The Role of Beta2-Glycoprotein I-Reactive T Cells in Antiphospholipid Syndrome

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ABSTRACT

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by the presence of autoantibodies to phospholipid (PL)-binding proteins, such as β2-glycoprotein I (β2GPI), and clinical manifestations including thrombosis and/or recurrent pregnancy loss. β2GPI-reactive T cells have been shown to be activated in patients with APS, but the mechanism responsible for this activation remains unclear. Recent studies have proposed that exposure of a cryptic epitope on β2GPI, as a consequence of binding to PL, leads to the activation of β2GPI-autoreactive T cells in APS patients. To test this hypothesis, we evaluated the development of β2GPI-reactive T cells in a murine model of aPL production. C57BL/6 mice were immunized repeatedly with human β2GPI in the presence of lipopolysaccharide (LPS) to induce aPL production. High levels of circulating aPL were observed as early as the second immunization, but splenic T cell reactivity to β2GPI was not detectable *in vitro* until after the fourth immunization. Splenic T cells from mice producing high levels of aPL proliferated in response to native human β2GPI, alone or bound to anionic PL, but PL-bound β2GPI appeared to be a more potent antigen. β2GPI-reactive T cells produced IL-2 and IFN-γ, but not IL-4 or IL-10, suggesting a T_H1 bias of this T cell response. These results demonstrate that T cell reactivity to β2GPI can develop in nonautoimmune individuals repeatedly exposed to this antigen in a proinflammatory context (e.g., LPS). Our data further suggest that the β2GPI-reactive T cells induced in this model have a T_H1 bias and may be more reactive to a PL-dependent epitope on β2GPI than to native β2GPI.

RÉSUMÉ

Le syndrome antiphospholipide (SAPL) est une maladie autoimmune caractérisée par la présence d'auto-anticorps antiphospholipides (aPL) dirigés contre des protéines liant les phospholipides anioniques dont la β2-glycoproteine I (β2GPI), ainsi que par des manifestations cliniques incluant la thrombose et la perte foetale récurrente. Il a été démontré que des lymphocytes T spécifiques à la β2GPI étaient activés chez les patients atteints du SAPL. Cependant, le mécanisme responsable de cette activation lymphocytaire reste nébuleux. Des études récentes ont proposé que l'exposition d'épitopes cryptiques de la β2GPI, suite à la liaison de cette glycoprotéine à des phospholipides anioniques, engendre l'activation de lymphocytes T autoréactifs spécifiques à la β2GPI chez les patients atteints du SAPL. Afin de vérifier cette hypothèse, nous avons évalué le développement de lymphocytes T spécifiques à la β2GPI dans un modèle murin de production d'aPL. Des souris C57BL/6 ont été immunisées à répétition avec de la β2GPI humaine en présence de lipopolysaccharide (LPS) dans le but d'induire la production d'aPL. Des titres élevés d'aPL circulants ont été observés dès la deuxième immunization, tandis que les lymphocytes T spécifiques à la β2GPI n'ont été détectés que suite à la quatrième immunisation. Ainsi, les lymphocytes T provenant de la rate des souris produisant des niveaux élevés d'aPL ont proliféré en réponse à la forme native de β2GPI et encore plus fortement en réponse au complexe β2GPI-PL. Ces lymphocytes T réactifs à la β2GPI ont démontré une production d'interleukine 2 et d'interféron gamma. Cependant, aucune interleukine 4 ou 10 n'ont été détectées insinuant un biais de type T_H1 pour cette réponse lymphocytaire. Ces résultats suggèrent que la réactivité des lymphocytes T à la β2GPI peut se développer chez des individus non-autoimmuns

exposés de façon récurrente à cet antigène dans un contexte pro-inflammatoire (ex.LPS). De plus, notre étude démontre que, dans notre modèle, les cellules T réactives à la β2GPI possèdent un biais T_H1 et réagissent plus fortement à un épitope PL dépendant qu'à la forme native de la β2GPI.

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ABBREVIATIONS

aCL	anticardiolipin antibody(ies)
ADP	adenosine diphosphate
AIDS	acquired immunodeficiency syndrome
anti-β2GPI	anti-β2-glycoprotein I antibody(ies)
APC	antigen presenting cells
aPL	antiphospholipid antibody(ies)
aPT	anti-phosphatidylserine/prothrombin antibody(ies)
APS	antiphospholipid syndrome
β2GPI	β2-glycoprotein I
BSA	bovine serum albumin
BrdU	bromodeoxyuridine
CAPS	catastrophic antiphospholipid syndrome
DC	dendritic cell
CFA	Complete Freund's Adjuvant
CL	cardiolipin
Con A	concanavalin A
DNA	deoxyribonucleic acid
DOPC	dioleoylphosphatidylcholine
DOPS	dioleoylphosphatidylserine
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
HSA	human serum albumin
HDL	high density lipoprotein
HLA	human leukocyte antigen
Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
IL-4	interleukin-4
IL-10	interleukin-10
IFN-γ	interferon-γ
i.v.	intravenous
KO	knockout
LA	lupus anticoagulant
LDL	low density lipoprotein
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
OD	optical density
oxLDL	oxidized low density lipoprotein
PAPS	primary antiphospholipid syndrome

PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
P*i*	inorganic phosphate
PI	phosphatidylinositol
PL	phospholipid(s)
PS	phosphatidylserine
PT	prothrombin
RT	room temperature
SAPS	secondary antiphospholipid syndrome
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	serine
SLE	systemic lupus erythematosus
TBS	Tris-buffered saline
TCR	T cell receptor
T_H	T helper
Trp	tryptophan
VDRL	Veneral Disease Research Laboratory
VLDL	very low density lipoprotein

CHAPTER I. INTRODUCTION AND LITERATURE REVIEW

SECTION 1. GENERAL FEATURES OF ANTIPHOSPHOLIPID SYDROME

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by the presence of antiphospholipid antibodies (aPL) and clinical manifestations such as thrombosis and/or recurrent pregnancy loss.[1] For a patient to be classified as having APS, they must meet at least one clinical criterion and at least one laboratory criterion (Table 1). Several other features associated with APS, but not included as classification criteria, include heart valve disease, livedo reticularis, thrombocytopenia, neuropathy and neurological manifestations. Many of these other features, although highly associated with APS, are not specific for the syndrome.

APS can occur alone, as primary APS (PAPS), or in association with other autoimmune disorders such as systemic lupus erythematosus (SLE), where it is referred to as secondary APS (SAPS). In rare cases of APS, patients will experience an acute form of the syndrome known as catastrophic APS (CAPS), experiencing multiple thrombotic events in a short period of time (weeks) that may result in death. While approximately 1 to 5% of the population produce aPL, the majority of these individuals do not present any clinical symptoms of APS.[2] It is difficult, however, to determine which individuals with aPL will go on to develop APS. It is estimated that 30% of aPL-positive SLE patients will develop APS and this risk increases to 50% at 20 years of follow-up.[2] Although the precise etiologic agent of APS is unknown, it has been suggested that both environmental and genetic factors contribute to the development of this syndrome.[3,4]

1.1 Antiphospholipid antibodies (aPL)

aPL are a class of autoantibodies that are heterogeneous in their affinity and specificity for their target antigens, but united in their recognition of phospholipids (PL), PL-binding proteins, or complexes of the two. aPL were first detected in 1906 in a serological laboratory test for *Treponema pallidum*, the etiological agent of syphilis.[5] Known as the Wassermann complement-fixation test, positivity was defined as reactivity to extracts from bovine hearts. The precise antigen of the Wasserman test was unknown at the time, but later identified as cardiolipin (CL) in 1941.[6] CL is exclusively found in bacterial and mitochondrial membranes and is the antigenic target of aPL in syphilis patients.[7-9] This discovery resulted in the development of the Venereal Disease Research Laboratory (VDRL) test for syphilis, an assay that uses a CL-cholesterol-lecithin mixture for the detection of antibodies present in the sera of patients with syphilis. At the beginning of the twentieth century, mass blood screening programs were initiated to prevent the spread of syphilis. This led to the observation that many SLE patients had a positive test for syphilis, but presented no clinical evidence of the disease.[10] Upon the development of a more sensitive immunoassay for the detection of anticardiolipin antibodies (aCL), strong correlations could be made between aCL titers, lupus anticoagulant antibodies (LA), and venous and arterial thrombosis in SLE patients.[11] In contrast, this was not found to be the case for anti-DNA antibodies, a prototypic autoantibody in patients with SLE.[11,12] APS was first referred to as 'the anticardiolipin syndrome' by Graham Hughes[13] and, in 1989, three independent groups published reports of APS existing as a distinct entity. These reports constituted the first recognition of PAPS.[14-16]

Since CL is a common bacterial membrane constituent, the VDRL test is not specific for *T. pallidum*.[6] Patients with other infectious diseases, such as malaria, tuberculosis, leprosy, hepatitis and acquired immunodeficiency syndrome (AIDS), also have antibodies that are reactive with CL.[17-21] Similarly, patients with SLE and APS have CL-reactive aPL. Early studies in this field investigated the differences between CL-reactive antibodies from patients with infectious diseases and those with SLE and/or APS. This has led to the definition of characteristics that distinguish 'infectious aPL' from 'autoimmune aPL'.[22-26] 'Infectious aPL' react directly with PL and bind to CL in the absence of serum proteins. On the other hand, 'autoimmune aPL' bind to CL only in the presence of the PL-binding protein, β2-glycoprotein I (β2GPI).[23,24] Since β2GPI binds to CL as well as other anionic PL, such as phosphatidylserine (PS) and phosphatidylinositol (PI), autoimmune aPL often recognize β2GPI bound to these PL as well. Furthermore, β2GPI inhibits the binding of infectious aPL to CL. Although 'infectious' and 'autoimmune' aPL differ in their binding characteristics, their appearance *in vivo* is not mutually exclusive. For example, patients with leprosy and human parvovirus B19 have been shown to possess both β2GPI-independent aCL and anti-β2GPI immunoglobulin G (IgG),[27-29] suggesting that infection may provide a context for the induction of some autoimmune aPL.

The discovery that autoimmune aPL recognize a PL-binding protein (e.g, β2GPI) bound to PL, rather than to PL alone, was a major milestone in the area of APS as it suggested that the protein, rather than the PL itself, was the antigenic target in these individuals. This discovery also led to the observation that some aPL bind directly to β2GPI, in the absence of PL.[24,30] The latter antibodies are referred to as 'anti-β2GPI', while antibodies recognizing CL-bound β2GPI are referred to as 'aCL'.

aPL can be categorized into three major categories: aCL, anti-β2GPI and LA (Figure 1). Although there are other types of aPL (such as antibodies to prothrombin [PT]), these three subsets of aPL are currently considered to be the most clinically relevant in APS. To fully understand the nature of aPL, it is important to be aware that their distinction is largely based on their method of detection. aCL and anti-β2GPI are detected by enzyme-linked immunosorbent assays (ELISA). Here, aCL are measured by their reactivity to β2GPI bound to CL coated on polystyrene plates and anti-β2GPI are measured by their reactivity to β2GPI bound directly to polystyrene plates. On the other hand, LA are detected by an *in vitro* coagulation assay that measures their ability to prolong clotting time *in vitro*. The term 'lupus anticoagulant' is misleading, however. Although these antibodies have anticoagulant activity in PL-dependent coagulation assays *in vitro*, they are associated with thrombosis in APS patients who produce these antibodies *in vivo*.[31,32] Furthermore, it should be noted, that aCL, anti-β2GPI and LA are not entirely distinct groups. That is, some aCL and anti-β2GPI have LA activity, while others do not. Similarly, some LA are dependent on β2GPI for their functional activity, while others depend on other PL-binding proteins (e.g., PT).[33,34] Due to the heterogeneity of LA antibodies, no one assay is both sensitive and specific enough to be used alone. Thus, several assays must be used in combination to detect LA and rule out the possibility of other coagulation abnormalities.[35] LA antibodies are more specific to APS, while aCL are more sensitive.[36,37] The value of studying the antigen specificity of aPL is that their interactions and downstream effects may explain their pathophysiology. However, at this time, no clear associations between particular aPL and clinical manifestations can be made.

1.2 PL-binding proteins

The distinction that autoimmune aPL are directed against PL-binding proteins, rather than PL themselves, marked an important point in the history of APS. β2GPI and PT are the two major antigenic targets of aPL in APS. Other target proteins include annexin V, protein C, protein S, factor XI, thrombomodulin and high molecular weight kininogen.[38,39] The following sections will primarily discuss β2GPI, as T cell reactivity to this protein is the focus of my thesis.

