells. β2GPI binding did not co-localize with annexin V, suggesting that these proteins bind to different epitopes on phosphatidylserine. These findings are consistent with those of Cocca et

al.[35], who showed that binding of antibody D56R/S76R single-chain variable fragments, which recognize the β2GPI/phosphatidylserine complex, coincided with annexin V binding only in early apoptotic cells. In later stages of apoptosis, binding of the antibody and annexin V localized to non-overlapping membrane domains. Our binding data show that apoptotic and necroptotic cells can both serve as targets for antibodies to β2GPI.

β2GPI binding to necroptotic cells suggests that these cells, like apoptotic cells, can provide a "scaffold" of cellular self-antigens taken up by phagocytic receptors that bind β2GPI[36] or β2GPI-reactive B cells[2,37]. To investigate the potential of dying cells to promote immunity to β2GPI, we evaluated the functional consequences of exposing antigen-presenting cells (i.e., macrophages and DCs) to apoptotic or necroptotic cells. We found that exposure to necroptotic cells increased production of TNF-α, a pro-inflammatory cytokine, in LPS-stimulated macrophages and DCs, while exposure to apoptotic cells decreased TNF-α production. Furthermore, immature BMDCs incubated with necroptotic, but not apoptotic, cells had increased expression of both MHC class II and costimulatory molecules. These data indicate that necroptotic cells provide a sufficient stimulus to immature APCs to enhance surface expression of moledules critical to antigen presentation. Indeed, necroptotic, but not apoptotic, cell-treated BMDCs enhanced the CD4 T cell response to β2GPI.

Previously, necroptotic cells have been shown to stimulate CD8[20] and gamma-delta[19] T cell responses. In the case of CD8 T cells, necroptotic NIH-3T3 cells promoted murine BMDC expression of CD86, CD40, and I-A^b (MHC class II) *in vitro*, and immunization of mice with ovalbumin-expressing necroptotic cells resulted in cross-priming of CD8 T cells *in vivo*[20]. To our knowledge, ours is the first study to demonstrate that necroptotic cells not only activate BMDCs to undergo maturation, but also promote an enhanced CD4 T cell response to an autoantigen (in this case, to β2GPI).

To address the physiological relevance of these findings, we used an induced model of murine SLE developed in our laboratory[2,26]. We asked whether induction of SLE by β2GPI and LPS was impacted in $RIPK3^{-/-}$ mice, which are deficient in necroptosis. Strikingly, $RIPK3^{-/-}$ mice were significantly protected from developing SLE-associated autoantibodies and pathology,

suggesting that necroptosis or another RIPK3-dependent mechanism is critical in the induction of SLE in this model.

There is a significant, albeit recent, literature about the role of necroptosis or other RIPK3-dependent processes in inflammatory pathologies, including autoimmune disease[10,12,15-17]. Evidence for necroptosis in inflammatory pathologies has come largely from studies using RIPK1 inhibitors[21] or *Ripk3*-deficient mice[12]. RIPK1 inhibitors have been shown to be protective in animal models of brain ischemia, myocardial infarction, renal ischemia-reperfusion, and multiple sclerosis (MS)[10,12,15-17], while mice genetically deficient in *Ripk3* or *Mlkl* (Mixed Lineage Kinase Domain-Like) (proteins critical for necroptosis) were protected in models of inflammatory bowel disease (IBD), and skin or liver inflammation[12]. However, recent identification of non-necroptotic functions of RIPK3, particularly apoptosis and inflammatory responses, indicates that RIPK3 dependence extends beyond necroptosis[38,39]. Findings in *Ripk3*-deficient mice are not always replicated in mice deficient in *Mlkl*, the executor molecule for necroptosis, indicating a role for RIPK3-dependent but necroptosis-independent pathways[11]. For example, *Mlkl* deficiency was less protective than *Ripk3* deficiency in a kidney ischemia-reperfusion injury model, and provided no benefit in a model of systemic inflammation[11]. Studies evaluating the impact of MLKL deficiency in our SLE model are currently underway.

Many of the models and human pathology studied to date involve tissue injury or inflammation, rather than autoimmune-mediated tissue damage. Far less is know about necroptosis in autoimmune diseases, particularly SLE. However, studies in MS and IBD shed light on the potential involvment of necroptosis in autoimmunity. Defective caspase-8 activation, combined with increased activation of RIPK1, RIPK3, and MLKL, were detected in cortical lesions of MS patients. Similarly, in murine models of MS, RIPK3 deficiency or treatment with a RIPK1 inhibitor was protective against oligodendrocyte cell death and MS-like disease symptoms[16]. In IBD, RIPK3 and MLKL were highly expressed in inflamed intestinal mucosa of pediatric patients[17], and *ex vivo* inhibition of necroptosis in colonic mucosal tissue explants reduced cytokine production and MLKL phosphorylation in these tissues[40]. Findings in these two organ-specific autoimmune diseases suggest that RIPK3 and MLKL activity are increased in the targeted organ, and that inhibition of necroptosis may prevent organ damage.

Unlike MS and IBD, SLE is a systemic autoimmune disease in which multiple organs are targeted. To date, there is a paucity of published data on necroptosis and other RIPK3-dependent processes in SLE. Two published reports suggest the presence of necroptosis-related markers in human and murine SLE, but neither study attempted to evaluate the role of necroptosis or other RIPK3-dependent processes in SLE autoantibody production and disease[22,41]. A third study[42] concluded that RIPK3 is not involved in the systemic autoimmunity observed in murine chronic graft versus host disease and pristane-induced SLE, but neither model developed glomerular or interstitial renal disease.

Necroptotic, but not apoptotic, cells release damage-associated molecular patterns (DAMPs) that induce maturation of dendritic cells *in vitro*[20] and inflammation *in vivo*[10-12]. We propose that the immunostimulatory effects of necroptotic cells may be key in inducing the strong CD4 T cell response to β2GPI that we observe in mice immunized with β2GPI and LPS **(Figure 6)**. Based on the association between SLE autoantibody production and a strong T cell response to β2GPI in this model[26], we hypothesize that β2GPI-reactive T cells provide help not only to anti-β2GPI-producing B cells, but also to B cells of other specificities that internalize necroptotic cells with β2GPI bound to their surface. We do not exclude the possibility that β2GPI-bound apoptotic cells may also be internalized by B cells and involved in epitope spread of the autoantibody response. Figure 6 shows an example of this possibility for dsDNA-specific B cells, but this would also apply to B cells specific for other SLE autoantigens expressed on the necroptotic cell surface. In this manner, multiple autoreactive B cells can be activated by a single β2GPI-specific T cell, leading to the emergence of autoantibodies observed in our induced model of SLE.

In conclusion, to our knowledge, our findings are the first to show that β2GPI binds to necroptotic cells and is recognized by anti-β2GPI antibodies when bound to these cells. We also show for the first time that RIPK3 is required for the induction of autoantibodies in a murine model of SLE. Finally, we demonstrate that necroptotic cells promote MHC class II and costimulatory molecule expression on dendritic cells, and enhance a CD4 T cell response to

β2GPI. Together with the model proposed above, our findings suggest that necroptosis and/or other RIPK3-dependent pathways may play a role in the induction of SLE.

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4.7.1 Footnotes

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The abbreviations used are: anti-CL, anti-cardiolipin; anti-β2GPI, anti-β2-glycoprotein I; anti-dsDNA, anti-double stranded DNA; aPL, anti-phospholipid antibodies; β2GPI, β2-glycoprotein I; BMDCs, bone marrow-derived dendritic cells; BMDMs, bone marrow-derived macrophages; ELISA, enzyme-linked immunoassay; MFI, mean fluorescence intensity; RIPK3, receptor-interacting serine/threonine-protein kinase 3; SLE, systemic lupus erythematosus.

4.8 REFERENCES

1. Casciola-Rosen LA, Anhalt G & Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J Exp Med* 1994; **179**: 1317-1330.

2. Levine JS, Subang R, Nasr SH, Fournier S, Lajoie G, Wither J *et al.* Immunization with an apoptotic cell-binding protein recapitulates the nephritis and sequential autoantibody emergence of systemic lupus erythematosus. *J Immunol* 2006; **177**: 6504-6516.

3. Jung JY & Suh CH. Incomplete clearance of apoptotic cells in systemic lupus erythematosus: pathogenic role and potential biomarker. *Int J Rheum Dis* 2015; **18**: 294-303.

4. Podolska MJ, Biermann MH, Maueroder C, Hahn J & Herrmann M. Inflammatory etiopathogenesis of systemic lupus erythematosus: an update. *J Inflamm Res* 2015; **8**: 161-171.

5. Getts DR, Turley DM, Smith CE, Harp CT, McCarthy D, Feeney EM *et al.* Tolerance induced by apoptotic antigen-coupled leukocytes is induced by PD-L1+ and IL-10-producing splenic macrophages and maintained by T regulatory cells. *J Immunol* 2011; **187**: 2405-2417.

6. Liu K, Iyoda T, Saternus M, Kimura Y, Inaba K & Steinman RM. Immune tolerance after delivery of dying cells to dendritic cells in situ. *J Exp Med* 2002; **196**: 1091-1097.

7. Su RJ, Epp A, Latchman Y, Bolgiano D, Pipe SW & Josephson NC. Suppression of FVIII inhibitor formation in hemophilic mice by delivery of transgene modified apoptotic fibroblasts. *Mol Ther* 2010; **18**: 214-222.

8. Galluzzi L, Bravo-San Pedro JM, Vitale I, Aaronson SA, Abrams JM, Adam D *et al.* Essential versus accessory aspects of cell death: recommendations of the NCCD 2015. *Cell Death Differ* 2015; **22**: 58-73.

9. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV *et al.* Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ* 2012; **19**: 107-120.

10. Linkermann A & Green DR. Necroptosis. *N Engl J Med* 2014; **370**: 455-465.

11. Newton K & Manning G. Necroptosis and Inflammation. *Annu Rev Biochem* 2016; **85**: 743-763.

12. Pasparakis M & Vandenabeele P. Necroptosis and its role in inflammation. *Nature* 2015; **517**: 311-320.

13. Vanden Berghe T, Grootjans S, Goossens V, Dondelinger Y, Krysko DV, Takahashi N *et al.* Determination of apoptotic and necrotic cell death in vitro and in vivo. *Methods* 2013; **61**: 117-129.

14. Gong YN, Guy C, Olauson H, Becker JU, Yang M, Fitzgerald P *et al.* ESCRT-III Acts Downstream of MLKL to Regulate Necroptotic Cell Death and Its Consequences. *Cell* 2017; **169**: 286-300 e216.

15. Gunther C, Martini E, Wittkopf N, Amann K, Weigmann B, Neumann H *et al.* Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. *Nature* 2011; **477**: 335-339.

16. Ofengeim D, Ito Y, Najafov A, Zhang Y, Shan B, DeWitt JP *et al.* Activation of necroptosis in multiple sclerosis. *Cell Rep* 2015; **10**: 1836-1849.

17. Pierdomenico M, Negroni A, Stronati L, Vitali R, Prete E, Bertin J *et al.* Necroptosis is active in children with inflammatory bowel disease and contributes to heighten intestinal inflammation. *Am J Gastroenterol* 2014; **109**: 279-287.

18. Aaes TL, Kaczmarek A, Delvaeye T, De Craene B, De Koker S, Heyndrickx L *et al.* Vaccination with Necroptotic Cancer Cells Induces Efficient Anti-tumor Immunity. *Cell Rep* 2016; **15**: 274-287.

19. Collins CC, Bashant K, Erikson C, Thwe PM, Fortner KA, Wang H *et al.* Necroptosis of Dendritic Cells Promotes Activation of gammadelta T Cells. *J Innate Immun* 2016; **8**: 479-492.

20. Yatim N, Jusforgues-Saklani H, Orozco S, Schulz O, Barreira da Silva R, Reis e Sousa C *et al.* RIPK1 and NF-kappaB signaling in dying cells determines cross-priming of CD8(+) T cells. *Science* 2015; **350**: 328-334.

21. Takahashi N, Duprez L, Grootjans S, Cauwels A, Nerinckx W, DuHadaway JB *et al.* Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. *Cell Death Dis* 2012; **3**: e437.

22. Fan H, Liu F, Dong G, Ren D, Xu Y, Dou J *et al.* Activation-induced necroptosis contributes to B-cell lymphopenia in active systemic lupus erythematosus. *Cell Death Dis* 2014; **5**: e1416.

23. Fenton K. The effect of cell death in the initiation of lupus nephritis. *Clin Exp Immunol* 2015; **179**: 11-16.

24. Price BE, Rauch J, Shia MA, Walsh MT, Lieberthal W, Gilligan HM *et al.* Anti-phospholipid autoantibodies bind to apoptotic, but not viable, thymocytes in a beta 2-glycoprotein I-dependent manner. *J Immunol* 1996; **157**: 2201-2208.

25. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA *et al.* Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* 2003; **349**: 1526-1533.

26. Salem D, Subang R, Okazaki Y, Laplante P, Levine JS, Kuwana M *et al.* beta2-Glycoprotein I-specific T cells are associated with epitope spread to lupus-related autoantibodies. *J Biol Chem* 2015; **290**: 5543-5555.