1.2.1 β2-glycoprotein I (β2GPI)

β2GPI is a 50 kDa PL-binding protein belonging to the complement control protein superfamily. Other members of this family include interleukin-2 receptor (IL-2R) and C4b binding protein.[40] β2GPI was first described in 1961 as a perchloric acid-soluble protein found in human plasma, but its function was unknown at the time.[41] It was not until years later, in 1990, when three groups simultaneously identified β2GPI as the cofactor responsible for the binding of aCL to CL.[23-25] β2GPI is one of the most abundant plasma proteins and is produced in the liver,[42] placenta[43] and intestinal tract.[44] It circulates in human plasma at a concentration of approximately 200 μg/mL. Although β2GPI is found mainly in its free form, more than 40% may be found in association with very low density lipoprotein (VLDL), high density lipoprotein (HDL) and postprandial chylomicrons.[44] As β2GPI satisfies all the classification criteria for an apolipoprotein, it has also been termed 'apolipoprotein H'.[45]

β2GPI exists as a single polypeptide chain of 345 amino acids that is unusually rich in cysteines, prolines and tryptophans. It possesses five glucosamine-containing oligosaccharides, all linked to asparagine residues, and five short consensus sequences to form an overall fish-hook like structure (Figure 2). These five highly conserved motifs of approximately 60 amino acids each, also known as sushi domains, may be the result of gene duplication.[46] Indeed, some homologous sets of cysteines form a "looped-back" configuration to link to other sets of homologous cysteines, forming its tertiary structure of five domains (I-V). Domain V, however, differs slightly from the other four domains. It possesses a cluster rich in positively charged lysines that allows for binding to anionic surfaces and a hydrophobic C-terminal loop that allows for insertion into the lipid bilayer.[47,48] Thus, Domain V possesses the PL-binding site and the region recognized by many aCL.[49] Mutational studies have shown that substitution of tryptophan (Trp)316 for serine (Ser)316 on Domain V results in the abolition of its PL-binding capacity.[50] Furthermore, proteolytic cleavage of β2GPI within Domain V results in the reduction of its antigenicity.[51] In addition to binding to anionic PL, β2GPI can bind to other negatively charged surfaces, such as heparin,[52] deoxyribonucleic acid (DNA)[53] and calmodulin,[54] as well as to the cell surfaces of endothelial cells[55] and activated platelets.[56,57]

The precise physiological function of β2GPI is unclear, but it has been reported to have metabolic roles such as increasing the enzymatic activity of lipoprotein lipase in the hydrolysis of triglyceride-PL emulsions in the presence of apolipoprotein CII (apoC-II)[58] as well as enhancing experimentally infused triglyceride removal from plasma in rats.[59] β2GPI has also been shown to bind to oxidized low density lipoprotein (LDL) (oxLDL). It has been proposed to have a protective function *in vivo* by coating the surface of

negatively charged cells or particles, allowing them to stay in the circulation without causing procoagulant activity.[60] In addition, i*n vitro* and *in vivo* studies have shown that β2GPI binds to apoptotic cells to promote their phagocytic uptake for clearance.[61,62]

In addition to being a major antigenic target in APS, *in vitro* studies have shown β2GPI to function both as a natural anticoagulant and procoagulant. Since negatively charged molecules trigger the intrinsic coagulation pathway,[63] it is thought that β2GPI can inhibit coagulation by sequestering anionic PL. Furthermore, β2GPI can inhibit adenosine diphosphate (ADP)-mediated aggregation of platelets[64] as well as impair thrombin generation.[64,65] On the other hand, β2GPI can function as a procoagulant by inhibiting activated protein C activity.[66] It must be kept in mind, however, that these observed effects occur *in vitro* and the net effect of β2GPI *in vivo* may be quite different. It has been proposed that in a diseased state, aPL may either inhibit the anticoagulant/procoagulant functions of β2GPI or potentiate them. aPL have been shown to enhance the inhibitory effect of β2GPI on thrombin generation[65,67] and the procoagulant effect of β2GPI on activated protein C activity.[65] In addition, aCL ablates the inhibitory effect by β2GPI on factor Xa generating activity of activated platelets.[68]

Much insight into the function of a protein often comes from individuals with genetic mutations or from the generation of deficient or knockout (KO) mice. To study the effect of β2GPI deficiency on plasma lipoprotein, metabolism and hemostasis in humans, Yasuda *et al*. surveyed the concentration of plasma β2GPI in apparently healthy individuals.[69] They found three individuals with a frameshift mutation in the β2GPI gene that resulted in undetectable serum levels of the protein. Moreover, the lipoprotein profiles of the three homozygous individuals appeared to have some abnormalities that increased their atherogenic risk, but data was insufficient to determine any cause-effect

relationships. Interestingly, heterozygous individuals with the same mutation showed significantly lower serum concentrations of β2GPI compared to those without the mutation, but had apparently normal lipid profiles. It appears that either low levels of β2GPI are sufficient to perform metabolic functions or that β2GPI has no significant role in lipoprotein function. Although, β2GPI deficiency alone is not a primary risk factor for thrombosis,[70] homozygous β2GPI deficiency may be an additional, if not an independent, risk factor for atherosclerosis and thrombosis.[69]

β2GPI-deficient mice show an impaired ability to generate thrombin, but have normal coagulation.[71] Furthermore, when β2GPI-deficient heterozygotes were crossed, β2GPI-null mice were born at significantly lower than predicted Mendelian ratios, suggesting that they might be less viable than wildtype littermates. However, there was no detectable difference in the weight, litter size and viability between β2GPI-deficient male or female pups and β2GPI-sufficient control mice. These findings indicate that β2GPI is not essential for successful reproduction, but suggest that it may be involved in early embryonic development or implantation.[71] Although studies of human and murine β2GPI deficiency have provided some insight into the physiological function of β2GPI, many questions remain given its many roles, some of which may be redundant. The matter is further complicated by its role in APS where its interaction with aPL and resulting pathology is poorly understood.

1.2.2 Prothrombin (PT)

PT is another major antigenic target of aPL, and antibodies to this PL-binding protein are detected in approximately 50-90% of patients with aPL.[72] Anti-PT antibodies

(aPT) were first shown to possess LA activity by Bevers in 1991.[33] It was later shown that the IgG fraction causing LA activity was directed against a complex of PT and PL.[34]

Prothrombin is a 72 kDa vitamin K-dependent glycoprotein present in human plasma at a concentration of approximately 100 μg/mL.[73,74] Also known as factor II, it is synthesized in the liver as a single polypeptide chain and undergoes γ-carboxylation on what is known as the Gla domain, located on fragment I of the protein.[73,75] The Gla-domain is essential for calcium-dependent binding of PS to PT.[76] PT is a zymogen that, when activated by the complex of activated factor X, activated factor V, PL and calcium, is cleaved to form thrombin. Thrombin, in turn, converts fibrinogen to fibrin, forming a blood clot.

Like β2GPI, aPT can be detected by ELISA where PT is the coated antigen on irradiated plates,[30] or by using PS-coated plates with PT as a cofactor.[77] The latter method of detection yields a greater number of positive patient samples, suggesting it is a more sensitive assay.[72] Furthermore, Atsumi *et al*. showed that antibodies to PS-bound PT, but not PT alone, significantly correlated with the clinical manifestations of APS, suggesting these antibodies may be a marker of the disease.[78]

1.3 Murine models of APS

There are several types of murine models of APS that have been developed over the past 20 years. These models fall into three major categories: (1) those that arise spontaneously; (2) those induced by passive transfer of antibody; and (3) those induced by active immunization. Given the complex nature and phenotypic diversity of APS,

each type of model can be used to address a different set of questions and no one model can answer all questions within APS.

1.3.1 Spontaneous models of disease

The two major murine models of spontaneously occurring APS are the MRL/lpr and NZW x BXSB F_1 (W/B F_1) mice, both well-known spontaneous models of SLE. The advantage of spontaneous models over induced ones is that they allow for the study of disease in a more physiological setting. However, these models often have other features that can interfere with disease analysis. For example, both spontaneous models of APS are models of SAPS, rather than PAPS. This means that these mice present features of another autoimmune disease (i.e., SLE), as well as APS. No spontaneous model of primary APS has been described to date.

MRL/lpr mice have the *lpr* (*l*ympho*pr*oliferative) gene, which is a mutant allele of Fas.[79] Lymphocytes in these mice cannot undergo Fas-mediated apoptosis, which is necessary for controlling lymphocyte activation.[80] Mutation of the Fas ligand gene, *gld* (*g*eneral *l*ymphoproliferative *d*isease), also results in autoimmunity. MRL/lpr mice produce anti-DNA antibodies and aCL; develop glomerulonephritis and thrombocytopenia; and have smaller than normal litters.[81] Furthermore, immunization of MRL/lpr mice with β2GPI and adjuvant results in the induction of increased levels of aPL and an acceleration in the appearance of autoimmune manifestations.[82]

NZW x BXSB F_1 (W/B F_1) male mice develop SLE-like disease, produce a wide range of autoantibodies and have a high incidence of thrombocytopenia and myocardial infarction.[83-86] Disease susceptibility in these mice has been shown to be linked to the Y

chromosome from the BXSB parental strain[87] and major histocompatibility (MHC) genes from the NZW parent.[88-91] The over-production of autoantibodies is at least partially due to the pre-activation of B cells *in vivo*.[92] W/B F_1 mice have also been shown to produce β2GPI-dependent aCL (mainly of the IgG isotype), with titers increasing with age.[86] These antibodies do not react to β2GPI or CL alone, apparently recognizing a novel epitope on β2GPI generated upon CL binding.

1.3.2 Transfer of aPL

An induced model of APS consists of transfer of purified aPL to mice. In this type of model, the *in vivo* effects of aPL of defined specificities can be studied and their role in causing pathology (e.g., pregnancy loss or thrombosis) may be evaluated. Passive transfer of polyclonal aCL IgG from APS patients has been shown to cause pregnancy loss in BALB/c mice.[93] Passive transfer of aPL has also been shown to affect thrombus formation. For example, passive transfer of human monoclonal IgG antibodies (CL- and phosphatidic acid [PA]- reactive) derived from a patient with recurrent thrombosis into CD1 mice resulted in increased thrombus generation in a model of controlled injury (applied pressure).[94] Active immunization of BALB/c mice with aPL and adjuvant has also been shown to result in PAPS with clinical and laboratory features including LA activity, thrombocytopenia, increased fetal resorptions and low embryo and placental weights.[95,96] It is noteworthy that passive transfer of a monoclonal IgG aPL from mice immunized with a 15-mer peptide (GDKV) containing the PL-binding site of β2GPI also resulted in enhanced thrombus formation and increased adherence of leukocytes to endothelial cells *in vivo*.[97] The latter findings, in particular, are consistent with the

hypothesis that the PL-binding site of β2GPI is immunogenic and that β2GPI-specific antibodies promote thrombosis in an *in vivo* model. While this model may be useful in determining the physiological impact of individual aPL, these mice do not possess β2GPI-specific B and T cells and therefore do not provide a model for the initiation or propagation of APS.

1.3.3 Immunization with β2GPI

Induction of APS by immunization with human (heterologous) β2GPI, in the presence of adjuvant (typically Complete Freund's Adjuvant [CFA]), in BALB/c mice has been demonstrated to result in the production of IgG aPL, thrombocytopenia and a high percentage of fetal resorptions.[98] Moreover, tolerance to murine (autologous) β2GPI has been shown to be broken in this model if one observes the mice several months following hyperimmunization.[99] Levine *et al*. demonstrated that immunization of BALB/c mice with β2GPI and lipopolysaccharide (LPS) (a non-specific simulator of the innate immune system) resulted in the production of high titers of anti-β2GPI, aCL, anti-phosphatidylethanolamine (PE), anti-phosphatidylserine (PS), and anti-phophatidylcholine (PC) antibodies as well as LA activity. Mice were no longer self-tolerant, produced other SLE-specific autoimmune aPL and developed glomerulonephritis.[100] The spontaneous models of SAPS and models of APS induced by transfer of aPL do not possess activated B or T cells, thus providing a very limited context in which to study APS. The induction of a strong and persistent β2GPI-specific B and T cell response, in the context of inflammation, is necessary to induce disease in healthy mice immunized with β2GPI and LPS.[100] Considering the strong β2GPI-specific

B and T cell response, the break in tolerance and sequential emergence of multiple SLE autoantigens, the model of Levine *et al*. provides a much more relevant model of aPL production. Although mice showed no evidence of thrombosis, despite high levels of aPL, this provides a valuable model in which the specificity of B and T cells for β2GPI and their roles in autoantibody production may be studied. Induction of thrombosis in this model, as in other aPL-related models,[101] will likely require an additional factor. A *two hit* hypothesis has been suggested to explain this phenomenon. Here, a *first hit*, such as the presence of aPL, and a *second hit*, such as endothelial activation or injury, may be necessary to induce thrombosis.[102] Showing this in a model of APS might help explain thrombotic episodes in APS patients.