27. Laplante P, Fuentes R, Salem D, Subang R, Gillis MA, Hachem A *et al.* Antiphospholipid antibody-mediated effects in an arterial model of thrombosis are dependent on Toll-like receptor 4. *Lupus* 2016; **25**: 162-176.

28. Balasubramanian K & Schroit AJ. Characterization of phosphatidylserine-dependent beta2-glycoprotein I macrophage interactions. Implications for apoptotic cell clearance by phagocytes. *J Biol Chem* 1998; **273**: 29272-29277.

29. Cocca BA, Seal SN, D'Agnillo P, Mueller YM, Katsikis PD, Rauch J *et al.* Structural basis for autoantibody recognition of phosphatidylserine-beta 2 glycoprotein I and apoptotic cells. *Proc Natl Acad Sci U S A* 2001; **98**: 13826-13831.

30. Pittoni V, Ravirajan CT, Donohoe S, MacHin SJ, Lydyard PM & Isenberg DA. Human monoclonal anti-phospholipid antibodies selectively bind to membrane phospholipid and beta2-glycoprotein I (beta2-GPI) on apoptotic cells. *Clin Exp Immunol* 2000; **119**: 533-543.

31. Levine JS, Subang R, Koh JS & Rauch J. Induction of anti-phospholipid autoantibodies by beta2-glycoprotein I bound to apoptotic thymocytes. *J Autoimmun* 1998; **11**: 413-424.

32. de Groot PG & de Laat B. Mechanisms of thrombosis in systemic lupus erythematosus and antiphospholipid syndrome. *Best Pract Res Clin Rheumatol* 2017; **31**: 334-341.

33. Weinlich R, Oberst A, Beere HM & Green DR. Necroptosis in development, inflammation and disease. *Nat Rev Mol Cell Biol* 2017; **18**: 127-136.

34. Newton K, Sun X & Dixit VM. Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *Mol Cell Biol* 2004; **24**: 1464-1469.

35. Cocca BA, Cline AM & Radic MZ. Blebs and apoptotic bodies are B cell autoantigens. *J Immunol* 2002; **169**: 159-166.

36. Maiti SN, Balasubramanian K, Ramoth JA & Schroit AJ. Beta-2-glycoprotein 1-dependent macrophage uptake of apoptotic cells. Binding to lipoprotein receptor-related protein receptor family members. *J Biol Chem* 2008; **283**: 3761-3766.

37. Salem D SR, Kuwana M, Levine JS, and Rauch J. T cells from induced and spontaneous models of SLE recognize a common T cell epitope in β2-glycoprotein I. . *Cell Mol Immunol (in press)*. 2018.

38. Orozco S & Oberst A. RIPK3 in cell death and inflammation: the good, the bad, and the ugly. *Immunol Rev* 2017; **277**: 102-112.

39. Wegner KW, Saleh D & Degterev A. Complex Pathologic Roles of RIPK1 and RIPK3: Moving Beyond Necroptosis. *Trends Pharmacol Sci* 2017; **38**: 202-225.

40. Negroni A, Colantoni E, Pierdomenico M, Palone F, Costanzo M, Oliva S *et al.* RIP3 AND pMLKL promote necroptosis-induced inflammation and alter membrane permeability in intestinal epithelial cells. *Dig Liver Dis* 2017; **49**: 1201-1210.

41. Honarpisheh M, Desai J, Marschner JA, Weidenbusch M, Lech M, Vielhauer V *et al.* Regulated necrosis-related molecule mRNA expression in humans and mice and in murine acute tissue injury and systemic autoimmunity leading to progressive organ damage, and progressive fibrosis. *Biosci Rep* 2016; **36**.

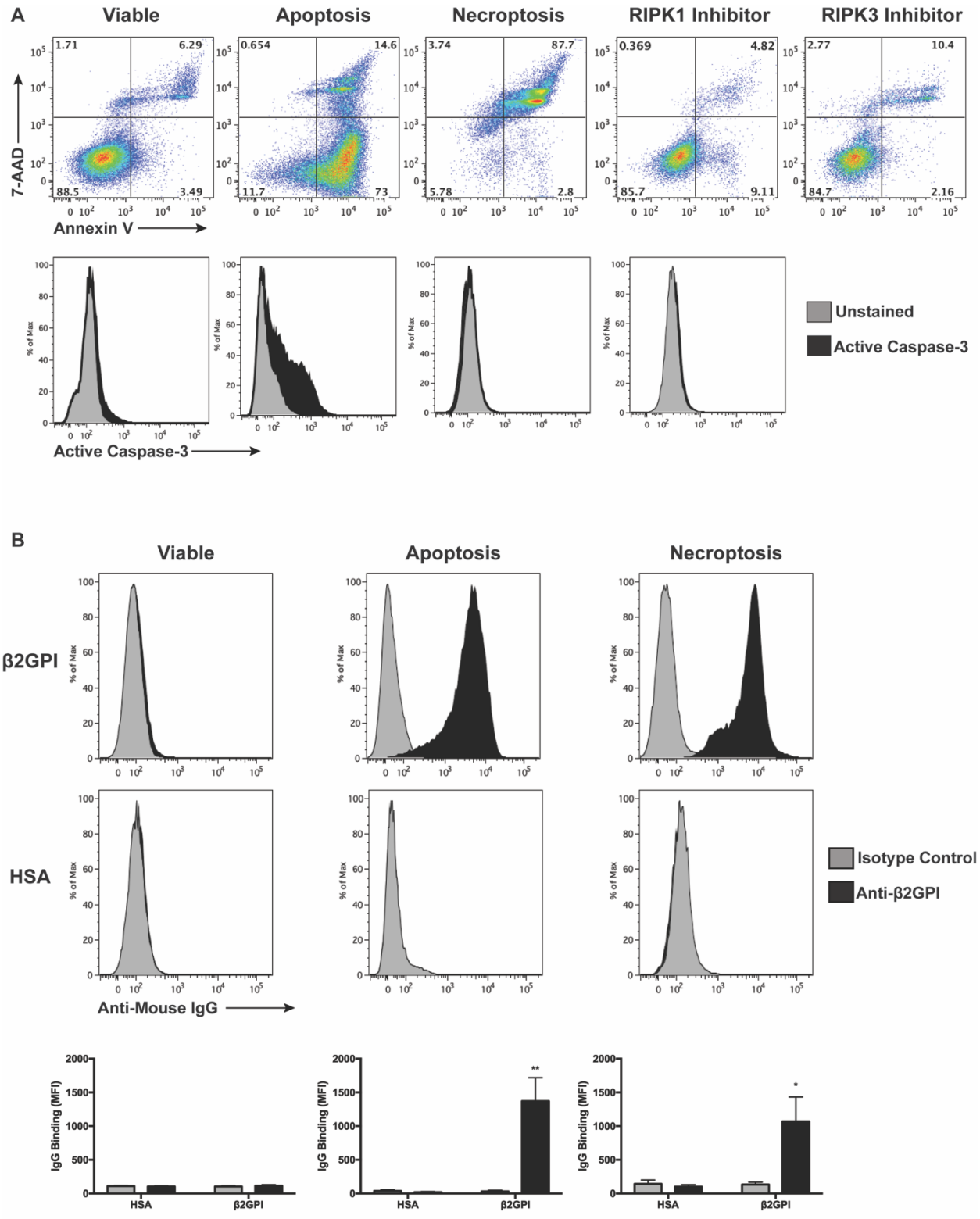
42. Corradetti C, Jog NR, Gallucci S, Madaio M, Balachandran S & Caricchio R. Immune-Mediated Nephropathy and Systemic Autoimmunity in Mice Does Not Require Receptor Interacting Protein Kinase 3 (RIPK3). *PLoS One* 2016; **11**: e0163611.

43. Downey J, Pernet E, Coulombe F, Allard B, Meunier I, Jaworska J *et al.* RIPK3 interacts with MAVS to regulate type I IFN-mediated immunity to Influenza A virus infection. *PLoS Pathog* 2017; **13**: e1006326.

44. Stockinger B, Zal T, Zal A & Gray D. B cells solicit their own help from T cells. *J Exp Med* 1996; **183**: 891-899.

45. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271-1277.

4.9 FIGURES & LEGENDS



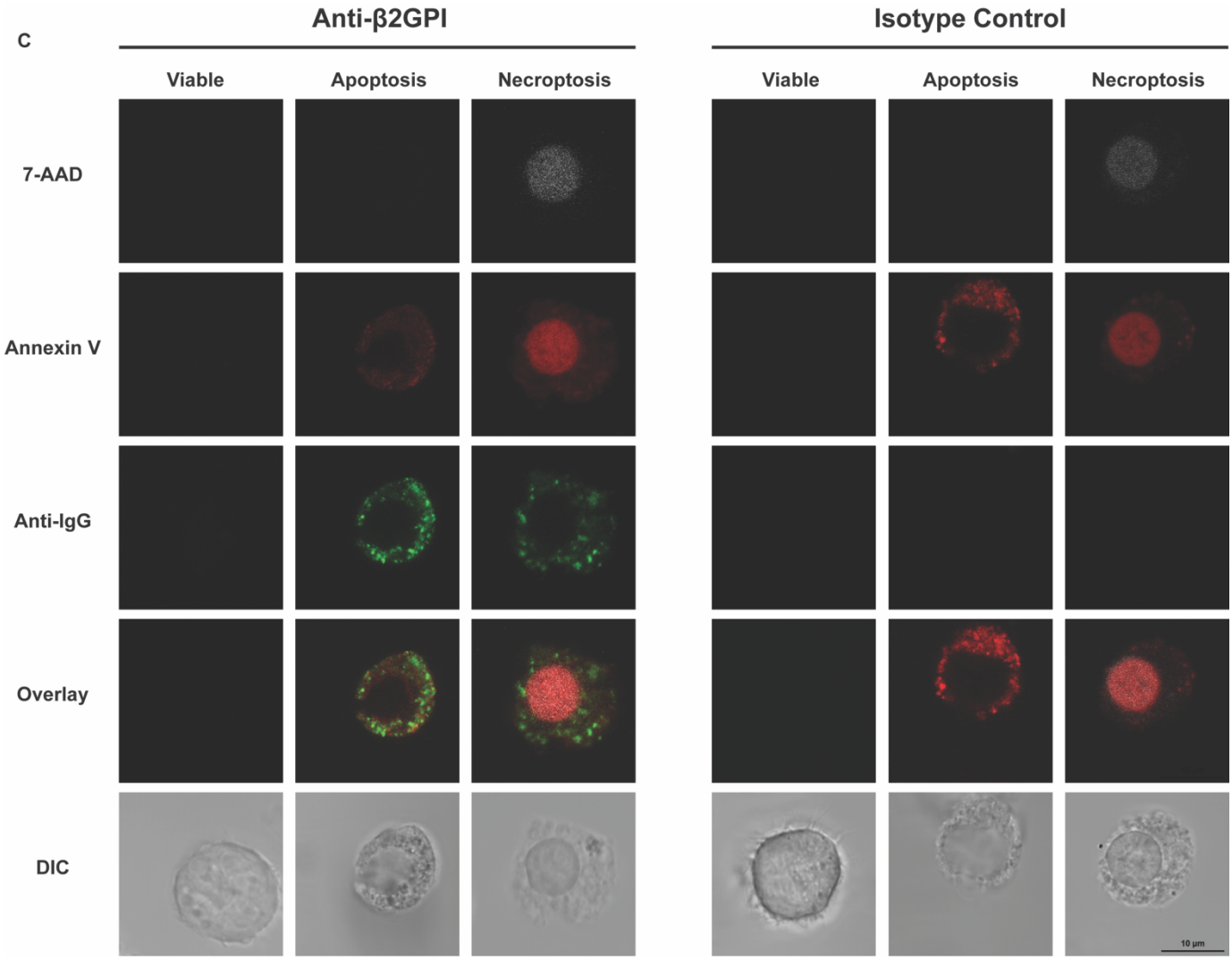


Figure 1. β2GPI binds to necroptotic and apoptotic cells. (A) L929 cells were cultured with vehicle (viable), or induced to undergo apoptosis (actinomycin-D and TNF-α) or necroptosis (z-VAD-fmk and TNF-α). Necroptosis induction was inhibited by treatment with Nec-1s (RIPK1 inhibitor) or GSK'872 (RIPK3 inhibitor). Cells were stained with 7-AAD and PE-annexin V **(top)**, or anti-active caspase-3 **(bottom)**, and analyzed by flow cytometry. **(B)** Viable, apoptotic, or necroptotic L929 cells were cultured with human β2GPI or human serum albumin (HSA) as control, and stained with a murine monoclonal anti-β2GPI antibody (black histogram) or an isotype control (grey histogram), followed by Alexa 488-conjugated anti-mouse IgG. **Top panel** shows a representative experiment; **bottom panel** shows the mean IgG binding (MFI) for three independent experiments (*$p<0.05$; **$p<0.01$). **(C)** Cells were treated as in panel B, and imaged by confocal microscopy. Merged fluorescent images (overlay) show cells stained with monoclonal anti-β2GPI or isotype control, followed by Alexa 488-conjugated anti-mouse IgG (green), annexin V (red), and 7-AAD (white). Images with individual stains and differential interference contrast (DIC) images are also shown for each condition.

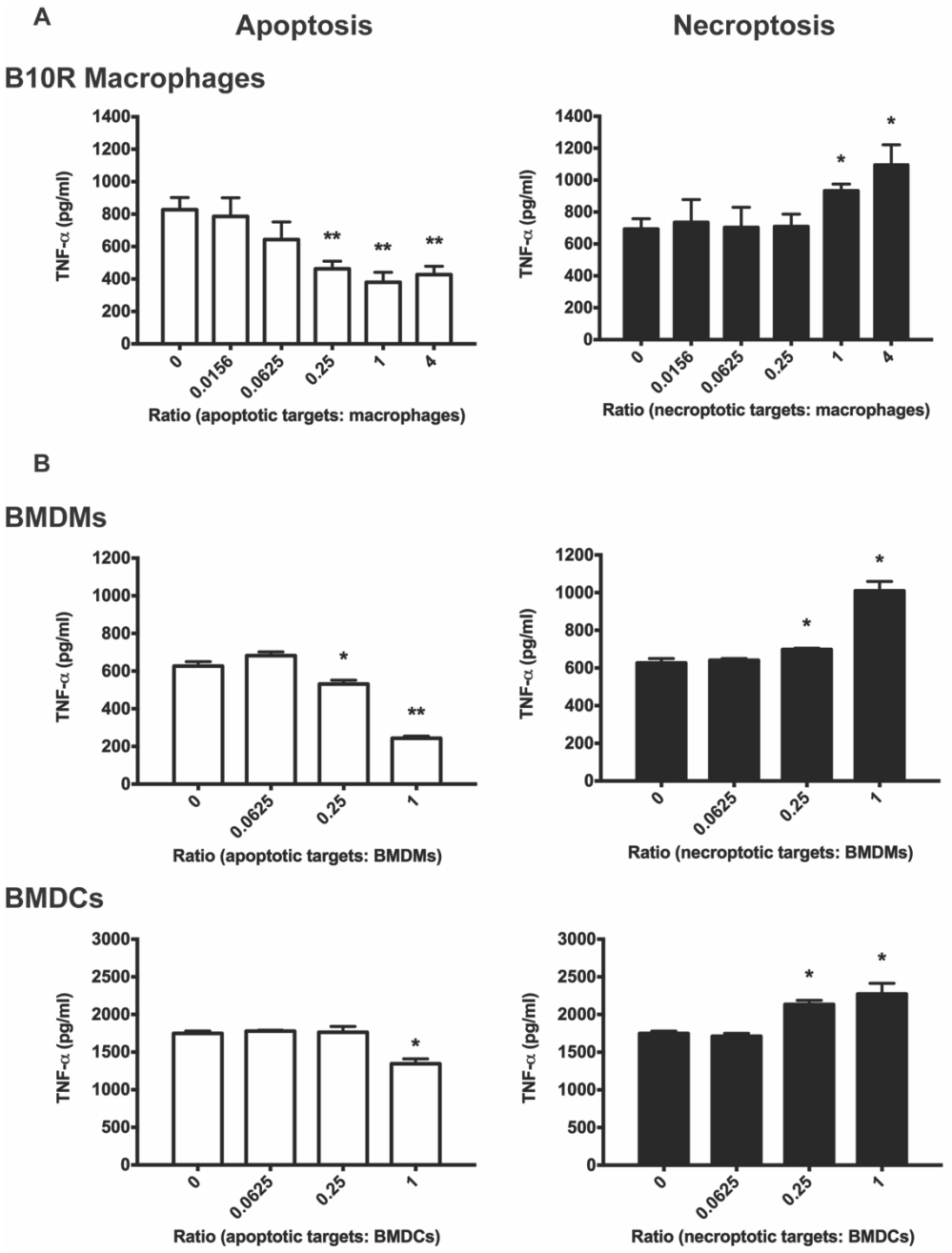


Figure 2. Necroptotic cells promote TNF-α production by antigen-presenting cells. Apoptotic or necroptotic L929 cells were incubated with a macrophage cell line (B10R cells) **(A)**; or primary antigen-presenting cells **(B)**. B10R cells **(A),** or murine bone marrow derived macrophages (BMDMs) **(B; top panel)** or dendritic cells (BMDCs) **(B; bottom panel)** were stimulated with LPS, and then monitored for cytokine (TNF-α) production by ELISA. In panel A, the graphs show the mean values and standard errors of duplicate samples, and are pooled data from 4 independent experiments for apoptotic cells and 5 independent experiments for necroptotic cells. Panel B shows representative data for two (BMDCs) or three (BMDMs) independent experiments, where 0 represents the control with no target cells added. *$p<0.05$, **$p<0.005$, and ***$p<0.0005$, compared to control (no cells).

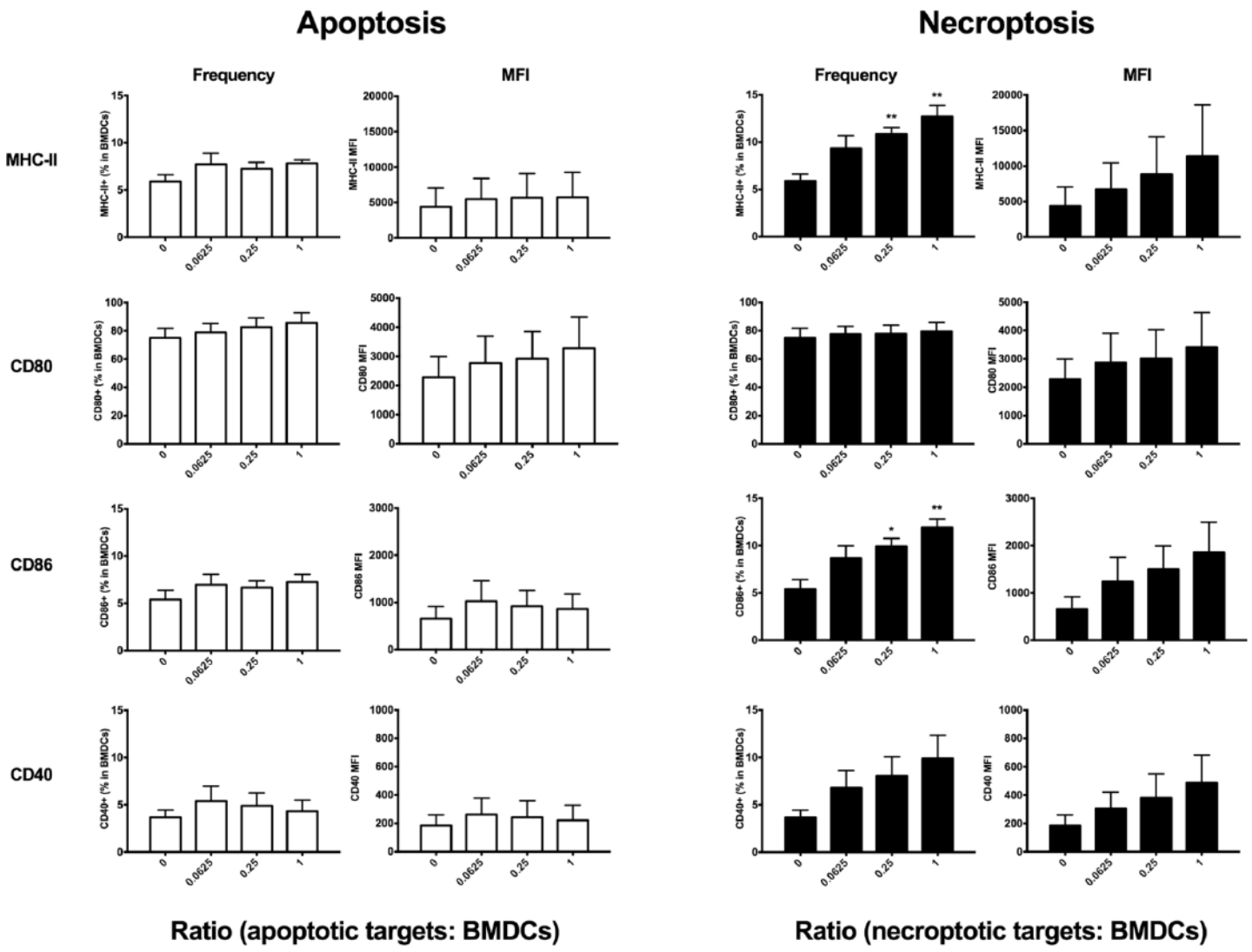


Figure 3. Necroptotic cells promote BMDC activation and antigen presentation of β2GPI. BMDCs were incubated with apoptotic or necroptotic L929 cells, stained for surface markers (MHC class II, CD80, CD86, and CD40), and analyzed by flow cytometry. **Left panels** (frequency) show the percentage of cells positive for MHC class II, CD80, CD86, or CD40 among $CD11c^{+}CD11b^{+}$ cells. **Right panels** (MFI) show the MFI of cells positive for MHC class II, CD80, CD86, or CD40 among $CD11c^{+}CD11b^{+}$ cells. The graphs show pooled data from 3 independent experiments. *$p<0.05$ and **$p<0.005$, compared to control (0, indicating no target cells).

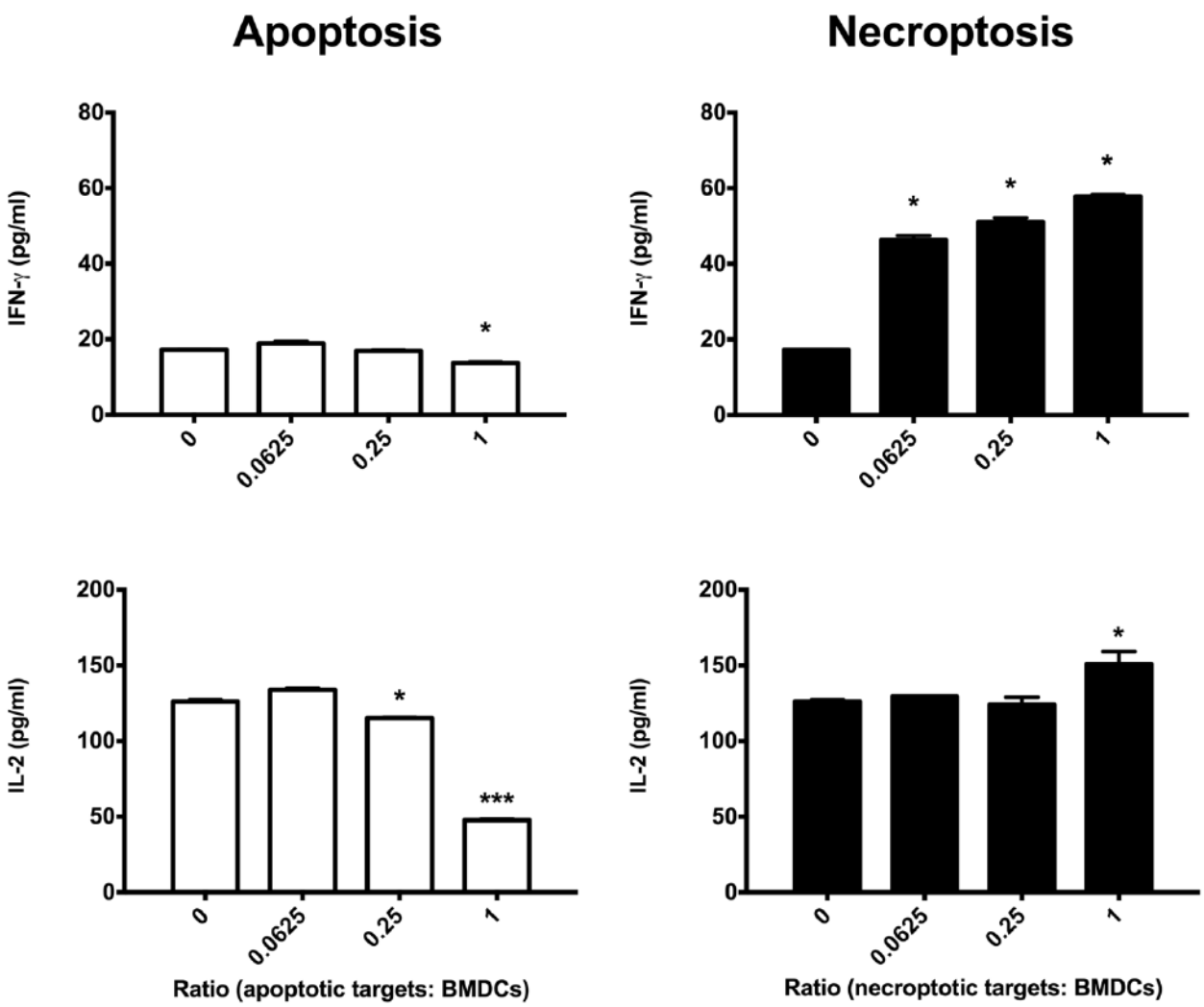


Figure 4. Necroptotic cells promote antigen presentation of β2GPI. BMDCs were incubated with apoptotic or necroptotic L929 cells, β2GPI and LPS, and β2GPI-reactive primary T cells. Bars show the mean IFN-γ or IL-2 production by T cells in duplicate samples, and are representative of 2 independent experiments. Apoptosis was induced by actinomycin D in panel A and staurosporine in panel B. *$p<0.05$ and ***$p<0.001$, compared to control (0, indicating no target cells).