Anionic PL are normally not exposed on the surface of viable cells. However, during the process of apoptosis, the plasma membrane of a cell undergoes redistribution of its PL. Here, anionic PL, such as PS, normally found in the inner leaflet of the plasma membrane, will redistribute to the outer leaflet.[103] Physiologically, exposure of PS allows for the phagocytosis of apoptotic cells via scavenger receptors for their subsequent elimination.[104] This minimizes the release of intracellular contents, which is necessary to limit inflammation.[105] However, exposed PS also allows for the binding of PL-binding proteins such as β2GPI and PT. It has been shown that aPL from APS patients bind to apoptotic, but not viable thymocytes, in a β2GPI-dependent manner. This suggests that β2GPI-bound apoptotic cells can serve as a potential target for aPL.[106] Furthermore, immunization of mice with β2GPI-bound apoptotic cells induces the production of anti-β2GPI and aCL antibodies and LA activity, demonstrating the ability of β2GPI-bound apoptotic cells to act as an immunogen *in vivo*. In accordance with this hypothesis, PL such as CL and PS, bound to β2GPI, were shown to be immunogenic in BALB/c mice,

inducing the production of anti-β2GPI, aCL and aPS antibodies.[107] The binding of foreign β2GPI to apoptotic cells (as in the model of Levine *et al*.) provides a potential immunogen for the activation of β2GPI-reactive T cells and generation of aPL *in vivo*. In contrast with spontaneous models of APS, these induced models of aPL allow one to address the question of *how* tolerance can be broken and autoantibodies produced. Clinical manifestations such as thrombosis, however, are not observed in any of these models. Determining the *second hit* required for pathology may be key to understanding APS and improving the treatment of patients.

SECTION 2. THE ROLE OF T CELLS IN ANTIPHOSPHOLIPID SYNDROME

Since APS was first defined in the early 1980's, much of the research has focused on the humoral aspects of the disease. Recently, the focus has shifted towards the cellular features of the disease, particularly with respect to disease initiation. The reason for this shift is largely based on the fact that high titers of autoantibodies of the IgG and/or immunoglobulin A (IgA) isotype are found in patients.[108,109] Isotype switching from immunoglobulin M (IgM) to IgG and IgA for antibodies to most protein antigens is known to require T cell help, thus implicating T cells in the initiation and/or exacerbation of disease. The first solid piece of evidence of the involvement of T cells in APS was demonstrated by the transfer of whole-population, but not T cell-depleted, bone marrow cells from mice with experimentally induced APS into irradiated naïve mice. This resulted in the induction of experimental APS, as defined by the production of aPL and clinical features of the syndrome.[110] Prior to that study, the same group showed that treatment of mice with experimental APS with anti-CD4 monoclonal antibody (mAb) resulted in decreased aPL titers and a reduction in some clinical features of APS and SLE.[111] Interestingly, the majority of the current research into T cell involvement in APS has been performed using peripheral blood mononuclear cells (PBMCs) or isolated T cells from humans rather than in mice. There are several limiting factors, however, to clinical research. First, although activated T cells may be found in the blood, the majority of β2GPI-reactive T cells will likely be found in the spleen, an organ that constantly samples the blood for antigen (e.g., β2GPI). Obviously, obtaining splenic tissue from patients for the purpose of experimental research is not ethical and, so, obviates investigating splenic lymphocytes in these individuals. Second, the phenotypic diversity

of patients with APS makes patient selection in human studies a difficult task and interpretation of the results of clinical investigations more complex. Nonetheless, some studies using human-derived PBMCs or T cells have been successful in characterizing the T cell response to β2GPI, both in terms of the antigen specificity and type of helper T response produced.

2.1 Antigen specificity of T cells in APS

Visvanathan and McNeil were the first to attempt to characterize the T cell response to β2GPI in patients using PBMCs[112]. They found cellular immunity to β2GPI in 44% of the 18 APS patients tested.[112] Termed "responders", the PBMCs of these patients proliferated in response to purified human β2GPI and to an even greater extent to β2GPI present in human serum whereas no response was observed in healthy controls, patients with other autoimmune diseases, or aCL-positive patients with no history of thrombosis or fetal loss. The β2GPI-reactive T cells, which were shown to be mainly of the CD4 subset and restricted by human leukocyte antigen (HLA) class II, produced high levels of interferon-γ (IFN-γ), but no interleukin-4 (IL-4), suggesting a T helper (T_H)-type 1 bias. Taking a slightly different approach, Hattori *et al*. demonstrated T cell reactivity to reduced β2GPI (dithiothreitol [DTT] treated), but not native β2GPI, in both APS and SLE patients and healthy individuals, whether positive or negative for anti-β2GPI.[113] Characterization of the T cell response showed that the β2GPI-reactive T cells were $CD4^+$ and HLA class II-restricted and that the T cell epitopes were located mainly on Domains IV and V of β2GPI. Furthermore, PBMC from patients with circulating anti-β2GPI demonstrated anti-β2GPI production *in vitro*, when they were stimulated with reduced

β2GPI. This anti-β2GPI production was blocked by depletion of $CD4^+$ T cells. *In vitro* anti-β2GPI production was not observed in patients who did not have detectable *in vivo* serum levels of anti-β2GPI. Interestingly, PBMCs from some of the patients (5/13) without detectable serum anti-β2GPI showed T cell proliferation to reduced β2GPI, suggesting that the lack of anti-β2GPI in these patients (both *in vitro* and *in vivo*) was due to an insufficient number of β2GPI-reactive B cells. It is not surprising that T cells from patients with APS and SLE, as well as healthy individuals, responded to reduced or recombinant β2GPI, as they are forms of β2GPI not normally found *in vivo*. Although the physiological relevance of β2GPI that is reduced *in vitro* remains unclear, it is possible that it possesses some similarities to the reduction that takes place during antigen processing, *in vivo*.[114]

2.2 Cryptic epitopes in autoimmunity

Since β2GPI is a protein present in abundance in plasma, β2GPI-reactive T cells should undergo deletion by negative selection during thymic development and tolerance should be maintained via mechanisms of peripheral tolerance. However, this is only true for those protein determinants that are efficiently processed and presented by antigen presenting cells (APC) to T cells (Figure 3). Of the numerous theoretical peptides that may be generated from a protein during processing in the physiological context of endosomes and lysosomes, only a fraction will be generated in reality (reviewed by Kuwana *et al*., 2004).[115] The pool of resultant peptides will exhibit a range of affinities for MHC molecules, thus competing for loading onto MHC molecules. Immunodominant peptides are those peptides that bind with high affinity to MHC molecules and will be

efficiently presented, at the cell surface, to T cells. From the perspective of the T cell, the affinity with which the T cell receptor (TCR) binds to the MHC-antigen complex will affect T cell activation. Taken together, the numerous factors influencing antigen processing and presentation, and T cell activation, result in a hierarchy of T cell determinants. Those peptides that are inefficiently processed and/or presented are termed "cryptic determinants" as they will be 'invisible' to T cells under normal circumstances.[116] T cells that are not exposed to self-determinants during thymic development or in the periphery will not undergo deletion and will remain part of the T cell repertoire. It is in this manner that some T cells escape mechanisms of central and peripheral tolerance, permitting the survival of self-reactive T cells that can give rise to autoimmunity. Human disease and murine models of autoimmunity are circumstances in which such self-reactive T cells may exist and be studied in isolation. The importance of autoreactive T cells in APS prompted the research in this thesis.

2.2.1 Cryptic T cell epitopes in APS

T cell epitope mapping is an important approach to understanding how T cells recognizing cryptic epitopes are activated in APS patients, while remaining at subthreshhold levels in healthy individuals. To address this issue, Arai *et al*. used a slightly different approach to studying T cells than previous reports.[117] Rather than using whole PBMCs, this group of investigators generated T cell clones from APS patients and mapped the T cell epitopes (determinants) of β2GPI. By incubating T cell clones with peptides, each comprised of two contiguous domains of the β2GPI molecule, they found that T cell clones mainly reacted to epitopes on Domains IV and V. Screening with

shorter peptides from Domain V showed that the immunodominant peptide was p276-290, which contains the PL-binding site. Furthermore, T cell recognition of p276-290 was shown to be restricted by the HLA class II allele DRB4*0103, also known as DR53. This finding is consistent with other data in the literature suggesting that particular HLA class II alleles predispose individuals to APS.[118] Moreover, it indicates that individuals possessing the DR53 allele likely present more efficiently the immunodominant peptide generated from processing of p276-290.[117] Finally, the affinity of this MHC molecule for this β2GPI-derived peptide suggests that particular MHC alleles such as this one will be found more frequently in APS patients than in the normal population. In fact, several studies have found associations between various HLA class II molecules and aPL within certain ethnic groups with APS and SLE.[119-121]

The MHC association in APS, however, is not at all clear and different groups have shown association of T cell reactivity with different HLA class II alleles. Ito *et al*. used a synthetically prepared β2GPI peptide library to stimulate T cells and generate T cell lines from PBMCs of APS patients.[122] They found four dominant epitopes in five individuals (four patients and one healthy control), one of which was the p244-264 region, located on Domain V. Overall, p244-264, a region of Domain V that does not include the PL-binding site, was the immunodominant epitope for the T cells from these patients. Interestingly, and in contrast to the Arai study, peptides containing the PL-binding site did not elicit T cell reactivity and, although DR molecules were shown to be involved, no particular HLA class II allele restriction was evident. Discrepancies between the peptides recognized by APS patients in the two studies may be due to the approach in the generation of the T cell lines/clones in the two studies; Arai *et al*. established T cell clones from PBMCs stimulated with recombinant full-length β2GPI, while Ito *et al*. used

synthetic β2GPI peptides or regions of β2GPI. The lack of a consensus between these studies also exemplifies how HLA class II restrictions may only partially account for disease susceptibility.

2.2.2 Physiological context of cryptic epitope exposure

One of the fundamental questions that arise when studying β2GPI is the nature of the anionic PL targeted by this protein *in vivo*. Research demonstrating that apoptotic cells can serve as an antigenic target, as well as an immunogen, for β2GPI-dependent[106] and PT-dependent[123] aPL, highly implicate apoptotic cells as important initiators and/or propagators of aPL production.[124] The redistribution of anionic PL, such as PS, from the inner to the outer leaflet of the plasma membrane during apoptosis allows for the binding of the PL-binding proteins β2GPI and PT.[106,123] CL is normally only found in the mitochondrial inner membrane,[7] however, apoptotic cells have been shown to redistribute their mitochondrial CL to the cell surface[125] and aCL IgG derived from APS patients have been shown to bind to the surface of apoptotic cells expressing CL.[126,127] Furthermore, the opsonization of aPL-bound apoptotic cells by macrophages and dendritic cells (DC) results in the secretion of proinflammatory cytokines and the enhanced ability of DC to activate MHC class II-restricted T cells. The generation of aPL specific for molecules normally hidden from the immune system suggests that their exposure may be an early step in initiating aPL production *in vivo*. Activation of T cells by exposure of these cryptic epitopes would lead to production of aPL and, potentially, generation of the inflammatory state and presentation of clinical symptoms observed in APS patients.

2.2.3 Cryptic B cell epitopes in APS

Cryptic epitopes can also be discussed from the point of view of the antibody response (i.e., the B cell). Cryptic B cell epitopes are not exposed under physiological conditions, but may be exposed if the antigen undergoes a conformational change that results in the accessibility of that epitope to an antibody. Changes in physiological conditions resulting in the exposure of a previously cryptic B cell epitope include oxidation, reduction, proteolytic cleavage, binding to a surface or other molecules (i.e., antibodies), and multimerization. Wagenknecht *et al*. proposed that β2GPI undergoes a conformational change upon PL binding, resulting in exposure of a neoepitope on β2GPI.[128] Indeed, it was later shown that interaction with PL does indeed induce a conformational change in β2GPI.[107,129,130] Prior to 1995, there was much controversy surrounding aPL and its antigens. It was thought by many that aPL could not recognize β2GPI in the absence of PL. This later proved to be inaccurate and largely due to the methodology of detection. Matsuura *et al*. showed that aCL recognize an epitope expressed by a conformational change in β2GPI that occurs when it interacts with an oxygen-substituted solid phase surface (e.g., γ-irradiated of microtiter plates).131,132 This conformational change induced by binding to γ-irradiated plates mimics that which occurs upon β2GPI binding to anionic PL. The same effect can also be achieved using carboxylated or polyvinylchloride plates, which are more hydrophilic in nature than untreated plates.133,134 Roubey *et al*. explained these findings by showing that aPL have a low affinity for β2GPI, but can bind β2GPI when the antigen density is sufficiently high enough for divalent binding.135 Specially treated microtiter plates allow for a

higher coating density of β2GPI, and make it clear why some groups were unable to detect aPL binding of β2GPI when using untreated plates.136,137

It is possible for aPL reactivity to change during the course of disease in patients with APS. That is, the epitopes targeted by autoantibodies early on in disease may not be the same epitopes targeted later in the disease. This is termed "epitope spread", and can also be used to define situations in which antibodies ultimately react with antigens other than those against which the antibodies were initially raised (Figure 4). Epitope spreading may be "intramolecular" (targeting new epitopes on the same molecule) or "intermolecular" (targeting new epitopes on other molecules). B cells bearing high-affinity Ig at the cell surface can specifically bind to and internalize antigen via receptor-mediated endocytosis. In this scenario, B cells can act as APC and process antigen into peptides that will bind to MHC class II molecules. In this manner, a B cell can present several different peptides to naïve T cells with specificities for epitopes other than the one initially bound by cell-surface immunolglobulin (Ig), resulting in the production of antibodies directed against new epitopes. Furthermore, antibody-bound internalized antigen may undergo alternative processing in the B cell, also resulting in the generation of a different set of peptides to be presented to naïve T cells.138

Several studies support the hypothesis of intramolecular epitope spread in APS. To better understand the pathophysiology of aCL, some groups have attempted to map the epitopes of β2GPI. It was initially thought that the putative epitope was found near the PL-binding region since the integrity of Domain V was shown to be crucial for aCL binding.[49] This was supported by research done using recombinant Domain V of β2GPI in direct-binding and inhibition studies with anti-β2GPI positive sera,139 however, there is evidence that aPL react with β2GPI epitopes located on domains other than Domain V.