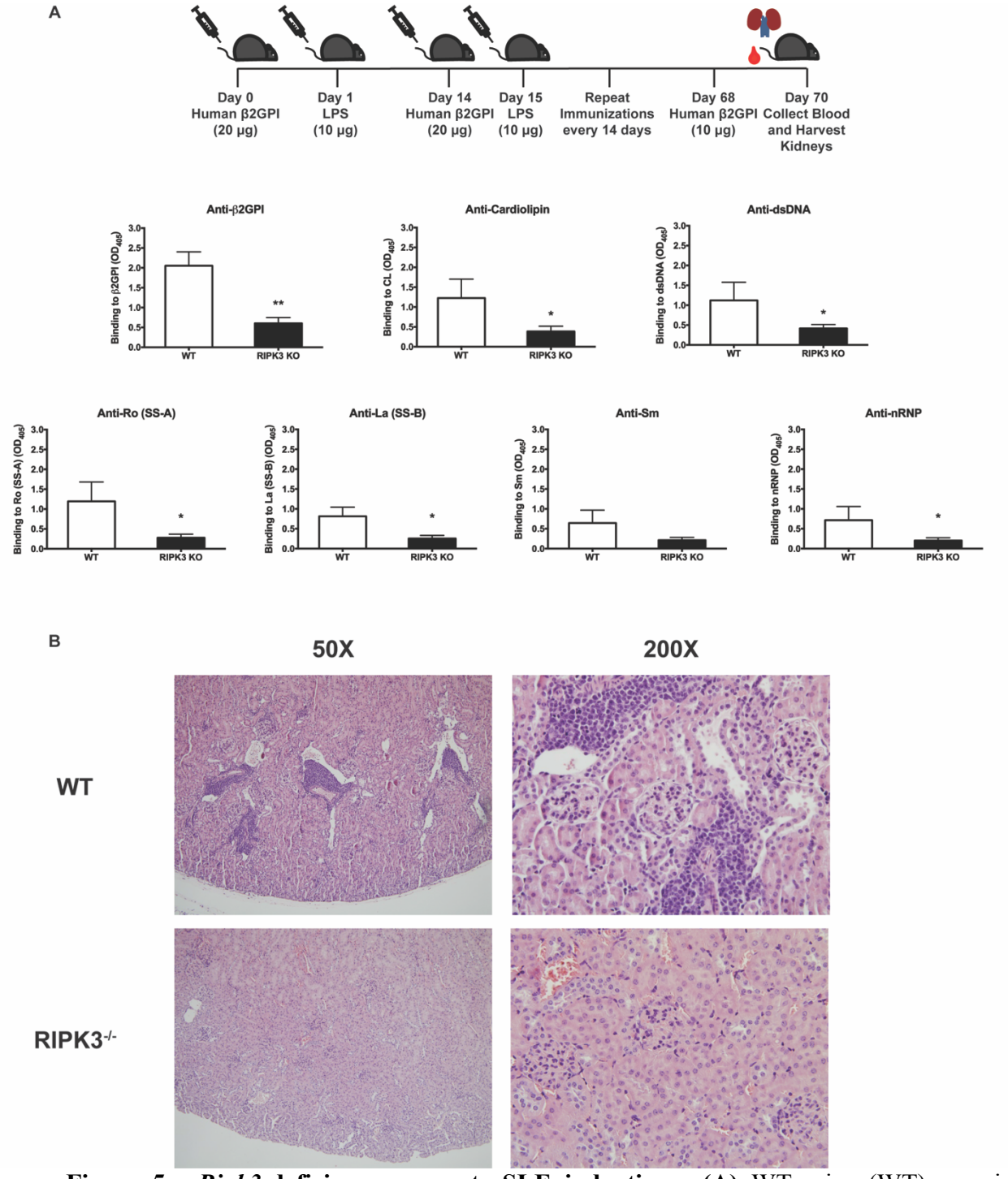


Figure 5. ***Ripk3*-deficiency prevents SLE induction.** **(A)** WT mice (WT) or mice deficient in *Ripk3* (RIPK3$^{-/-}$), were immunized with β2GPI and LPS. Serum autoantibodies were detected by ELISA (bars indicate the mean value ± standard error per group [n=4-6 mice/group]). *p<0.05 and **p<0.005, compared to WT. **(B)** The images show kidney sections stained with H&E from representative WT (n=4) and RIPK3$^{-/-}$ (n=2) mice immunized with β2GPI and LPS. Original magnification: 50x **(left)**: 200x **(right)**.

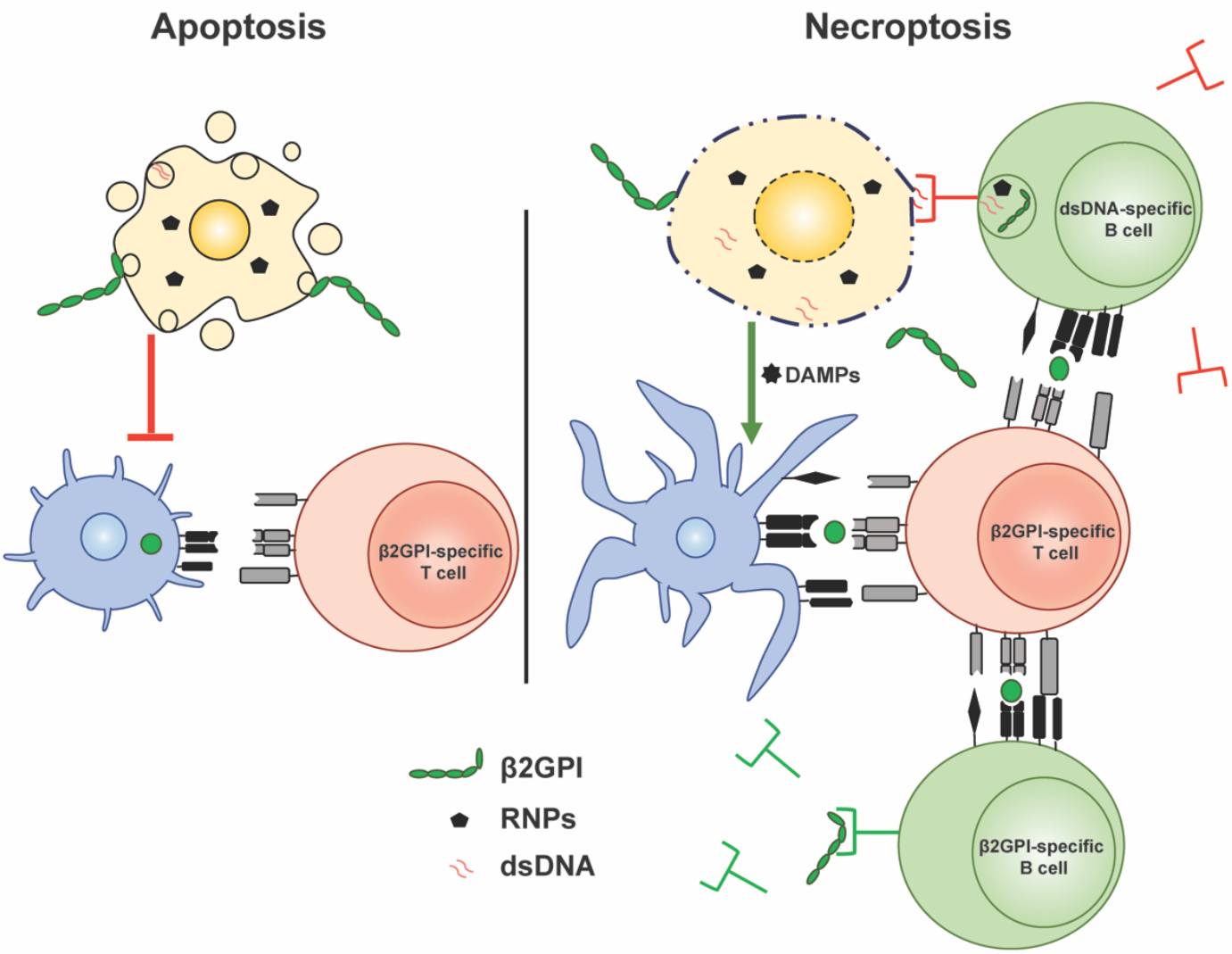
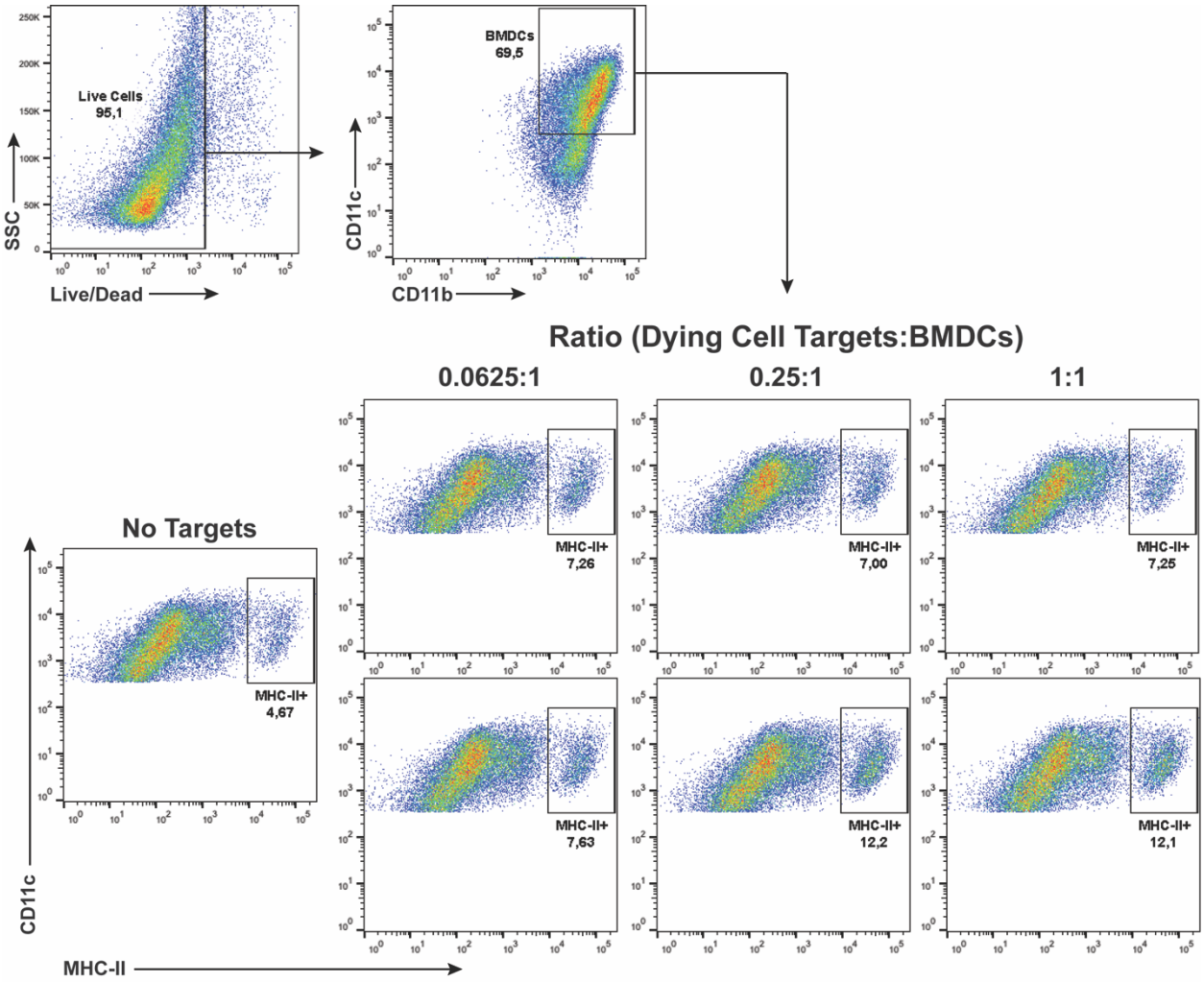


Figure 6. Proposed model for the effects of apoptosis and necroptosis on SLE induction. This schematic diagram summarizes our findings showing immunostimulatory effects of necroptotic, but not apoptotic, cells on antigen-presenting cells. We focus on how these two forms of cell death may impact the immunogenicity of human β2GPI, a protein that binds to both apoptotic and necroptotic cells (Fig.1) and induces murine SLE (Fig. 6). **Left panel:** Human β2GPI bound to apoptotic cells does not promote dendritic cell activation. **Right panel:** β2GPI bound to necroptotic cells promotes dendritic cell activation (upregulation of costimulatory molecule and MHC class II expression), and β2GPI presentation to β2GPI-reactive T cells. We propose that the immunostimulatory effects of necroptotic cells (through the release of DAMPs) may be key in inducing the strong CD4 T cell response to β2GPI that we observe in mice immunized with β2GPI and LPS. We further hypothesize that a β2GPI-specific T cell can provide help to any B cell that has internalized a necroptotic cell with human β2GPI bound to it. This is shown here for β2GPI-specific and dsDNA-specific B cells, but would also apply to B cells specific for other SLE autoantigens. In this manner, different autoreactive B cells can be activated by a single β2GPI-specific T cell, and lead to the emergence of multiple autoantibodies and pathology in SLE.



Supplemental Figure 1. Gating strategy for evaluating the effect of dying cell targets on BMDCs. BMDCs were incubated with apoptotic or necroptotic L929 cells, stained for MHC class II, CD40, CD80, and CD86, and analyzed by flow cytometry. Representative flow cytometry data are shown here for MHC class II staining. Top panels show the gating strategy: live cells were first gated and then $CD11c^{+}CD11b^{+}$ cells were then selected as our BMDC population. Bottom panels show the percentage of cells positive for MHC class II and CD11c among $CD11c^{+}CD11b^{+}$ BMDCs incubated with different ratios of apoptotic or necroptotic cell targets.

CHAPTER 5

SUMMARY AND FUTURE PERSPECTIVES

5.1 SUMMARY

Genetics and the environment both play a role in the development of SLE, but the factors responsible for initiating and promoting disease progression remain unclear. T cells are pivotal in the progression to SLE in that they not only provide help to autoreactive B cells that produce autoantibodies, but also contribute to organ pathology. However, little is currently known about the antigen specificity of autoreactive T cells in SLE. In this thesis, we characterize the antigen specificity of β2GPI-reactive T cells and investigate their role in the development of SLE. Using murine models of SLE, we evaluated the immune epitopes recognized by β2GPI-reactive T cells, and the association of this response with epitope spread to multiple SLE autoantibodies and progression of disease. We also evaluated the impact of apoptotic and necroptotic cell death on the development of a β2GPI-reactive T cell response. Based on our findings, we propose a mechanism by which loss of tolerance to β2GPI can result in epitope spread to multiple SLE autoantibodies and renal disease.

In Chapter 2, we show that mice of different strains developed high levels of SLE autoantibodies when immunized with β2GPI and LPS, irrespective of their MHC class II haplotype. We aimed to identify common T cell epitopes shared across MHC class II haplotypes, initially using a peptide library spanning Domain I-II of β2GPI. We identified epitopes that were MHC class II-restricted, notably peptide 23 that was recognized both by T cells from H-2^b-bearing mice and T cell clones from a HLA-DRB1*0403 (DR4)-bearing patient with autoimmune disease. However, none of the Domain I-II epitopes were shared across the murine MHC class II haplotypes examined. Nevertheless, in all mouse strains, the generation of a β2GPI-reactive T cell response was associated with epitope spread to SLE-related autoantibodies independent of epitope specificity or MHC class II restriction. In Chapter 3, we expanded our peptide library to include Domains I through V (i.e., the entire sequence of β2GPI), and mapped β2GPI T cell epitopes in induced and spontaneous murine SLE models. We identified a β2GPI T cell epitope (peptide 31) in Domain III that was recognized by T cells from H-2^b- and H-2^k-bearing mice with induced SLE, as well as H-2^k-bearing MRL/*lpr* mice that develop SLE spontaneously. β2GPI-reactive T cells from both models showed a bias towards an inflammatory phenotype: Th1 in induced SLE and Th17 in spontaneous SLE. The finding that both induced and spontaneous models of SLE share a common β2GPI T cell epitope suggests a common

immunological origin for the two models. In Chapter 4, we investigated the origin of the β2GPI-reactive T cell response. Cell death (particularly apoptosis) has long been associated with development of SLE. However, given the potentially immunosuppressive nature of apoptotic cells, we compared apoptotic cells with necroptotic cells, which also expresses autoantigens but release damage associated molecular patterns (DAMPs) that are highly pro-inflammatory and potentially immunogenic. We showed that necroptotic cells, like apoptotic cells, can bind β2GPI. Moreover, necroptotic, but not apoptotic, cells were immunostimulatory and provided a microenvironment in which β2GPI-reactive T cells were activated. Finally, SLE induction in mice was greatly diminished in *Ripk3*-deficient mice, which lack necroptosis. We propose a mechanism by which β2GPI-bound necroptotic cells promote MHC class II and co-stimulatory marker expression through the release of DAMPs, resulting in activated MHCs (Chapter 4; Figure 6). Activated APCs that have taken up β2GPI-bound necroptotic cells then present MHC class II-bound β2GPI peptides to β2GPI-reactive T cells, and trigger T cell activation and proliferation. These β2GPI-reactive T cells can provide help to B cells reactive with β2GPI, but also to other autoreactive B cells that have internalized β2GPI-bound necroptotic cells (e.g., via receptors for individual autoantigens on their surface) and present β2GPI on their surface. The latter mechanism is referred to as epitope spread, and can explain the multiple different autoantibodies present in SLE.

5.2 FUTURE PERSPECTIVES

Studies linking genetic susceptibility to SLE in different populations have revealed a significant association with HLA class II genes, particularly HLA-DR4 alleles[9]. HLA-DR4 alleles have also been associated with susceptibility to other autoimmune diseases, including rheumatoid arthritis[138], T1D[139], and APS[127,128,140]. Specifically, the HLA-DRB1*0403 allele has been linked to development of aPL in large cohorts of European and Japanese patients with SLE and/or APS[127,128,140]. Our finding showing recognition of peptide 23 by a T cell clone from an APS patient in the context of the HLA-DRB1*0403 allele may explain how β2GPI-reactive T cells become activated in SLE and APS patients with this MHC haplotype, potentially leading to epitope spread to both aPL and SLE autoantibodies. This finding in T cell clones from a single individual with APS requires replication in other HLA-DRB1*0403-bearing patients with APS

and/or SLE. Furthermore, we have not yet examined whether human T cells or T cell clones recognize other β2GPI epitopes (e.g., peptide 31) we identified in mice. Screening peptides 23 and 31, along with other β2GPI-derived peptides, for recognition by peripheral blood T cells from SLE and APS patients would provide evidence for the presence of β2GPI-reactive T cells in these human diseases and identify immunodominant epitopes.

Other studies focusing on β2GPI-reactive T cells in human APS and SLE have revealed immunodominant $CD4^+$ T cell epitopes and identified their MHC class II restrictions. Table 5.1 summarizes these findings, together with those of our laboratory. Notably, the presence of β2GPI-reactive T cells has been implicated in the development of atherosclerotic plaques, thrombi, and/or fetal loss in some of these patients (Figure 5.1). In the majority of these studies and, in accordance with our findings, β2GPI-reactive T cells displayed a primarily Th1 phenotype, as detected by the production of pro-inflammatory IFN-γ and TNF-α cytokines.

We propose that β2GPI-reactive T cells are crucial for B cell epitope spread in murine SLE, and that these T cells provide help to autoreactive B cells that have taken up dying cells with cell-bound β2GPI. We suggest that necroptotic cells may play an important role in initiating epitope spread though their ability to bind β2GPI and to create a highly proinflammatory/immunogenic context. However, we cannot exclude the possibility that β2GPI-bound apoptotic cells may also be involved in the propagation of epitope spread, and act as a source or "scaffold" of SLE autoantigens. Similar findings by Yatim et al.[141] have shown that necroptotic cells, but not apoptotic cells, enhance DC cross-priming function and activation of $CD8^+$ T cells. Here, we demonstrate that β2GPI-bound necroptotic cells enhance the activation of β2GPI-reactive $CD4^+$ T cells, and thus have the potential to drive B cell epitope spread to multiple SLE autoantibodies.

Necroptosis has been associated with the development of inflammatory pathologies in both mice and humans[94]. In most cases, the association was established either through pharmacological or genetic inhibition of key proteins (RIPK1 or RIPK3) involved in necroptosis. In murine models of systemic inflammatory response syndrome (SIRS), brain and kidney ischaemia-reperfusion injury (IRI), Huntington's disease and MS, treatment with RIPK1

inhibitor (Nec-1 or Nec-1s) has significantly reduced pathology[142-146]. However, some studies have suggested that Nec-1 may have some off-target effects and the use of the more specific Nec-1s inhibitor should be used[147]. Furthermore, targeting RIPK3, which is downstream of RIPK1, would be more precise in targeting necroptosis. RIPK3 inhibitors that are effective *in vivo* are needed.

We have shown that *Ripk3*-deficient mice immunized with β2GPI and LPS have diminished SLE autoantibody production and renal pathology. RIPK3 is a master regulator of necroptosis, but can also regulate apoptosis and inflammation. For example, RIPK3, has been shown to associate with RIPK1, FADD, FLIP and caspase-8 to induce apoptosis through kinase-independent processes[148]. Furthermore, RIPK3 also regulates non-cell death-related inflammatory processes, and can activate certain inflammasomes, leading to processing and production of pro-inflammatory cytokines IL-1β and IL-18[149,150]. Moriwaki et al.[151] have shown that *Ripk3* is essential for caspase-1 and 18-mediated production of IL-1β and IL-18 by BMDCS in response to LPS. We therefore cannot rule out the possibility that necroptosis-independent effects of *Ripk3* deficiency play a role in reducing pathology in induced SLE. To confirm the role of RIPK3-dependent necroptosis in our model, we are currently studying the induction of SLE in *Mlkl*-deficient and $Ripk3^{51A/K51A}$ mutant mice (kinase-inactive RIPK3*)*. *Mlkl*-deficient and $Ripk3^{51A/K51A}$ mutant mice are deficient in necroptosis, but have all other RIPK3-dependent processes intact. Induction of SLE in mice deficient in both apoptosis and necroptosis (*Mlkl*/*Casp8*-deficient mice) will also be assessed, as mice deficient solely in apoptosis (e.g., *Casp8*-deficient) are not viable[152].

Finally, the role for RIPK3-dependent mechanisms in the development of human SLE remains to be explored. There is a paucity of published data in this area. GWAS data from previous studies show variations of expression of genes related to dead cell clearance, inflammatory pathways, and lymphocyte signaling in SLE patients compared to healthy controls[9]. However, these studies failed to examine the expression of genes in involved in necroptosis induction. We have preliminary gene expression data for CD4 T cells from 44 rheumatic disease patients (SLE, RA, and systemic sclerosis), compared to eight healthy controls, that show an increased log fold change (logFC) in *MLKL* expression (Rauch et al.,

unpublished data). Furthermore, there was a decreased logFC in apoptosis-related genes like *FADD*, *CASP3*, and *CASP8*. Caspase-8 directly inhibits RIPK3 activation and downstream necroptosis induction, and caspase-8 inhibition allows necroptosis to occur[94]. Further analysis of gene expression data in other cell types from this cohort of patients, as well as additional gene expression studies focusing on SLE patients need to be completed to confirm our preliminary data. Findings indicating a genetic predisposition for increased necroptosis (and/or decreased apoptosis) in SLE patients would provide support for cell death playing a role in the development of SLE. Preliminary studies from our group have shown an increase in the frequency of annexin V^+7-AAD^+ (i.e. necrotic or necroptotic) in $CD19^+$ cells (B cells) in PBMCs of SLE patients, compared to healthy controls (Rauch et al., unpublished data). These findings add to the limited published data on necroptosis in SLE-derived cells, which show an increase in necroptosis-related genes in B cells of SLE patients[97]. Although it is premature to draw any conclusions from these preliminary findings, our current data provide support for further investigating a role for necroptosis in human SLE.

5.3 IMPLICATIONS AND CONCLUDING REMARKS

In this thesis, we provide evidence for a mechanism by which a β2GPI-reactive T cell response leads to the development of murine SLE. As in human SLE, both genetic and environmental factors are implicated in this proposed mechanism. We provide evidence that necroptosis-related genes are involved in the development of both murine and human SLE. An increase in necroptosis, by genetic predisposition or infection, may provide the necessary microenvironment that leads to loss of tolerance to certain self-antigens like β2GPI. We have shown that certain MHC class II alleles are associated with the development of specific β2GPI-reactive T cell responses. In particular, one T cell epitope on Domain III of β2GPI (peptide 31) is recognized by $CD4^+$ T cells in both induced and spontaneous murine models of SLE, and could potentially be an immunodominant epitope in murine SLE. Studies evaluating recognition of this and other β2GPI-derived peptides by human T cells from SLE patients, and their MHC class II allele restrictions, will provide important information on the specificity and role of β2GPI-reactive T cells in SLE. Further work is also required to identify the roles of RIPK3 and necroptosis in murine and human SLE. If implicated in initiating the disease, treating murine

SLE with RIPK3 or more selective necroptosis inhibitors, could provide proof of concept for novel therapeutic interventions in human SLE. Understanding the mechanisms responsible for the initiation and progression of SLE is critical to the discovery of new and innovative therapeutic interventions, and, ultimately, to prevent the development of SLE.