George *et al*. demonstrated, using β2GPI domain deletion mutants to inhibit anti-β2GPI activity, that the target site for some IgG are found on Domain IV of β2GPI.[140] A recent report demonstrated that β2GPI-dependent LA antibodies recognize an epitope on Domain I that is exposed upon anionic PL-binding and that these antibodies are highly correlated with thrombosis.[141,142] Igarashi *et al*. argue that the epitope is absent from Domain I, but that Domain IV may play a critical role in the exposure of a cryptic epitope.[143] In accordance with this, Iverson *et al*. demonstrated that anti-β2GPI activity of 11 IgG antibodies purified from APS patients were inhibited by β2GPI domain deletion mutants containing Domain I, not Domain IV.144 Furthermore, a large cohort study showed a three-fold selectivity of anti-β2GPI for domain deletion mutants containing Domain I over mutants lacking Domain I, supporting the hypothesis that the immunodominant epitope may be found on the amino terminal domain of β2GPI.145 Later studies by Iverson *et al*. refined their techniques to demonstrate that Domain I possesses a dominant target epitope by performing single point mutation analysis. They show that mutations in the p40-43 region (on Domain I), containing a patch of positively charged residues, greatly affects binding of aPL to β2GPI.146 On the other hand, Koike *et al*. generated β2GPI variants with point mutations on Domain IV that were generally less reactive with APS patient sera.147 Iverson *et al*. repeated the production of the mutants produced by Koike *et al*. and showed them to be markedly less reactive with affinity purified antibodies from APS patients, even though these antibodies had been previously reported to recognize epitopes on Domain I.148 Taken together, Iverson *et al*. conclude that an intact Domain IV is necessary for the exposure of epitopes on Domain I. Although the precise immunodominant epitope still remains unknown, determining the

influence of antibodies of differing specificities on pathology is imperative to understanding APS.

2.3 A model of aPL production for the study of APS

The idea that autoreactive T cells become activated when exposed to cryptic epitopes is one of the most convincing hypotheses proposed to date to explain the production of aPL in APS.149 Kuwana *et al*. were the first to clearly demonstrate a mechanism of activation of pathogenic β2GPI-reactive T cells against cryptic epitopes using T cell lines reactive with the β2GPI peptide (p276-290), which contains the PL-binding domain.149 They showed that binding of β2GPI to anionic PL results in enhanced generation of cryptic T cell determinants as a direct consequence of antigen processing. In their study, autologous DCs, pulsed with a mixture of native human β2GPI and dioleoylphosphatidylserine (DOPS) or CL, stimulated HLA class II-restricted p276-290-reactive T cell lines derived from APS patients to proliferate and specifically produce IFN-γ. Furthermore, DR53-restricted peripheral blood T cells from healthy individuals, incubated with DCs that had been pulsed with PS-bound β2GPI, produced IFN-γ in response to p276-290. In contrast, no T cell response was observed to native β2GPI alone. These data provide evidence that T cells recognize the PL-binding site of β2GPI.[117] Taken together with studies of aPL induction by immunization with PL-[107] or apoptotic cell-bound[124] β2GPI, or the GDKV peptide[97] (containing the PL-binding site), these data strongly support the hypothesis that exposure of a cryptic epitope on Domain V is a major factor influencing the activation of self-reactive T cells in APS. Furthermore, the

microenvironment of the apoptotic cell provides a potential physiological context for the binding of β2GPI and subsequent activation of T cells.[106,123,124]

In our laboratory, immunization with heterologous β2GPI, in the presence of LPS, has been shown to be an effective inducer of aPL in a murine model.[100] Based on the high sequence homology between human and murine β2GPI (76% identities, 87% positives [CAA72190[42]; CAA41113150; as performed by NCBI BLASTP V2.2.17151]), we hypothesize that mice immunized repeatedly with human β2GPI and LPS will demonstrate a break in self-tolerance and produce aPL reactive with murine β2GPI. We further propose that β2GPI-reactive T cells derived from these mice recognize cryptic epitopes exposed on PL-bound β2GPI, as has been demonstrated in patients who produce aPL.

SECTION 3. RATIONALE AND OBJECTIVES

The humoral immune response to autoantigens has been studied extensively in patients with APS; however, the cellular immune response to these same antigens has been largely overlooked. The presence of β2GPI-reactive IgG autoantibodies in APS patients clearly implicates β2GPI-specific T cell help, but it is unclear how these self-reactive T cells become activated. There is some evidence suggesting that β2GPI-specific T cells recognize cryptic epitopes expressed on modified or PL-bound β2GPI, but not on the native form of the protein. However, the immune stimulus or mechanism that results in the activation and expansion of β2GPI-reactive T cells remains unknown.

To examine the origin and role of β2GPI-reactive T cells in APS, we have used a murine model of aPL induction. C57BL/6 mice were immunized with heterologous β2GPI in the presence of an activator of the innate immune system (LPS), an immunization protocol known to induce high levels of aPL. Splenic T cells were isolated from aPL-producing mice and studied for their recognition of native and PL-bound β2GPI.

The main objectives of this study were: (1) to characterize the β2GPI-reactive T cell response in a murine model of aPL production, in terms of the T cell proliferative and cytokine responses to native and PL-bound β2GPI; and (2) to compare the kinetics of the B cell and T cell responses to β2GPI in mice producing aPL.

Table 1. Classification criteria for antiphospholipid syndrome.

Clinical Criteria	Laboratory Criteria
Vascular Thrombosis[1]	Lupus anticoagulant antibodies (LA) in plasma[2]
Pregnancy Morbidity[3]	Anticardiolipin antibodies (aCL) of IgG and/or IgM isotype in serum or plasma[3,4]
	Anti-β2-glycoprotein antibodies (anti-β2GPI) of IgG and/or IgM isotype in serum or plasma[3,4]

[1]One or more clinical episodes of arterial, venous or small vessel thrombosis.
[2]Present on two or more occasions, at least 12 weeks apart.
[3]As outlined by the International consensus statement on an update of the classification criteria for definite Antiphospholipid Syndrome.[1]
[4]Present in medium or high titer measured by a standardized ELISA.

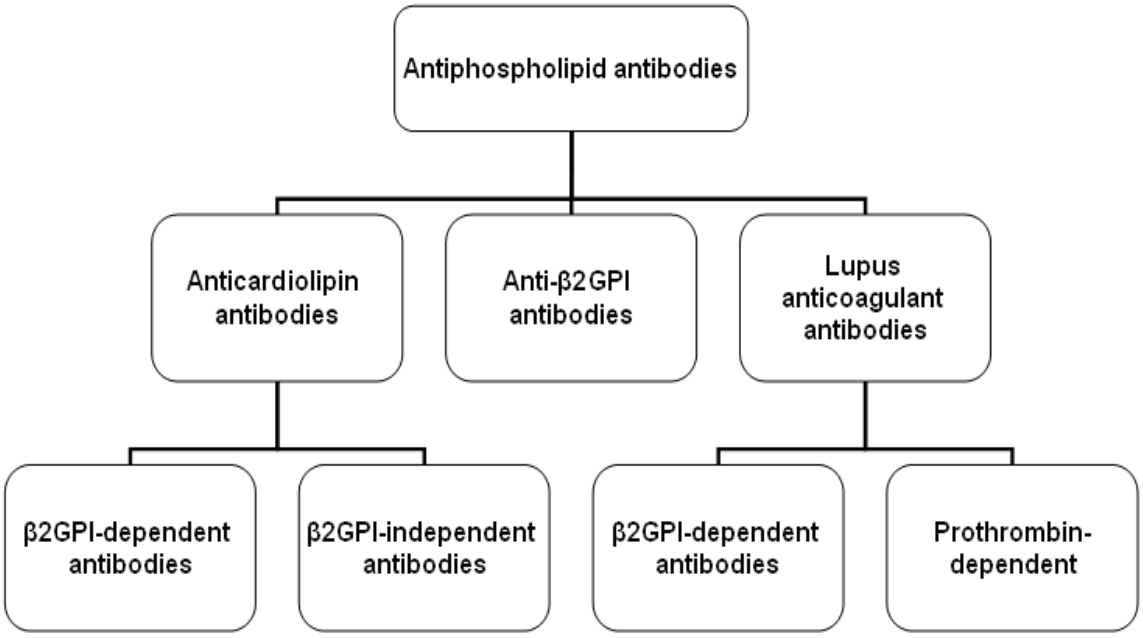

Figure 1. Schematic diagram representing the three major categories of aPL and their subsets. The three major groups of aPL are anticardiolipin, anti-β2GPI, and lupus anticoagulant antibodies. The subsets indicate the phospholipid-binding protein specificity of the aPL.

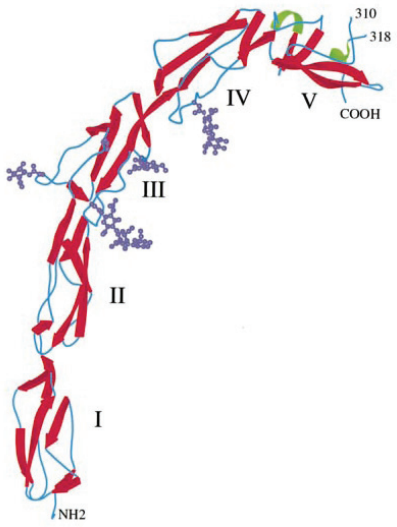

Figure 2. Ribbon diagram of the PL-binding protein, β2GPI. The tertiary structure of β2GPI, which forms a fish-hook like structure, consists of five domains (Domains I – V). The PL-binding domain is located on Domain V.

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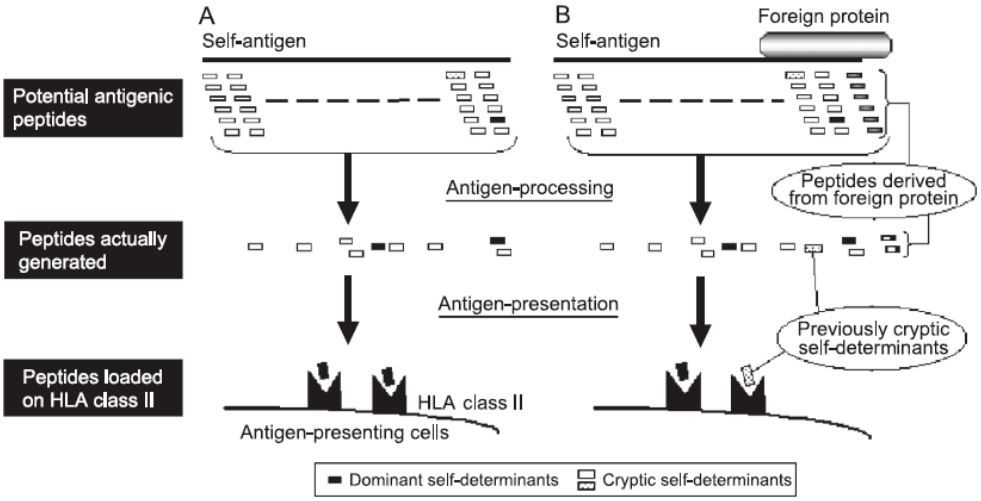

Figure 3. Cryptic epitopes expressed when there is modulation of antigen processing and presentation. Processing of self-antigens by APC results in the generation of numerous theoretical peptides, however, only those with a high affinity for HLA class II molecules will be presented at the cell surface (A). Binding of self-antigen to foreign proteins (or other molecules (e.g., PL) can modulate antigen processing and presentation to result in the generation of previously cryptic epitopes, or increase the concentration at which they are presented (B).