Table 1. β2GPI CD4+ T cell epitopes identified in APS and SLE

Peptide sequence	Domain	Disease	Source (MHC class II)	Publication
^{1}MISPVLILFSSFLCHVAIAG^{20}	I	APS	Human PBMCs (DRB3*02:02)†	de Moerloose et al. (2017)
^{26}PDDLPFSTVVPLKTF^{40}	I	Induced SLE	129S1 (I-A^{b})	Salem et al. (2015)
^{31}FSTVVPLKTFYEPGE^{45}	I	Induced SLE	BALB/c (I-A^{d}/I-E^{d})	Salem et al. (2015)
^{111}NTGFYLNGADSAKCT^{125}	II	PAPS Induced SLE	Human PBMCs (DRB1*04:03) C57BL/6 (I-A^{b}), 129S1(I-A^{b})	Salem et al. (2015)
^{154}ECLPQHAMFGNDTITCTTHGN^{174}	III	SAPS	Human PBMCs❖	Ito et al. (2000)
^{159}SAGNNSLYRDTAVFECLP^{176}	III	Induced SLE Spontaneous SLE	C57BL/6 (I-A^{b}), C3H/HeN (I-A^{k}/I-E^{k}) MRL/lpr (I-A^{k}/I-E^{k})	Salem et al. (2018)
^{165}LYRDTAVFECLPQHAMFG^{182}	III	Induced SLE Spontaneous SLE	C57BL/6 (I-A^{b}), C3H/HeN (I-A^{k}/I-E^{k}) MRL/lpr (I-A^{k}/I-E^{k})	Salem et al. (2018)
^{208}PSRPDNGFVNYPAKPTLY^{225}	IV	Induced SLE Spontaneous SLE	C3H/HeN (I-A^{k}/I-E^{k}) MRL/lpr (I-A,/I-E^{k})	Salem et al. (2018)
^{256}AMPSCKASCKVPVKKATV^{273}	IV/V	Induced SLE	C3H/HeN (I-A^{k}/I-E^{k})	Salem et al. (2018)
^{244}SCKLPVKKATVVYQGERVKIQ^{264}	V	SAPS, SLE	Human PBMCs (DRB1*04:03, DRB4*01:03)†	Ito et al. (2000)
^{247}VPVKKATVVYQGERV^{261}	V	PAPS	Human PBMCs (DRB1*04:03, DRB4*01:03)	Arai et al. (2001)
^{276}KVSFFCKNKEKKCSY^{290}	V	PAPS, SAPS	Human PBMCs (DRB4*01:03)	Arai et al. (2001)

APS, anti-phospholipid syndrome; SAPS, secondary APS; SLE, systemic lupus erythematosus. The numbering of amino acids in the studies by Salem et al. (2015, 2018) and de Moerloose et al. (2017) includes the 19-amino acid leader sequence. In studies by Salem et al. (2015, 2017), murine T cells were derived from spleen.
† Prominent HLA restrictions are noted here, but additional restrictions were found; ❖HLA restriction was not defined.

Table 5.1. β2GPI CD4^{+} T cell epitopes identified in APS and SLE. This table summarizes the published β2GPI CD4^{+} T cell epitopes identified in patients with primary APS, secondary APS, and SLE alone, as well as in murine SLE. The amino acid sequence of the epitope, as well as the domain it is located, are shown together with the source of the T cells (human or mouse) and their MHC class II restriction. Reproduced from Rauch, J., Salem, D., Subang, R., Kuwana, M., & Levine, J.S. β2-Glycoprotein I-reactive T cells in autoimmune disease. *Frontiers in Immunology* 2018, in press.

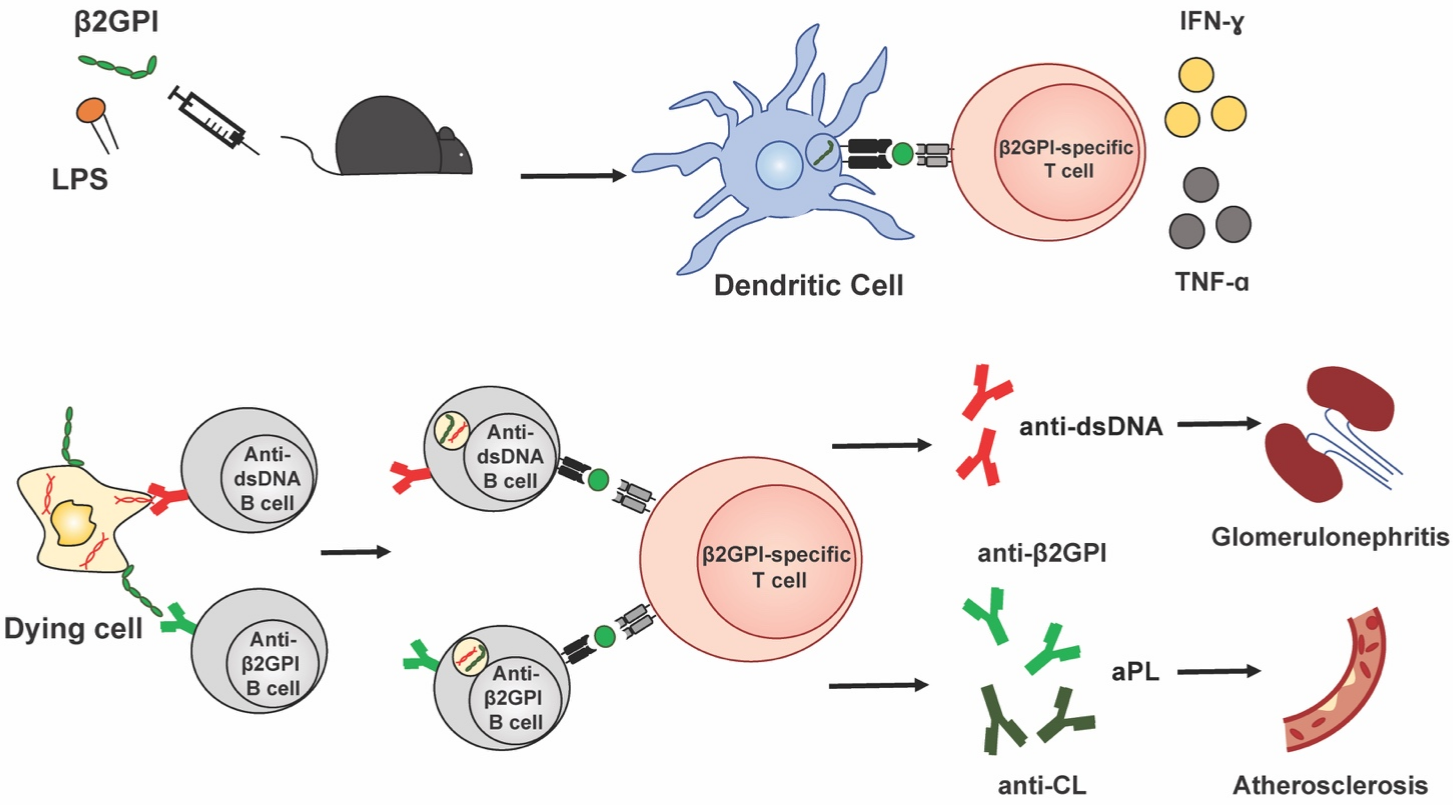


Figure 5.1. β2GPI-reactive T cells promote autoantibody production and pathology in APS and SLE. This simplified schematic diagram illustrates a possible mechanism by which β2GPI-reactive T cells promote the development of multiple serological and clinical outcomes. β2GPI-reactive $CD4^+$ T cells would provide help to autoantigen-specific B cells that have taken up dying cells and present MHC class II-bound β2GPI peptides on their surface. For example, B cells specific for SLE-associated autoantigens (e.g., dsDNA) expressed on the surface of dying cells would receive T cell help from β2GPI-reactive T cells, and secrete class-switched autoantibodies (anti-dsDNA) against these autoantigens. Similarly, this would be the case for β2GPI-reactive autoantibodies, including anti-β2GPI and anti-CL. In addition, pro-inflammatory cytokines (e.g., IFN-γ and TNF-α) produced by the β2GPI-reactive T cells would impact other cells and tissues, either locally in a paracrine manner or at a distance in an endocrine manner. Depending on the autoantibodies and cytokines produced, different pathologies could arise (e.g., thrombosis or atherothrombosis with aPL, and glomerulonephritis with anti-dsDNA). In this way, β2GPI-reactive T cells could be a driving force for autoantibody production and pathology in both APS and SLE. Reproduced from Rauch, J., Salem, D., Subang, R., Kuwana, M., & Levine, J.S. β2-Glycoprotein I-reactive T cells in autoimmune disease. *Frontiers in Immunology* 2018, in press.

CHAPTER 6

REFERENCES

1. Bernatsky S, Joseph L, Pineau CA, Tamblyn R, Feldman DE & Clarke AE. A population-based assessment of systemic lupus erythematosus incidence and prevalence--results and implications of using administrative data for epidemiological studies. *Rheumatology (Oxford)* 2007; **46**: 1814-1818.

2. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; **40**: 1725.

3. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271-1277.

4. Stojan G & Petri M. Epidemiology of systemic lupus erythematosus: an update. *Curr Opin Rheumatol* 2018; **30**: 144-150.

5. Bernatsky S, Boivin JF, Joseph L, Manzi S, Ginzler E, Gladman DD *et al.* Mortality in systemic lupus erythematosus. *Arthritis Rheum* 2006; **54**: 2550-2557.

6. Zhu TY, Tam LS & Li EK. Cost-of-illness studies in systemic lupus erythematosus: A systematic review. *Arthritis Care Res (Hoboken)* 2011; **63**: 751-760.

7. Tsokos GC. Systemic lupus erythematosus. *N Engl J Med* 2011; **365**: 2110-2121.

8. Cui Y, Sheng Y & Zhang X. Genetic susceptibility to SLE: recent progress from GWAS. *J Autoimmun* 2013; **41**: 25-33.

9. Bentham J, Morris DL, Cunninghame Graham DS, Pinder CL, Tombleson P, Behrens TW *et al.* Genetic association analyses implicate aberrant regulation of innate and adaptive immunity genes in the pathogenesis of systemic lupus erythematosus. *Nat Genet* 2015; **47**: 1457-1464.

10. Tsokos GC, Lo MS, Costa Reis P & Sullivan KE. New insights into the immunopathogenesis of systemic lupus erythematosus. *Nat Rev Rheumatol* 2016; **12**: 716-730.

11. Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P *et al.* A revised estimate of twin concordance in systemic lupus erythematosus. *Arthritis Rheum* 1992; **35**: 311-318.

12. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA *et al.* Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* 2003; **349**: 1526-1533.

13. Kaufman DL, Clare-Salzler M, Tian J, Forsthuber T, Ting GS, Robinson P *et al.* Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 1993; **366**: 69-72.

14. McRae BL, Kennedy MK, Tan LJ, Dal Canto MC, Picha KS & Miller SD. Induction of active and adoptive relapsing experimental autoimmune encephalomyelitis (EAE) using an encephalitogenic epitope of proteolipid protein. *J Neuroimmunol* 1992; **38**: 229-240.

15. McRae BL, Vanderlugt CL, Dal Canto MC & Miller SD. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. *J Exp Med* 1995; **182**: 75-85.

16. Vanderlugt CL & Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol* 2002; **2**: 85-95.

17. Munoz LE, Lauber K, Schiller M, Manfredi AA & Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nat Rev Rheumatol* 2010; **6**: 280-289.

18. Doria A, Canova M, Tonon M, Zen M, Rampudda E, Bassi N *et al.* Infections as triggers and complications of systemic lupus erythematosus. *Autoimmun Rev* 2008; **8**: 24-28.

19. Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. *Nat Rev Immunol* 2006; **6**: 823-835.

20. McClain MT, Heinlen LD, Dennis GJ, Roebuck J, Harley JB & James JA. Early events in lupus humoral autoimmunity suggest initiation through molecular mimicry. *Nat Med* 2005; **11**: 85-89.

21. Poole BD, Scofield RH, Harley JB & James JA. Epstein-Barr virus and molecular mimicry in systemic lupus erythematosus. *Autoimmunity* 2006; **39**: 63-70.

22. Suurmond J & Diamond B. Autoantibodies in systemic autoimmune diseases: specificity and pathogenicity. *J Clin Invest* 2015; **125**: 2194-2202.

23. Waldman M & Madaio MP. Pathogenic autoantibodies in lupus nephritis. *Lupus* 2005; **14**: 19-24.

24. Mok CC & Lau CS. Pathogenesis of systemic lupus erythematosus. *J Clin Pathol* 2003; **56**: 481-490.

25. Cozzani E, Drosera M, Gasparini G & Parodi A. Serology of Lupus Erythematosus: Correlation between Immunopathological Features and Clinical Aspects. *Autoimmune Dis* 2014; **2014**: 321359.

26. Su D, Liu R, Li X & Sun L. Possible novel biomarkers of organ involvement in systemic lupus erythematosus. *Clin Rheumatol* 2014; **33**: 1025-1031.

27. Kampmeier RH. A serodiagnostic reaction in syphilis: the work of Wassermann, Neisser, and Bruck. *Sex Transm Dis* 1978; **5**: 163-164.

28. Pangborn MC. A New Serologically Active Phospholipid from Beef Heart. *Proceedings of the Society for Experimental Biology and Medicine* 1941; **48**: 484-486.

29. Schlame M. Cardiolipin synthesis for the assembly of bacterial and mitochondrial membranes. *J Lipid Res* 2008; **49**: 1607-1620.

30. Haserick JR & Long R. [Systemic lupus erythematosus preceded by false-positive serologic tests for syphilis: presentation of five cases]. *Ann Intern Med* 1952; **37**: 559-565.

31. Asherson RA, Khamashta MA, Ordi-Ros J, Derksen RH, Machin SJ, Barquinero J *et al.* The "primary" antiphospholipid syndrome: major clinical and serological features. *Medicine (Baltimore)* 1989; **68**: 366-374.