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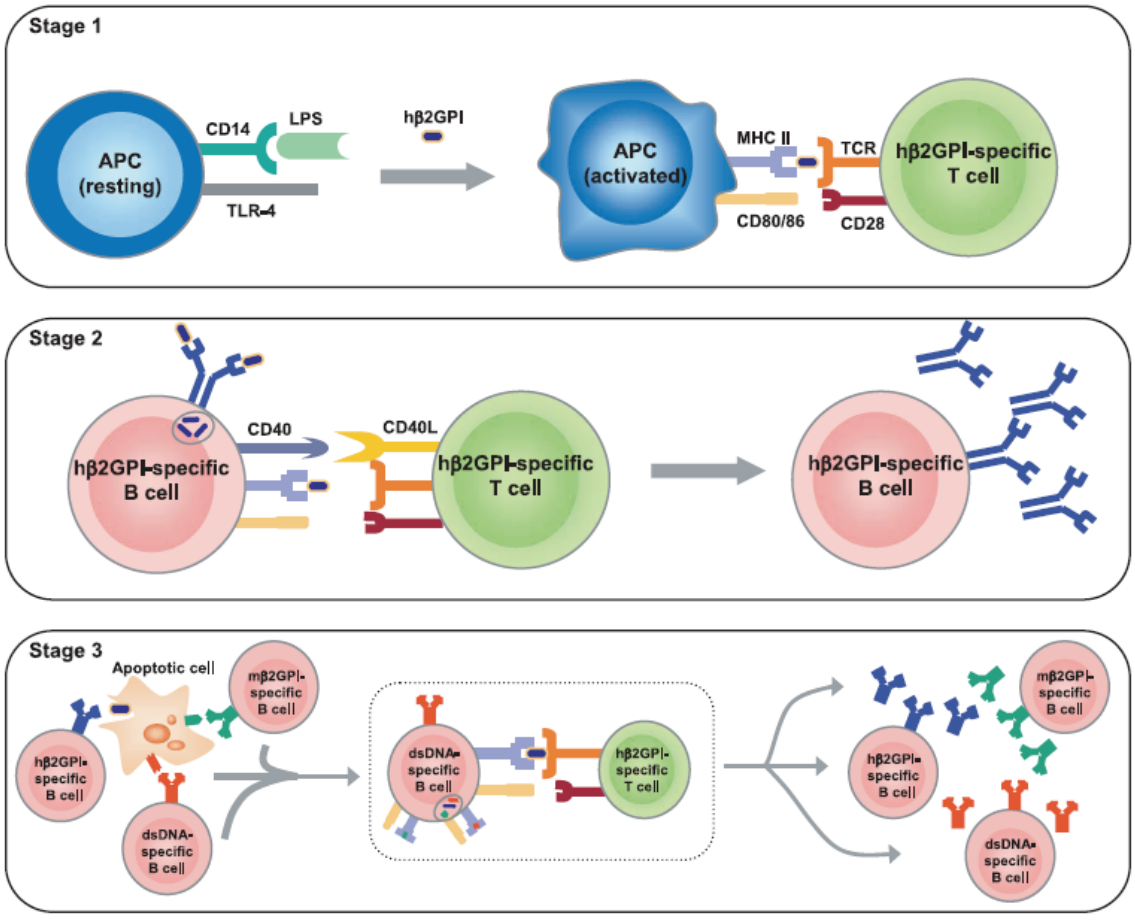


Figure 4. Schematic representation of intermolecular epitope spread in a murine model of aPL production. Stage 1: Activation of resting APC occurs by the binding of LPS via its receptor, TLR4, in the presence of human β2GPI to result in general systemic inflammation. Activated APC then present human β2GPI to activate human β2GPI-specific T cells. Stage 2: Activated β2GPI-specific T cells help β2GPI-spcecific B cells to produce anti-human β2GPI aPL. Stage 3: B cells can act as APC to recognize antigens on the surface of apoptotic cells (e.g., PL-bound β2GPI), internalize them via their surface Ig, process and present several apoptotic cell-associated antigens to multiple T cells of differing specificities. These activated T cells can now provide help to self-reactive B cells to produce autoantibodies.

CHAPTER II. MATERIALS AND METHODS

Materials

Unless stated otherwise, all chemicals were obtained commercially and used without further purification. β2GPI (apolipoprotein H) purified from human serum was obtained from Crystal Chem Inc. (Downers Grove, IL) and LPS was obtained from List Biological Laboratories (Campbell, CA). PL (bovine heart cardiolipin [CL], dioleoylphosphatidylserine [PS], and dioleoylphosphatidylcholine [PC]) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL).

Mice and Immunizations

All experiments were approved by the McGill University Animal Care Committee (see Appendix). Specific pathogen-free C57BL/6 mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and were maintained under specific pathogen-free conditions for all experiments. Female mice (10-12 weeks old) were bled for pre-immune (heparinized) plasma, and were immunized intravenously (i.v.) with 100 μL of either 10 mM HEPES buffer (150 mM NaCl, pH 7.4) or human β2GPI (20 μg/mouse for the first immunization; 10 μg/mouse for subsequent immunizations) followed 24 h later by injection of 100 μL of LPS (10 μg/mouse) (Figure 1a). All immunogens were prepared in 10 mM HEPES buffer. Mice were immunized at two-week intervals and received a total of two or four immunizations. Mice were bled 12 days post-immunization, except when they were used for T cell studies (Figure 1b). In the latter case, the mice were bled seven days post-immunization (immediately prior to sacrifice).

Measurement of aCL and Anti-β2GPI

Antibody reactivity to β2GPI bound to CL (referred to as "aCL") was detected on CL-coated plates in the presence of 10% fetal calf serum (FCS) (i.e., bovine β2GPI). Antibody reactivity to native human β2GPI, detected on human β2GPI-coated plates in the absence of CL, is referred to as "anti-β2GPI".[124] For the aCL ELISA, Immulon-2 plates (Dynatech Laboratories, Chantilly, VA) were coated with either 90 μg/mL of CL or gelatin in 10 mM phosphate-buffered saline (PBS), pH 7.3, and left uncovered to dry for 16 h at 37ºC. For the anti-β2GPI ELISA, Greiner high-binding plates (Bellco Glass, Vineland, NJ) were coated with 15 μg/mL of β2GPI in PBS. For aCL and anti-β2GPI ELISAs, coated plates were blocked with PBS containing 10% FCS and 0.5% gelatin for 2 h at 4ºC, and washed three times with 0.01 M Tris-buffered saline (TBS), pH 7.4. Murine plasma samples were diluted in PBS containing 10% FCS and 0.3% gelatin. Samples were added to the coated wells in duplicate and incubated for 3 h at 25ºC. The plates were washed three times with TBS, and bound antibody was detected by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Southern Biotechnology Associates, Birmingham, AL), diluted 1/1000 in PBS containing 0.4% bovine serum albumin (BSA), for 16 h at 4ºC. Plates were washed thee times with TBS and developed with *p*-nitrophenol phosphate for approximately 20 min at 37ºC and the optical density (OD) at 405 nm was read using an ELISA reader (model EL800 Universal Microplate Reader, Bio-Tek Instruments, Winooski, VT). Murine plasma known to be negative for, or to contain high levels of, aCL and anti-β2GPI served as negative and positive controls, respectively, for all ELISAs.

Depletion of bovine β2GPI from FCS

Before use in culture, FCS was depleted of bovine β2GPI to remove any endogenous source of β2GPI. Bovine β2GPI was depleted from FCS by two sequential absorptions on a HiTrap Heparin column (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was regenerated with 2.0 M NaCl, pH 7.4 containing 20 mM Tris between absorptions.

Preparation of β2GPI-Phospholipid Complexes

Appropriated volumes of PL (DOPS, DOPC, or DOPS combined with DOPC at a molar ratio of 3:7), dissolved in choloroform (CH_3Cl) at a concentration of 25 mg/mL, were dried down under nitrogen (N_2) gas for 5 min, resuspended in sterile HEPES buffer, pH 7.4, and hydrated for 1 h at 37ºC with vortexing at 15 min intervals. The concentration (wt/vol) of each PL suspension was then adjusted to 3.0 μmol/mL and the suspensions were sonicated for 5 min at 37ºC. Accurate molar concentrations of the PL suspensions were determined using the Bartlett assay for inorganic phosphate.[152] Aliquots of each PL suspension were then frozen at -70ºC. For each T cell experiment, frozen aliquots from the same preparation of PL were thawed for 30 min at 37ºC and sonicated for 5 min at 37°C. PL vesicles were mixed 1:1 (vol/vol) with HEPES buffer or native human β2GPI (300 μg/mL) and then incubated for 30 min at 25ºC before use. The vesicles were then ultracentrifuged (178, 000 x *g*) for 25 min in an airfuge (Beckman Instruments, Palo Alto, CA), and the supernatant containing unbound human β2GPI was aspirated and stored at -20°C for protein determination. The vesicles were resuspended in complete RPMI medium (RPMI supplemented with 10% β2GPI-depleted FCS, 0.1% L-glutamine, 0.1%

HEPES, 0.1% non-essential amino acids, 0.1% penicillin/streptomycin, and 55 mM 2-mercaptoethanol).

Protein Determination

For each preparation of vesicles and each experiment, the quantity of β2GPI remaining in the supernatants from the β2GPI-vesicle mixtures was determined by the MicroBCA Protein Assay kit (Pierce, Rockford, IL). Samples for protein determination were prepared in HEPES buffer, pH 7.4, containing 2% sodium dodecyl sulphate (SDS), which dissolves any residual lipid in the supernatant that could interfere with the protein assay, as recommended by the manufacturer. The percentage (%) of β2GPI bound to each type of vesicle was calculated as follows:

$$\frac{\text{[Initial concentration of β2GPI added] – [protein remaining in the supernatant]}}{\text{[Initial concentration of β2GPI added]}} \times 100\%$$

T Cell Proliferation Assay

Spleens were dissected from sacrificed mice and homogenized into single cell suspensions in PBS under sterile conditions. Splenic T cells were isolated using a mouse T cell isolation kit (StemCell Technologies, Vancouver, BC) according to the manufacturer's specifications. For each T cell experiment, one β2GPI/LPS-immunized mouse was compared with one control LPS-immunized mouse. Following isolation, T cells were resuspended in complete RPMI and plated at 1×10^5 cells/well (100 μL/well) in 96-well plates (Becton Dickinson, Franklin Lakes, NJ). Splenocytes used as APC were obtained from unimmunized female C57BL/6 mice (3 – 6 months) and were treated with

mitomycin C (Sigma Aldrich, St.-Louis, MO) at 50 μg/mL for 20 min at 37°C in the presence of 10% CO_2, The cells were washed twice and added to the wells at a concentration of 4 x 10^5 cells/well (100 μL/well). Antigens were then added to each well (20 μL/well) at the following concentrations (i.e., final concentration in the cell mixture): (1) PL vesicles or buffer that had been preincubated with native human β2GPI at 0.15 μM lipid and 15 μg/mL protein; (2) native human β2GPI or human serum albumin (HSA) at 30 μg/mL; and (3) concanavalin A (con A) at 1 μg/mL or 5 μg/mL. HSA and con A served as negative and positive controls, respectively. Cell proliferation was assessed using a colorimetric bromodeoxyuridine (BrdU) ELISA (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's specifications. Briefly, BrdU labeling solution was added to the T cell cultures after 96 h of incubation at 37°C (10% CO_2) and incubated for an additional 24 h at 37ºC (10% CO_2). The cells were centrifuged, the supernatants aspirated, and the plates dried for 60 min at 60ºC. The cells were then fixed and the DNA denatured using the Fix-Denat reagent for 60 min at 25ºC. The cells were incubated with anti-BrdU-POD antibody conjugate for 2 h at 37ºC, and washed three times with washing reagent. The TMB substrate was added and incubated for 30 min. The reaction was stopped by the addition of 1M H_2SO_4 and the plates were read at 450 nm using an ELISA reader (model EL800 Universal Microplate Reader, Bio-Tek Instruments, Winooski, VT).

Measurement of Cytokine Production

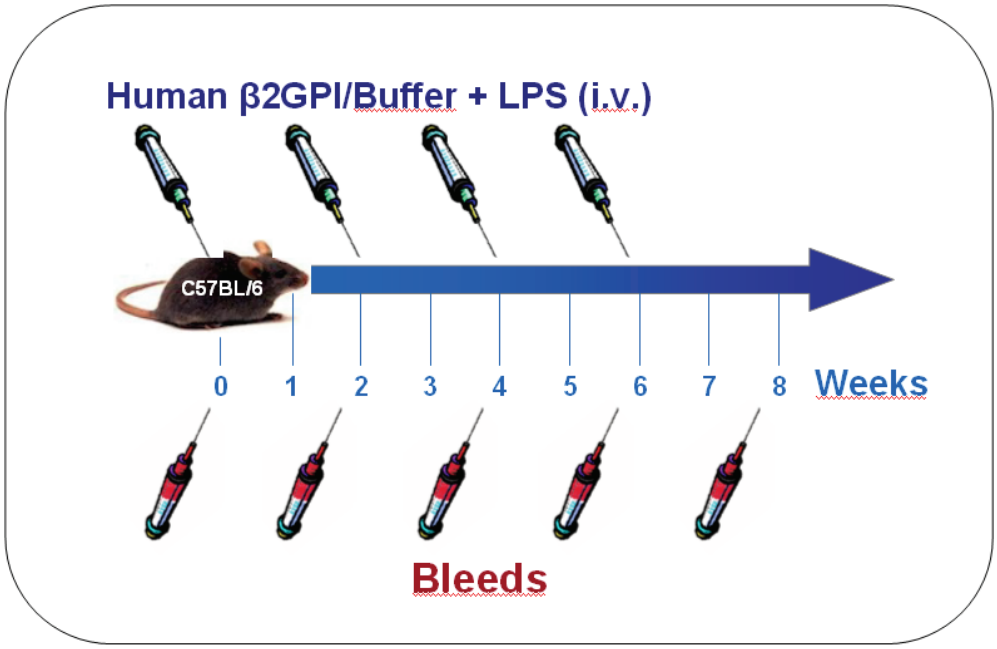
T cells (1 x 10^6 cells/well, 1 mL) isolated from immunized mice as described above, were co-cultured with antigen-presenting cells (4 x 10^6 cells/well, 1 mL) in the presence of antigen for 48 h at 37ºC (10% CO_2). Cytokines in the culture supernatants were measured using cytokine-specific ELISA kits (BD Biosciences, Mississauga, ON)

according to the manufacturer's specifications. Cytokine concentrations were determined from standard curves produced using known concentrations of recombinant cytokines from the cytokine kits.

Statistical Analyses

Statistical significance was determined by a two-tailed unpaired t test with Welch correction using InStat 3.0 (GraphPad Software, San Diego, CA).

a)



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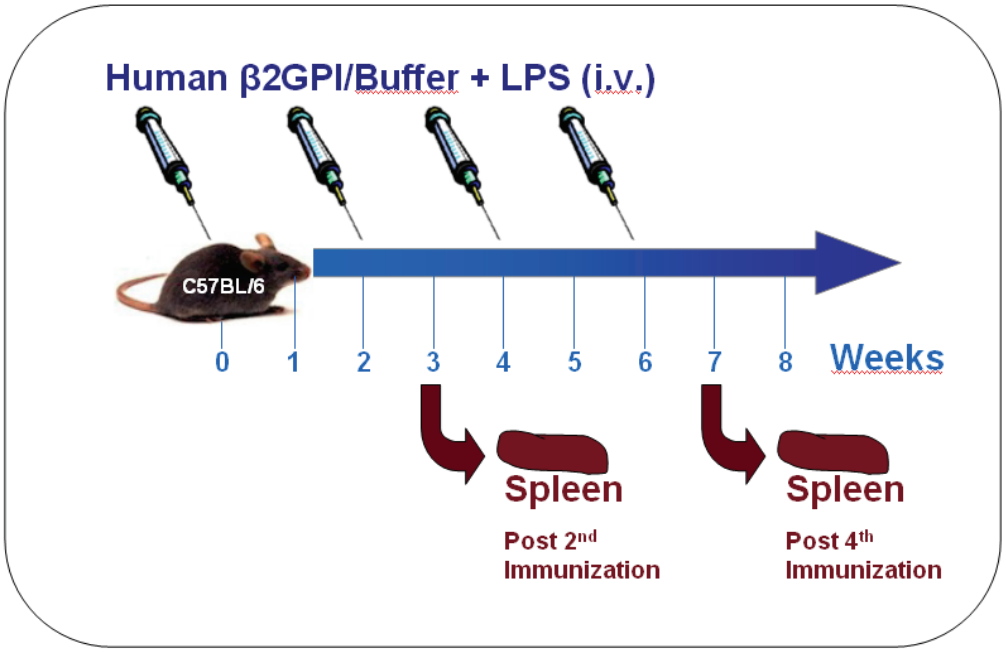


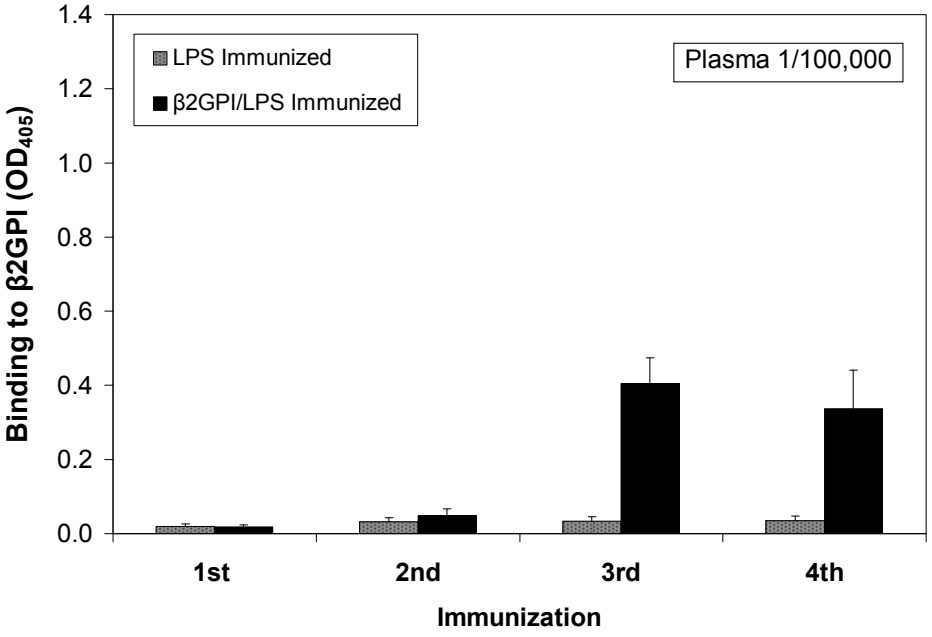
Figure 1. Schematic diagram representing the immunization and T cell isolation schedules. (a) Mice were immunized with human β2GPI + LPS or with HEPES + LPS every two weeks and bled 12 days post-immunization. (b) Mice were sacrificed for T cell studies seven days post-immunization, after two or four immunizations.

CHAPTER III. RESULTS

Immunization with β2GPI/LPS induces the production of aPL

Immunization of C57BL/6 mice with purified human β2GPI and LPS induced the production of high levels of aCL and anti-β2GPI IgG (Figure 1). Antibody reactivity is shown at high dilutions (1/100 000 and 1/5000 for anti-β2GPI and aCL, respectively) of the plasma to allow comparisons between the four post-immunization bleeds. Although these high dilutions result in apparent negativity or very low reactivity of early bleeds (first and second), these plasma samples had significant antibody titers measurable at lower dilutions (e.g., 1/100). In contrast, control mice immunized with LPS only produced low or insignificant levels of these aPL. The immunization schedule was performed as previously described by our laboratory (Levine *et al*.) with the exception of the timing of the LPS injection.[100] Due to the high mortality observed when β2GPI and LPS were injected on the same day (Levine *et al*.; Subang, personal communication), mice in this study were injected with LPS 24 h after immunization with β2GPI. This modified immunization protocol resulted in survival of all of the mice. Antibody titers increased with each subsequent immunization (Figure 1a). Although there appears to be a decrease in the antibody titers of both aCL and anti-β2GPI after the fourth immunization (Figure 1b), it is important to note that plasma samples for the second and fourth timepoints were taken from mice at seven days post-immunization (immediately prior to sacrifice for T cell experiments) and not at 12 days post-immunization (as for the first and third timepoints). Overall, levels of anti-β2GPI were approximately 20 times higher than those of aCL, as estimated from the plasma dilution required to produce similar antibody binding (OD_{405}) in the two assays.

a)



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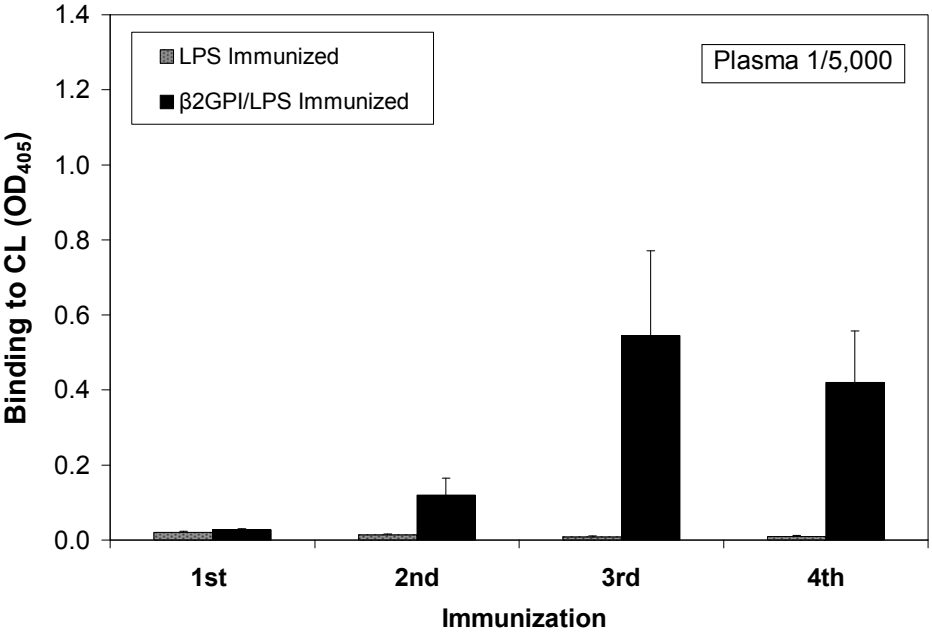


Figure 1. Induction of anti-β2GPI and aCL IgG responses in mice immunized with β2GPI/LPS. Plasma dilutions (as indicated) from mice immunized with LPS or β2GPI/LPS were evaluated by ELISA for IgG antibodies to (a) β2GPI or (b) CL. Mice immunized with β2GPI in the presence of LPS produced significantly elevated titers of anti-β2GPI ($p < 0.0001$) and aCL ($p < 0.02$) IgG, compared to mice immunized with LPS alone, after the third immunization. The data represent the mean OD_{405} ± SEM for each group of mice ($n = 8 – 15$ mice/group).

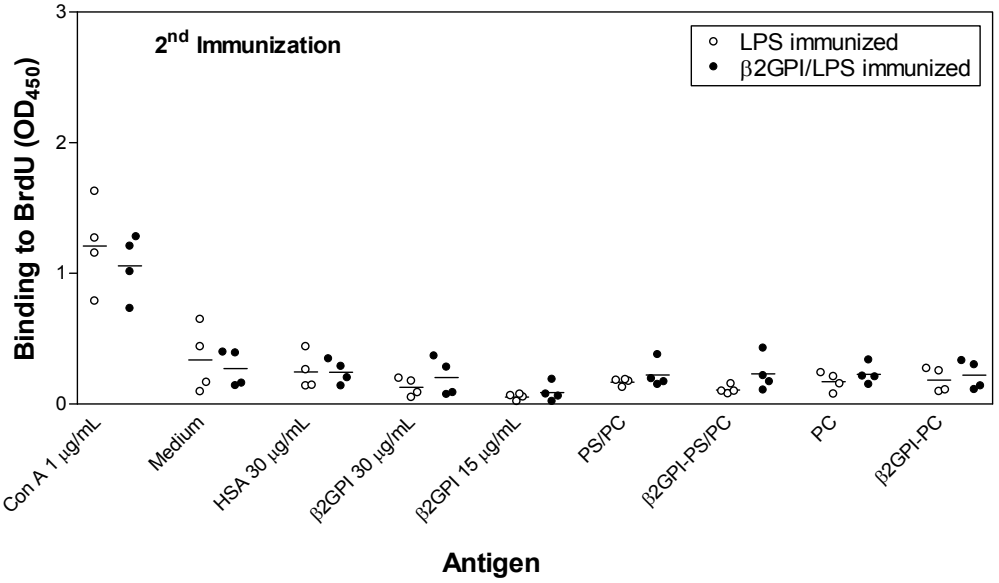
Immunization with β2GPI/LPS induces a T cell response to native β2GPI

In order to determine whether the mice immunized with β2GPI and LPS had a significant T cell response to β2GPI, purified splenic T cells were isolated from these mice and evaluated for proliferation and interleukin-2 (IL-2) production to native and PL-bound β2GPI. Mice immunized with LPS alone served as controls in each experiment. The T cell response was evaluated using two concentrations of β2GPI (15 and 30 μg/mL). Control antigens included culture medium and HSA (30 μg/mL) as negative controls, and con A (a polyclonal activator of T cells) as the positive control.