32. Hughes GR. The anticardiolipin syndrome. *Clin Exp Rheumatol* 1985; **3**: 285-286.

33. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; **4**: 295-306.

34. Santiago MB, Cossermelli W, Tuma MF, Pinto MN & Oliveira RM. Anticardiolipin antibodies in patients with infectious diseases. *Clin Rheumatol* 1989; **8**: 23-28.

35. McNeil HP, Simpson RJ, Chesterman CN & Krilis SA. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H). *Proc Natl Acad Sci U S A* 1990; **87**: 4120-4124.

36. Galli M, Comfurius P, Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJ *et al.* Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. *Lancet* 1990; **335**: 1544-1547.

37. Miyakis S, Giannakopoulos B & Krilis SA. Beta 2 glycoprotein I--function in health and disease. *Thromb Res* 2004; **114**: 335-346.

38. Laplante P, Amireault P, Subang R, Dieude M, Levine JS & Rauch J. Interaction of beta2-glycoprotein I with lipopolysaccharide leads to Toll-like receptor 4 (TLR4)-dependent activation of macrophages. *J Biol Chem* 2011; **286**: 42494-42503.

39. Sheng Y, Reddel SW, Herzog H, Wang YX, Brighton T, France MP *et al.* Impaired thrombin generation in beta 2-glycoprotein I null mice. *J Biol Chem* 2001; **276**: 13817-13821.

40. Yasuda S, Tsutsumi A, Chiba H, Yanai H, Miyoshi Y, Takeuchi R *et al.* beta(2)-glycoprotein I deficiency: prevalence, genetic background and effects on plasma lipoprotein metabolism and hemostasis. *Atherosclerosis* 2000; **152**: 337-346.

41. Steinkasserer A, Estaller C, Weiss EH, Sim RB & Day AJ. Complete nucleotide and deduced amino acid sequence of human beta 2-glycoprotein I. *Biochem J* 1991; **277 (Pt 2)**: 387-391.

42. de Groot PG & Meijers JC. beta(2) -Glycoprotein I: evolution, structure and function. *J Thromb Haemost* 2011; **9**: 1275-1284.

43. de Laat B, Pengo V, Pabinger I, Musial J, Voskuyl AE, Bultink IE *et al.* The association between circulating antibodies against domain I of beta2-glycoprotein I and thrombosis: an international multicenter study. *J Thromb Haemost* 2009; **7**: 1767-1773.

44. Nojima J, Motoki Y, Hara K, Sakata T, Tsuneoka H & Ichihara K. Detection of antibodies against domain 1 of beta2-glycoprotein I is key in predicting thromboembolic complications in patients with systemic lupus erythematosus. *Thromb Res* 2017; **153**: 83-84.

45. Morel L. Genetics of SLE: evidence from mouse models. *Nat Rev Rheumatol* 2010; **6**: 348-357.

46. Dubois EL, Horowitz RE, Demopoulos HB & Teplitz R. NZB/NZW mice as a model of systemic lupus erythematosus. *JAMA* 1966; **195**: 285-289.

47. Perry D, Sang A, Yin Y, Zheng YY & Morel L. Murine models of systemic lupus erythematosus. *J Biomed Biotechnol* 2011; **2011**: 271694.

48. Morel L, Croker BP, Blenman KR, Mohan C, Huang G, Gilkeson G *et al.* Genetic reconstitution of systemic lupus erythematosus immunopathology with polycongenic murine strains. *Proc Natl Acad Sci U S A* 2000; **97**: 6670-6675.

49. Boackle SA, Holers VM, Chen X, Szakonyi G, Karp DR, Wakeland EK *et al.* Cr2, a candidate gene in the murine Sle1c lupus susceptibility locus, encodes a dysfunctional protein. *Immunity* 2001; **15**: 775-785.

50. Lee YH, Ji JD & Song GG. Fcgamma receptor IIB and IIIB polymorphisms and susceptibility to systemic lupus erythematosus and lupus nephritis: a meta-analysis. *Lupus* 2009; **18**: 727-734.

51. Merino R, Fossati L & Izui S. The lupus-prone BXSB strain: the Yaa gene model of systemic lupus erythematosus. *Springer Semin Immunopathol* 1992; **14**: 141-157.

52. Hudgins CC, Steinberg RT, Klinman DM, Reeves MJ & Steinberg AD. Studies of consomic mice bearing the Y chromosome of the BXSB mouse. *J Immunol* 1985; **134**: 3849-3854.

53. Merino R, Fossati L, Lacour M, Lemoine R, Higaki M & Izui S. H-2-linked control of the Yaa gene-induced acceleration of lupus-like autoimmune disease in BXSB mice. *Eur J Immunol* 1992; **22**: 295-299.

54. Nagata S. Mutations in the Fas antigen gene in lpr mice. *Semin Immunol* 1994; **6**: 3-8.

55. Watson ML, Rao JK, Gilkeson GS, Ruiz P, Eicher EM, Pisetsky DS *et al.* Genetic analysis of MRL-lpr mice: relationship of the Fas apoptosis gene to disease manifestations and renal disease-modifying loci. *J Exp Med* 1992; **176**: 1645-1656.

56. Izui S, Kelley VE, Masuda K, Yoshida H, Roths JB & Murphy ED. Induction of various autoantibodies by mutant gene lpr in several strains of mice. *J Immunol* 1984; **133**: 227-233.

57. Kelley VE & Roths JB. Interaction of mutant lpr gene with background strain influences renal disease. *Clin Immunol Immunopathol* 1985; **37**: 220-229.

58. Vidal S, Kono DH & Theofilopoulos AN. Loci predisposing to autoimmunity in MRL-Fas lpr and C57BL/6-Faslpr mice. *J Clin Invest* 1998; **101**: 696-702.

59. Haraldsson MK, Louis-Dit-Sully CA, Lawson BR, Sternik G, Santiago-Raber ML, Gascoigne NR *et al.* The lupus-related Lmb3 locus contains a disease-suppressing Coronin-1A gene mutation. *Immunity* 2008; **28**: 40-51.

60. Punwani D, Pelz B, Yu J, Arva NC, Schafernak K, Kondratowicz K *et al.* Coronin-1A: immune deficiency in humans and mice. *J Clin Immunol* 2015; **35**: 100-107.

61. Carlsten H, Tarkowski A, Holmdahl R & Nilsson LA. Oestrogen is a potent disease accelerator in SLE-prone MRL lpr/lpr mice. *Clin Exp Immunol* 1990; **80**: 467-473.

62. Roubinian JR, Talal N, Greenspan JS, Goodman JR & Siiteri PK. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice. *J Exp Med* 1978; **147**: 1568-1583.

63. Yung RL. Mechanisms of lupus: the role of estrogens. *Clin Exp Rheumatol* 1999; **17**: 271-275.

64. Lawson BR, Prud'homme GJ, Chang Y, Gardner HA, Kuan J, Kono DH *et al.* Treatment of murine lupus with cDNA encoding IFN-gammaR/Fc. *J Clin Invest* 2000; **106**: 207-215.

65. Schwarting A, Paul K, Tschirner S, Menke J, Hansen T, Brenner W *et al.* Interferon-beta: a therapeutic for autoimmune lupus in MRL-Faslpr mice. *J Am Soc Nephrol* 2005; **16**: 3264-3272.

66. Zagury D, Le Buanec H, Mathian A, Larcier P, Burnett R, Amoura Z *et al.* IFNalpha kinoid vaccine-induced neutralizing antibodies prevent clinical manifestations in a lupus flare murine model. *Proc Natl Acad Sci U S A* 2009; **106**: 5294-5299.

67. Biswas PS, Gupta S, Chang E, Song L, Stirzaker RA, Liao JK *et al.* Phosphorylation of IRF4 by ROCK2 regulates IL-17 and IL-21 production and the development of autoimmunity in mice. *J Clin Invest* 2010; **120**: 3280-3295.

68. Herber D, Brown TP, Liang S, Young DA, Collins M & Dunussi-Joannopoulos K. IL-21 has a pathogenic role in a lupus-prone mouse model and its blockade with IL-21R.Fc reduces disease progression. *J Immunol* 2007; **178**: 3822-3830.

69. Ahuja A, Shupe J, Dunn R, Kashgarian M, Kehry MR & Shlomchik MJ. Depletion of B cells in murine lupus: efficacy and resistance. *J Immunol* 2007; **179**: 3351-3361.

70. Liu W, Szalai A, Zhao L, Liu D, Martin F, Kimberly RP *et al.* Control of spontaneous B lymphocyte autoimmunity with adenovirus-encoded soluble TACI. *Arthritis Rheum* 2004; **50**: 1884-1896.

71. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR *et al.* Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N Engl J Med* 2004; **350**: 2572-2581.

72. Merrill JT, Neuwelt CM, Wallace DJ, Shanahan JC, Latinis KM, Oates JC *et al.* Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. *Arthritis Rheum* 2010; **62**: 222-233.

73. Navarra SV, Guzman RM, Gallacher AE, Hall S, Levy RA, Jimenez RE *et al.* Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. *Lancet* 2011; **377**: 721-731.

74. Giles JR, Kashgarian M, Koni PA & Shlomchik MJ. B Cell-Specific MHC Class II Deletion Reveals Multiple Nonredundant Roles for B Cell Antigen Presentation in Murine Lupus. *J Immunol* 2015; **195**: 2571-2579.

75. Satoh M & Reeves WH. Induction of lupus-associated autoantibodies in BALB/c mice by intraperitoneal injection of pristane. *J Exp Med* 1994; **180**: 2341-2346.

76. Satoh M, Kumar A, Kanwar YS & Reeves WH. Anti-nuclear antibody production and immune-complex glomerulonephritis in BALB/c mice treated with pristane. *Proc Natl Acad Sci U S A* 1995; **92**: 10934-10938.

77. Satoh M, Richards HB, Shaheen VM, Yoshida H, Shaw M, Naim JO *et al.* Widespread susceptibility among inbred mouse strains to the induction of lupus autoantibodies by pristane. *Clin Exp Immunol* 2000; **121**: 399-405.

78. Reeves WH, Lee PY, Weinstein JS, Satoh M & Lu L. Induction of autoimmunity by pristane and other naturally occurring hydrocarbons. *Trends Immunol* 2009; **30**: 455-464.

79. Calvani N, Satoh M, Croker BP, Reeves WH & Richards HB. Nephritogenic autoantibodies but absence of nephritis in Il-12p35-deficient mice with pristane-induced lupus. *Kidney Int* 2003; **64**: 897-905.

80. Richards HB, Satoh M, Jennette JC, Croker BP, Yoshida H & Reeves WH. Interferon-gamma is required for lupus nephritis in mice treated with the hydrocarbon oil pristane. *Kidney Int* 2001; **60**: 2173-2180.

81. Nacionales DC, Kelly-Scumpia KM, Lee PY, Weinstein JS, Lyons R, Sobel E *et al.* Deficiency of the type I interferon receptor protects mice from experimental lupus. *Arthritis Rheum* 2007; **56**: 3770-3783.

82. Thibault DL, Graham KL, Lee LY, Balboni I, Hertzog PJ & Utz PJ. Type I interferon receptor controls B-cell expression of nucleic acid-sensing Toll-like receptors and autoantibody production in a murine model of lupus. *Arthritis Res Ther* 2009; **11**: R112.

83. Lee PY, Li Y, Kumagai Y, Xu Y, Weinstein JS, Kellner ES *et al.* Type I interferon modulates monocyte recruitment and maturation in chronic inflammation. *Am J Pathol* 2009; **175**: 2023-2033.

84. Blank M, Faden D, Tincani A, Kopolovic J, Goldberg I, Gilburd B *et al.* Immunization with anticardiolipin cofactor (beta-2-glycoprotein I) induces experimental antiphospholipid syndrome in naive mice. *J Autoimmun* 1994; **7**: 441-455.

85. Levine JS, Branch DW & Rauch J. The antiphospholipid syndrome. *N Engl J Med* 2002; **346**: 752-763.

86. Levine JS, Subang R, Nasr SH, Fournier S, Lajoie G, Wither J *et al.* Immunization with an apoptotic cell-binding protein recapitulates the nephritis and sequential autoantibody emergence of systemic lupus erythematosus. *J Immunol* 2006; **177**: 6504-6516.

87. Casciola-Rosen LA, Anhalt G & Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J Exp Med* 1994; **179**: 1317-1330.

88. Casciola-Rosen L & Rosen A. Ultraviolet light-induced keratinocyte apoptosis: a potential mechanism for the induction of skin lesions and autoantibody production in LE. *Lupus* 1997; **6**: 175-180.

89. Francis L & Perl A. Infection in systemic lupus erythematosus: friend or foe? *Int J Clin Rheumtol* 2010; **5**: 59-74.

90. Colonna L, Lood C & Elkon KB. Beyond apoptosis in lupus. *Curr Opin Rheumatol* 2014; **26**: 459-466.

91. Nagata S. Apoptosis and Clearance of Apoptotic Cells. *Annu Rev Immunol* 2018; **36**: 489-517.

92. Baumann I, Kolowos W, Voll RE, Manger B, Gaipl U, Neuhuber WL *et al.* Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis Rheum* 2002; **46**: 191-201.

93. Munoz LE, van Bavel C, Franz S, Berden J, Herrmann M & van der Vlag J. Apoptosis in the pathogenesis of systemic lupus erythematosus. *Lupus* 2008; **17**: 371-375.