After two immunizations with β2GPI and LPS, no significant response to β2GPI was observed even though T cells responded well to con A (Figure 2a). This finding is consistent with the relatively low aCL and anti-β2GPI IgG titers (Figure 1) observed in these mice. After four immunizations, however, 7/9 mice immunized with β2GPI/LPS had high T cell proliferative responses to β2GPI (Figure 2b). This response increased with the concentration of β2GPI added and was higher at 30 μg/ml than at 15 μg/mL. This response was specific for β2GPI, as T cells from the same mice responded only minimally to similar concentrations of HSA. In contrast, no β2GPI-specific T cell response was observed in mice immunized with LPS alone, and T cells from these mice responded similarly to β2GPI, HSA, or medium. These T cells were functional, however, as they were as reactive to con A as T cells from mice immunized with β2GPI and LPS. Of note, T cells from mice immunized with β2GPI/LPS had a higher basal level of proliferation than T cells from LPS-immunized mice, likely due to the more generalized autoimmune response known to be induced in these mice by this immunization protocol (Levine *et al*.).[100] IL-2 functions in an autocrine manner as a growth factor for T cells

and is a more specific indicator of antigen-specific activation than the proliferation assay. IL-2 responses (Figure 3) were very similar to the proliferative responses, but exhibited lower background levels and less variability.

a)



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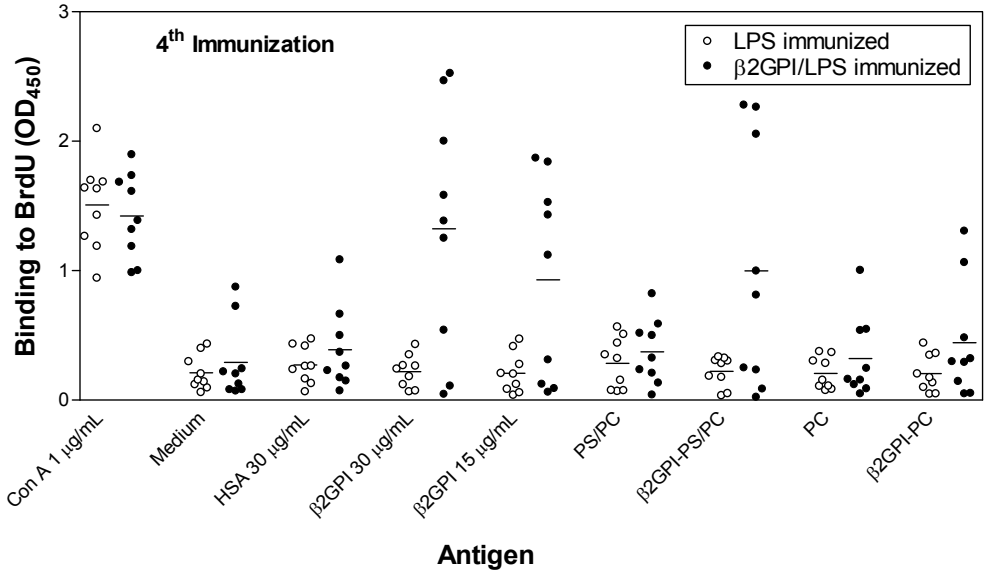
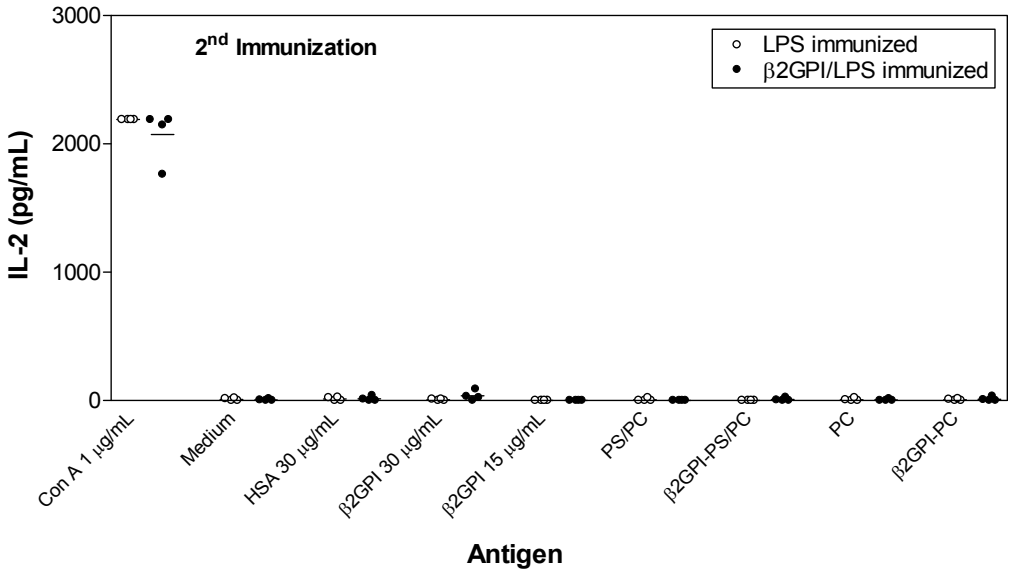


Figure 2. Induction of a T cell response to native and PL-bound β2GPI in mice immunized with β2GPI/LPS. Proliferation of splenic T cells from mice immunized with LPS or β2GPI/LPS was measured using a colorimetric bromodeoxyuridine (BrdU) cell proliferation ELISA. T cells were co-cultured with mitomycin C-treated splenocytes and antigen for 96 h. Proliferation of T cells to β2GPI, alone or bound to PS/PC, in mice immunized with β2GPI/LPS compared with mice immunized with LPS alone was significant after the 4th, but not the 2nd, immunization: $p < 0.003$ for β2GPI (30 μg/mL), $p < 0.02$ for β2GPI (15 μg/mL) and $p < 0.03$ for β2GPI-PS/PC. Each point represents the mean OD_{450} of triplicate samples for each mouse and the bar represents the mean OD_{450} for each group of mice (n = 4 – 9 mice/group).

a)



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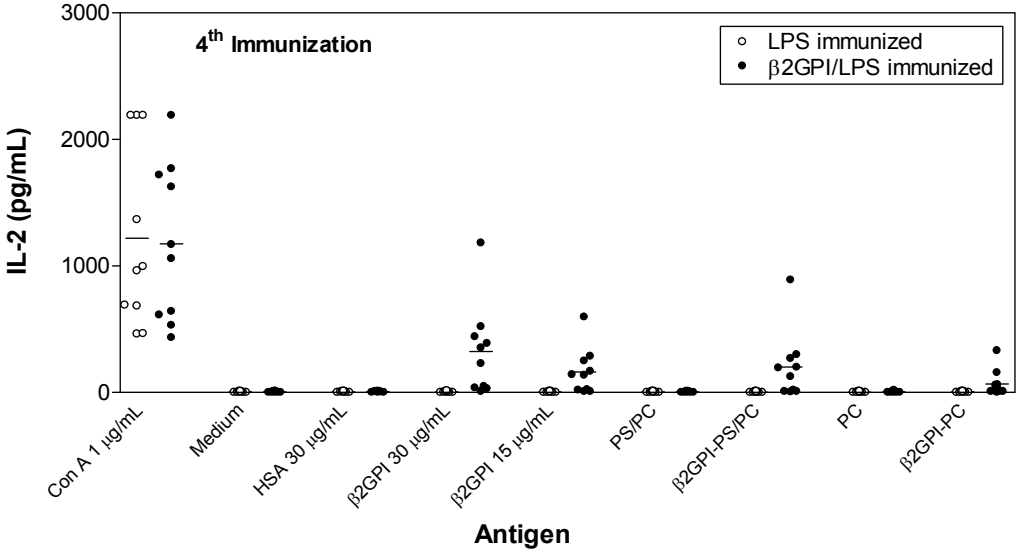


Figure 3. IL-2 production of T cells from mice immunized with β2GPI/LPS in response to native and PL-bound β2GPI. Supernatants were obtained from splenic T cells co-cultured with mitomycin C-treated splenocytes and antigen for 48 h. IL-2 production was quantitated by ELISA. IL-2 production in response to β2GPI, alone or bound to PS/PC in mice immunized with β2GPI/LPS, compared with mice immunized with LPS alone was significant after the 4th, but not the 2nd, immunization: $p < 0.01$ for β2GPI (30 μg/mL), $p < 0.01$ for β2GPI (15 μg/mL) and $p < 0.03$ for β2GPI-PS/PC. Each point represents the mean IL-2 concentration (pg/mL) of duplicate samples for each mouse and the bar represents the mean concentration for each group of mice (n = 4 – 10 mice/group).

Immunization with β2GPI/LPS induces a T cell response to PL-bound β2GPI

To determine whether binding of native β2GPI to anionic PL alters T cell recognition of this protein or induces the exposure of new T cell epitopes, PL vesicles consisting of either a mixture of PS and PC, PS alone, or PC alone were incubated with β2GPI prior to testing. To ensure that the response was to vesicle-bound, and not free, β2GPI, unbound β2GPI was removed from the PL suspension by centrifugation and washing of the PL vesicles. The amount of β2GPI bound to the PL vesicles was determined by quantitating the protein remaining in the supernatant following incubation with the PL vesicles (Table 1). β2GPI binding to the different PL vesicles varied depending on the content of anionic PL, as expected. The amount of β2GPI bound to PS/PC vesicles was greater than that bound to vesicles containing neutral PL (PC): 4.7 μg/mL β2GPI versus 2.7 μg/mL, respectively. The quantitative difference in the amount of β2GPI bound to the different vesicles is especially evident when evaluated as the percentage of β2GPI added to the vesicles, and was 63% for PS/PC and 35.6% for PC.

T cell responses to PS/PC and PC-bound β2GPI were evaluated by proliferation and IL-2 assays and were compared to responses to β2GPI in the same assay. Similar to the findings with native β2GPI alone, there was no measurable T cell response (proliferative or IL-2 production) to PL-bound β2GPI after two immunizations (Figures 2 and 3). However, after four immunizations, mice immunized with β2GPI/LPS had mean levels of proliferation (Figure 2) to PS/PC-bound β2GPI that were similar to those to β2GPI (15 μg/mL), despite the much lower concentration of β2GPI (4.7 μg/mL) in the PS/PC-bound antigen preparation. Although variable proliferative responses were observed with PC-bound β2GPI in these mice, the mean proliferation was similar to that

seen with medium or HSA. Responses to con A were strong and similar in both β2GPI/LPS and LPS-immunized mice.

The IL-2 responses (Figure 3) in the β2GPI/LPS-immunized mice were even clearer, with lower background levels and less variability between mice. After four immunizations, T cells from 6/9 mice immunized with β2GPI/LPS produced IL-2 in response to either PS/PC-bound or native β2GPI. These findings demonstrate that T cells from mice immunized with β2GPI/LPS recognize native β2GPI both alone and bound to PS/PC. PC-bound β2GPI did not activate these T cells, indicating that PS was required. T cell responses to both native and PL-bound β2GPI were restricted to mice immunized with β2GPI/LPS, as responses in LPS-immunized mice were similar to those observed with medium or HSA.

Table 1. Binding of β2GPI to phospholipid vesicles.

Phospholipid (PL) vesicle*	β2GPI bound to PL (μg/mL)†	% β2GPI bound to PL
PS/PC	4.7	63.0
PC	2.7	35.6

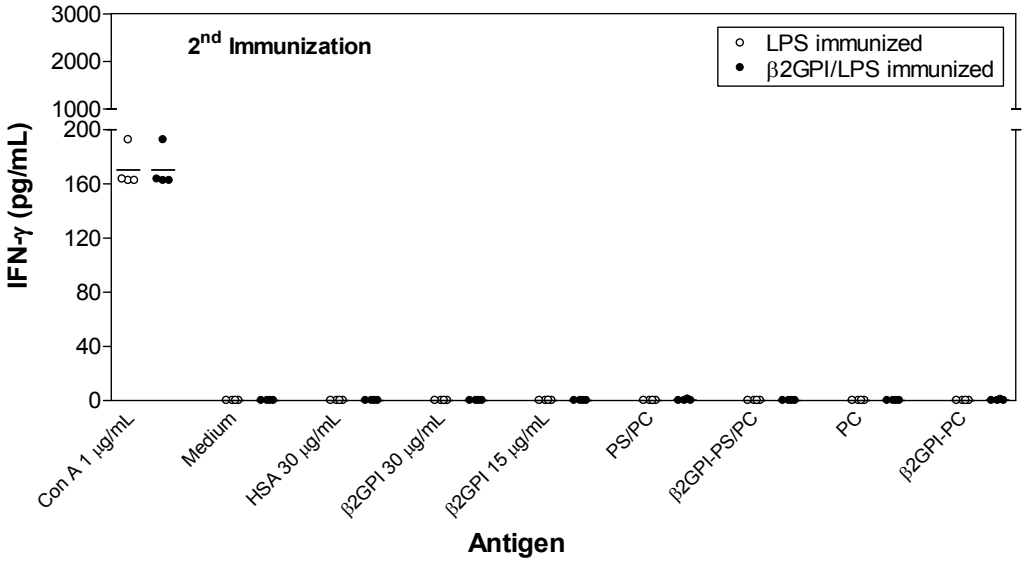
* PL vesicle denotes the vesicle that was incubated with β2GPI.