94. Linkermann A & Green DR. Necroptosis. *N Engl J Med* 2014; **370**: 455-465.

95. Khan N, Lawlor KE, Murphy JM & Vince JE. More to life than death: molecular determinants of necroptotic and non-necroptotic RIP3 kinase signaling. *Curr Opin Immunol* 2014; **26**: 76-89.

96. Weinlich R, Oberst A, Beere HM & Green DR. Necroptosis in development, inflammation and disease. *Nat Rev Mol Cell Biol* 2017; **18**: 127-136.

97. Fan H, Liu F, Dong G, Ren D, Xu Y, Dou J *et al.* Activation-induced necroptosis contributes to B-cell lymphopenia in active systemic lupus erythematosus. *Cell Death Dis* 2014; **5**: e1416.

98. Honarpisheh M, Desai J, Marschner JA, Weidenbusch M, Lech M, Vielhauer V *et al.* Regulated necrosis-related molecule mRNA expression in humans and mice and in murine acute tissue injury and systemic autoimmunity leading to progressive organ damage, and progressive fibrosis. *Biosci Rep* 2016; **36**.

99. Corradetti C, Jog NR, Gallucci S, Madaio M, Balachandran S & Caricchio R. Immune-Mediated Nephropathy and Systemic Autoimmunity in Mice Does Not Require Receptor Interacting Protein Kinase 3 (RIPK3). *PLoS One* 2016; **11**: e0163611.

100. Krishnan S, Farber DL & Tsokos GC. T cell rewiring in differentiation and disease. *J Immunol* 2003; **171**: 3325-3331.

101. Moulton VR & Tsokos GC. T cell signaling abnormalities contribute to aberrant immune cell function and autoimmunity. *J Clin Invest* 2015; **125**: 2220-2227.

102. Sunahori K, Juang YT & Tsokos GC. Methylation status of CpG islands flanking a cAMP response element motif on the protein phosphatase 2Ac alpha promoter determines CREB binding and activity. *J Immunol* 2009; **182**: 1500-1508.

103. Brusko TM, Putnam AL & Bluestone JA. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. *Immunol Rev* 2008; **223**: 371-390.

104. Nambiar MP, Fisher CU, Warke VG, Krishnan S, Mitchell JP, Delaney N *et al.* Reconstitution of deficient T cell receptor zeta chain restores T cell signaling and augments T cell receptor/CD3-induced interleukin-2 production in patients with systemic lupus erythematosus. *Arthritis Rheum* 2003; **48**: 1948-1955.

105. Crispin JC, Keenan BT, Finnell MD, Bermas BL, Schur P, Massarotti E *et al.* Expression of CD44 variant isoforms CD44v3 and CD44v6 is increased on T cells from patients with systemic lupus erythematosus and is correlated with disease activity. *Arthritis Rheum* 2010; **62**: 1431-1437.

106. Steinmetz OM, Turner JE, Paust HJ, Lindner M, Peters A, Heiss K *et al.* CXCR3 mediates renal Th1 and Th17 immune response in murine lupus nephritis. *J Immunol* 2009; **183**: 4693-4704.

107. Schmidt T, Paust HJ, Krebs CF, Turner JE, Kaffke A, Bennstein SB *et al.* Function of the Th17/interleukin-17A immune response in murine lupus nephritis. *Arthritis Rheumatol* 2015; **67**: 475-487.

108. Kyttaris VC, Kampagianni O & Tsokos GC. Treatment with anti-interleukin 23 antibody ameliorates disease in lupus-prone mice. *Biomed Res Int* 2013; **2013**: 861028.

109. Kyttaris VC, Zhang Z, Kuchroo VK, Oukka M & Tsokos GC. Cutting edge: IL-23 receptor deficiency prevents the development of lupus nephritis in C57BL/6-lpr/lpr mice. *J Immunol* 2010; **184**: 4605-4609.

110. Koga T, Ichinose K & Tsokos GC. T cells and IL-17 in lupus nephritis. *Clin Immunol* 2017; **185**: 95-99.

111. Vincent FB, Northcott M, Hoi A, Mackay F & Morand EF. Clinical associations of serum interleukin-17 in systemic lupus erythematosus. *Arthritis Res Ther* 2013; **15**: R97.

112. Wong CK, Ho CY, Li EK & Lam CW. Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. *Lupus* 2000; **9**: 589-593.

113. Wong CK, Lit LC, Tam LS, Li EK, Wong PT & Lam CW. Hyperproduction of IL-23 and IL-17 in patients with systemic lupus erythematosus: implications for Th17-mediated inflammation in auto-immunity. *Clin Immunol* 2008; **127**: 385-393.

114. Akahoshi M, Nakashima H, Tanaka Y, Kohsaka T, Nagano S, Ohgami E *et al.* Th1/Th2 balance of peripheral T helper cells in systemic lupus erythematosus. *Arthritis Rheum* 1999; **42**: 1644-1648.

115. Blanco P, Ueno H & Schmitt N. T follicular helper (Tfh) cells in lupus: Activation and involvement in SLE pathogenesis. *Eur J Immunol* 2016; **46**: 281-290.

116. Linterman MA & Vinuesa CG. T follicular helper cells during immunity and tolerance. *Prog Mol Biol Transl Sci* 2010; **92**: 207-248.

117. Odegard JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C *et al.* ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. *J Exp Med* 2008; **205**: 2873-2886.

118. Muller S & Wallace DJ. The importance of implementing proper selection of excipients in lupus clinical trials. *Lupus* 2014; **23**: 609-614.

119. Monneaux F, Briand JP & Muller S. B and T cell immune response to small nuclear ribonucleoprotein particles in lupus mice: autoreactive CD4(+) T cells recognize a T cell epitope located within the RNP80 motif of the 70K protein. *Eur J Immunol* 2000; **30**: 2191-2200.

120. Monneaux F, Dumortier H, Steiner G, Briand JP & Muller S. Murine models of systemic lupus erythematosus: B and T cell responses to spliceosomal ribonucleoproteins in MRL/Fas(lpr) and (NZB x NZW)F(1) lupus mice. *Int Immunol* 2001; **13**: 1155-1163.

121. Monneaux F, Lozano JM, Patarroyo ME, Briand JP & Muller S. T cell recognition and therapeutic effect of a phosphorylated synthetic peptide of the 70K snRNP protein administered in MR/lpr mice. *Eur J Immunol* 2003; **33**: 287-296.

122. Greidinger EL, Foecking MF, Schafermeyer KR, Bailey CW, Primm SL, Lee DR *et al.* T cell immunity in connective tissue disease patients targets the RNA binding domain of the U1-70kDa small nuclear ribonucleoprotein. *J Immunol* 2002; **169**: 3429-3437.

123. Kattah NH, Newell EW, Jarrell JA, Chu AD, Xie J, Kattah MG *et al.* Tetramers reveal IL-17-secreting CD4+ T cells that are specific for U1-70 in lupus and mixed connective tissue disease. *Proc Natl Acad Sci U S A* 2015; **112**: 3044-3049.

124. George J, Afek A, Gilburd B, Blank M, Levy Y, Aron-Maor A *et al.* Induction of early atherosclerosis in LDL-receptor-deficient mice immunized with beta2-glycoprotein I. *Circulation* 1998; **98**: 1108-1115.

125. Visvanathan S & McNeil HP. Cellular immunity to beta 2-glycoprotein-1 in patients with the antiphospholipid syndrome. *J Immunol* 1999; **162**: 6919-6925.

126. Hattori N, Kuwana M, Kaburaki J, Mimori T, Ikeda Y & Kawakami Y. T cells that are autoreactive to beta2-glycoprotein I in patients with antiphospholipid syndrome and healthy individuals. *Arthritis Rheum* 2000; **43**: 65-75.

127. Ito H, Matsushita S, Tokano Y, Nishimura H, Tanaka Y, Fujisao S *et al.* Analysis of T cell responses to the beta 2-glycoprotein I-derived peptide library in patients with anti-beta 2-glycoprotein I antibody-associated autoimmunity. *Hum Immunol* 2000; **61**: 366-377.

128. Arai T, Yoshida K, Kaburaki J, Inoko H, Ikeda Y, Kawakami Y *et al.* Autoreactive CD4(+) T-cell clones to beta2-glycoprotein I in patients with antiphospholipid syndrome: preferential recognition of the major phospholipid-binding site. *Blood* 2001; **98**: 1889-1896.

129. Kuwana M, Matsuura E, Kobayashi K, Okazaki Y, Kaburaki J, Ikeda Y *et al.* Binding of beta 2-glycoprotein I to anionic phospholipids facilitates processing and presentation of a cryptic epitope that activates pathogenic autoreactive T cells. *Blood* 2005; **105**: 1552-1557.

130. Yamaguchi Y, Seta N, Kaburaki J, Kobayashi K, Matsuura E & Kuwana M. Excessive exposure to anionic surfaces maintains autoantibody response to beta(2)-glycoprotein I in patients with antiphospholipid syndrome. *Blood* 2007; **110**: 4312-4318.

131. Jung JY & Suh CH. Incomplete clearance of apoptotic cells in systemic lupus erythematosus: pathogenic role and potential biomarker. *Int J Rheum Dis* 2015; **18**: 294-303.

132. Liu K, Iyoda T, Saternus M, Kimura Y, Inaba K & Steinman RM. Immune tolerance after delivery of dying cells to dendritic cells in situ. *J Exp Med* 2002; **196**: 1091-1097.

133. Pasparakis M & Vandenabeele P. Necroptosis and its role in inflammation. *Nature* 2015; **517**: 311-320.

134. Levine JS, Subang R, Koh JS & Rauch J. Induction of anti-phospholipid autoantibodies by beta2-glycoprotein I bound to apoptotic thymocytes. *J Autoimmun* 1998; **11**: 413-424.

135. Tolomeo T, Rico De Souza A, Roter E, Dieude M, Amireault P, Subang R *et al.* T cells demonstrate a Th1-biased response to native beta2-glycoprotein I in a murine model of anti-phospholipid antibody induction. *Autoimmunity* 2009; **42**: 292-295.

136. Schwarzenbacher R, Zeth K, Diederichs K, Gries A, Kostner GM, Laggner P *et al.* Crystal structure of human beta2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. *EMBO J* 1999; **18**: 6228-6239.

137. Wree A, Broderick L, Canbay A, Hoffman HM & Feldstein AE. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 627-636.

138. Stastny P, Ball EJ, Khan MA, Olsen NJ, Pincus T & Gao X. HLA-DR4 and other genetic markers in rheumatoid arthritis. *Br J Rheumatol* 1988; **27 Suppl 2**: 132-138.

139. Erlich H, Valdes AM, Noble J, Carlson JA, Varney M, Concannon P *et al.* HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the type 1 diabetes genetics consortium families. *Diabetes* 2008; **57**: 1084-1092.

140. Galeazzi M, Sebastiani GD, Tincani A, Piette JC, Allegri F, Morozzi G *et al.* HLA class II alleles associations of anticardiolipin and anti-beta2GPI antibodies in a large series of European patients with systemic lupus erythematosus. *Lupus* 2000; **9**: 47-55.

141. Yatim N, Jusforgues-Saklani H, Orozco S, Schulz O, Barreira da Silva R, Reis e Sousa C *et al.* RIPK1 and NF-kappaB signaling in dying cells determines cross-priming of CD8(+) T cells. *Science* 2015; **350**: 328-334.

142. Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N *et al.* Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol* 2005; **1**: 112-119.

143. Duprez L, Takahashi N, Van Hauwermeiren F, Vandendriessche B, Goossens V, Vanden Berghe T *et al.* RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. *Immunity* 2011; **35**: 908-918.

144. Linkermann A, Brasen JH, Darding M, Jin MK, Sanz AB, Heller JO *et al.* Two independent pathways of regulated necrosis mediate ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* 2013; **110**: 12024-12029.

145. Ofengeim D, Ito Y, Najafov A, Zhang Y, Shan B, DeWitt JP *et al.* Activation of necroptosis in multiple sclerosis. *Cell Rep* 2015; **10**: 1836-1849.

146. Zhu S, Zhang Y, Bai G & Li H. Necrostatin-1 ameliorates symptoms in R6/2 transgenic mouse model of Huntington's disease. *Cell Death Dis* 2011; **2**: e115.

147. Takahashi N, Duprez L, Grootjans S, Cauwels A, Nerinckx W, DuHadaway JB *et al.* Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. *Cell Death Dis* 2012; **3**: e437.

148. Mandal P, Berger SB, Pillay S, Moriwaki K, Huang C, Guo H *et al.* RIP3 induces apoptosis independent of pronecrotic kinase activity. *Mol Cell* 2014; **56**: 481-495.

149. Newton K & Manning G. Necroptosis and Inflammation. *Annu Rev Biochem* 2016; **85**: 743-763.

150. Orozco S & Oberst A. RIPK3 in cell death and inflammation: the good, the bad, and the ugly. *Immunol Rev* 2017; **277**: 102-112.

151. Moriwaki K, Bertin J, Gough PJ & Chan FK. A RIPK3-caspase 8 complex mediates atypical pro-IL-1beta processing. *J Immunol* 2015; **194**: 1938-1944.

152. Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R *et al.* RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* 2011; **471**: 368-372.