†The quantity of β2GPI that was bound to each PL vesicle (PS/PC, or PC) was determined by protein assay (see Materials and Methods). The percentage (%) of β2GPI bound is based on the initial concentration (15 μg/mL) of β2GPI added to the PL vesicles. These data represent the mean values for 10 individual experiments.

Immunization with β2GPI/LPS elicits a T_H1 response

The T_H orientation of the T cell response can be an important indicator of its potential contribution to disease pathogenesis. To investigate the T_H orientation in our model of aPL induction, we evaluated the production of T_H1 (IFN-γ) and T_H2 (IL-4, IL-10) cytokines in response to native and PL-bound β2GPI. Mice immunized with β2GPI/LPS, compared with LPS-immunized controls, produced mean levels of cytokines that were significantly elevated for IFN-γ ($p < 0.04$) (Figure 4), but low or insignificant for IL-4 and IL-10 (Figures 5 and 6). This profile is characteristic of a T_H1 type T cell response. The bias towards a T_H1 type response is clearly demonstrated by calculation of the ratio of IFN-γ to IL-4 produced by T cells from the β2GPI/LPS immunized mice (Figure 7). The ratio was maximal 35.3 for native β2GPI at 30 μg/mL, ~28.3 for native β2GPI at 15 μg/mL and ~15.4 for PS/PC-bound β2GPI. In contrast, the IFN-γ to IL-4 ratio for the T cell response to the negative control antigen (PC-bound β2GPI) was 4.2.

a)



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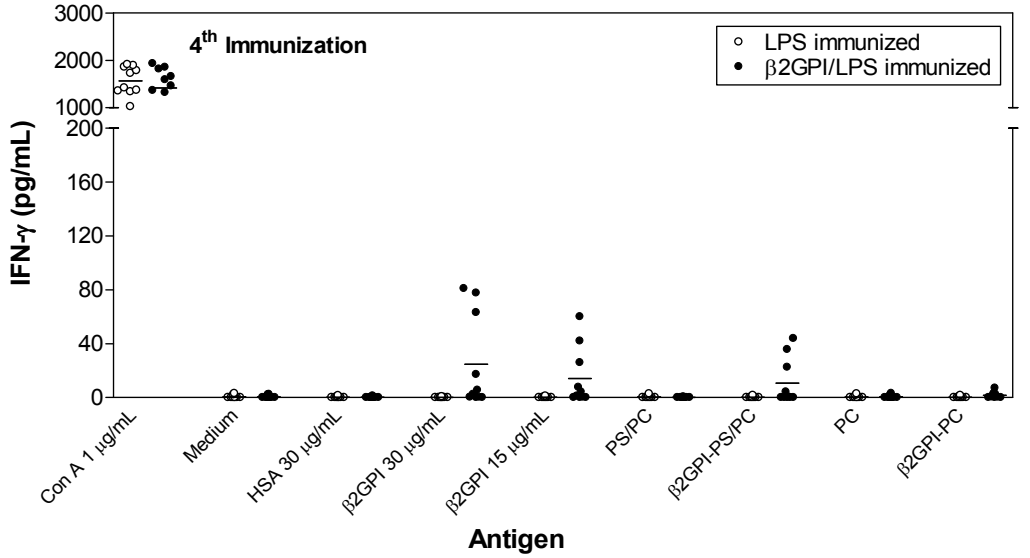
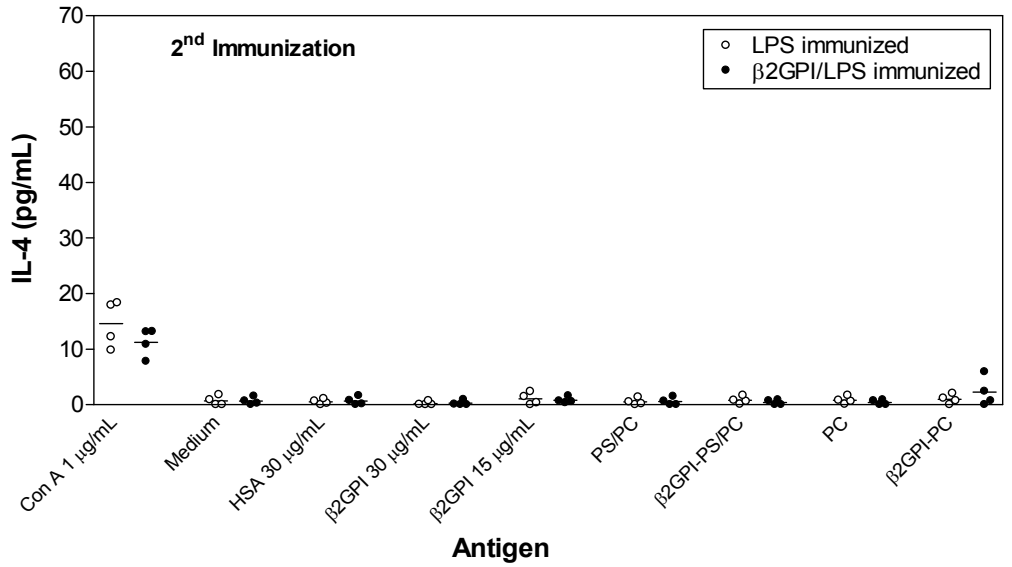


Figure 4. IFN-γ production of T cells from mice immunized with β2GPI/LPS in response to native and PL-bound β2GPI. Supernatants were obtained from splenic T cells co-cultured with mitomycin C-treated splenocytes and antigen for 48 h. IFN-γ production was quantitated by ELISA. IFN-γ production in response to β2GPI, alone or bound to PS/PC, was significant in mice immunized with β2GPI/LPS compared with mice immunized with LPS alone after the 4th, but not the 2nd, immunization: $p < 0.04$ for β2GPI (30 μg/mL), $p < 0.03$ for β2GPI (15 μg/mL) and $p < 0.03$ for β2GPI-PS/PC. Each point represents the mean IFN-γ concentration (pg/mL) of duplicate samples for each mouse and the bar represents the mean concentration for each group of mice (n = 4 – 10 mice/group).

a)



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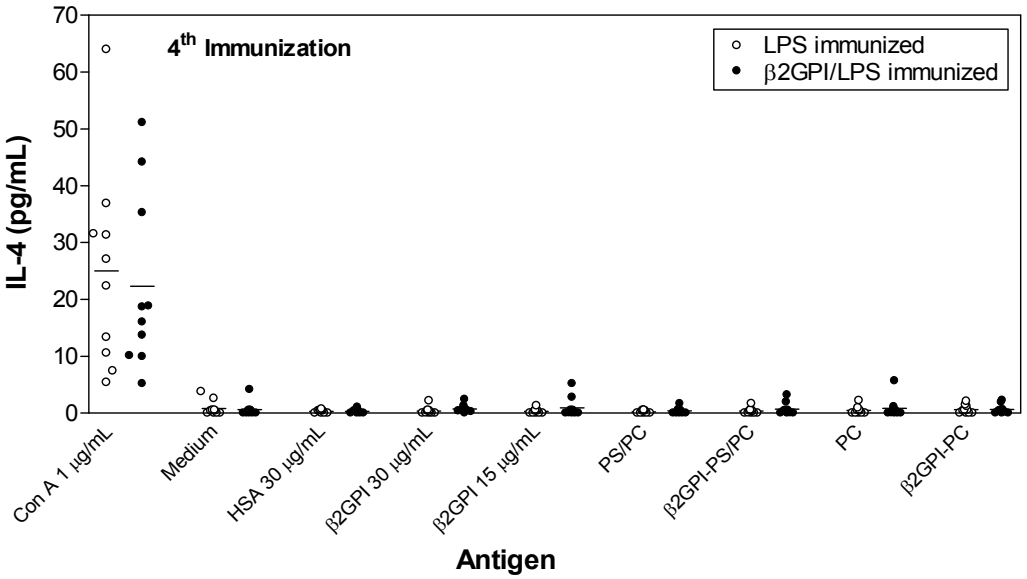
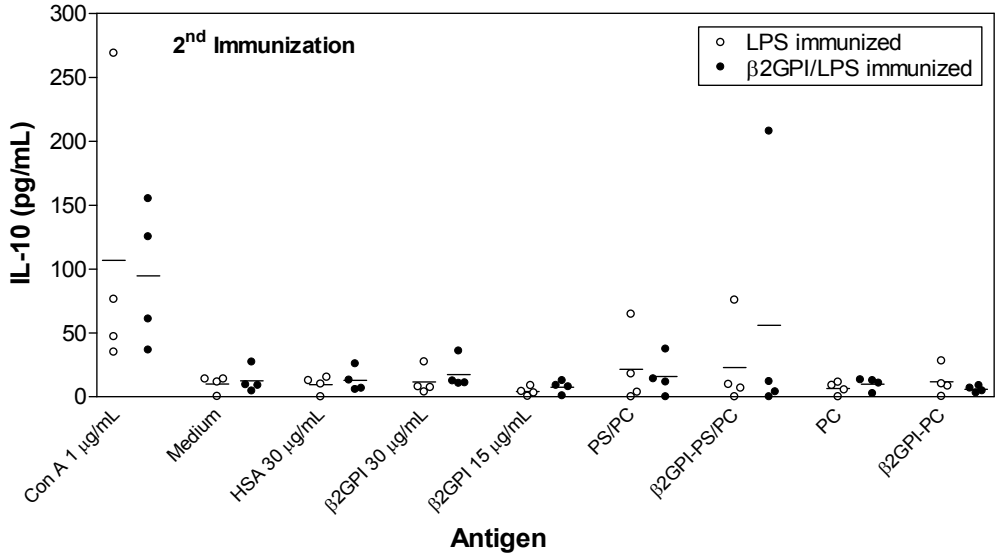


Figure 5. IL-4 production of T cells from mice immunized with β2GPI/LPS in response to native and PL-bound β2GPI. Supernatants were obtained from splenic T cells co-cultured with mitomycin C-treated splenocytes and antigen for 48 h. IL-4 production was quantitated by ELISA. Little or no significant IL-4 production was detected in mice immunized with β2GPI/LPS, compared with mice immunized with LPS alone, after 4 immunizations: $p \geq 0.05$ for all antigens. Each point represents the mean IL-4 concentration (pg/mL) of duplicate samples for each mouse and the bar represents the mean concentration for each group of mice (n = 4 – 10 mice/group).

a)



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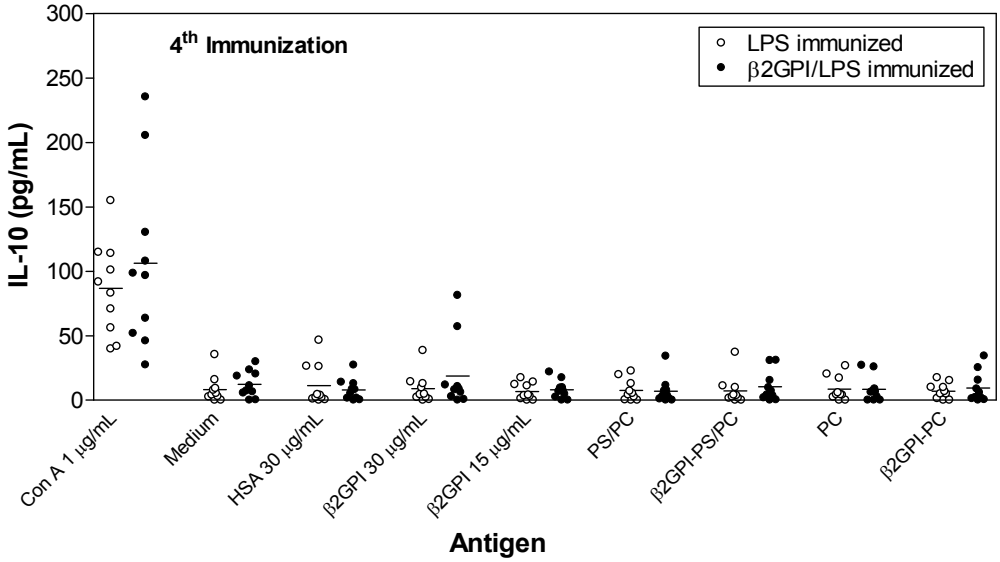


Figure 6. IL-10 production of T cells from mice immunized with β2GPI/LPS in response to native and PL-bound β2GPI. Supernatants were obtained from splenic T cells co-cultured with mitomycin C-treated splenocytes and antigen for 48 h. IL-10 production was quantitated by ELISA. Little or no significant IL-10 production was detected in mice immunized with β2GPI/LPS, compared with mice immunized with LPS alone, after 4 immunizations: $p \geq 0.05$ for all antigens by. Each point represents the mean IL-10 concentration (pg/mL) of duplicate samples for each mouse and the bar represents the mean concentration for each group of mice (n = 4 – 10 mice/group).

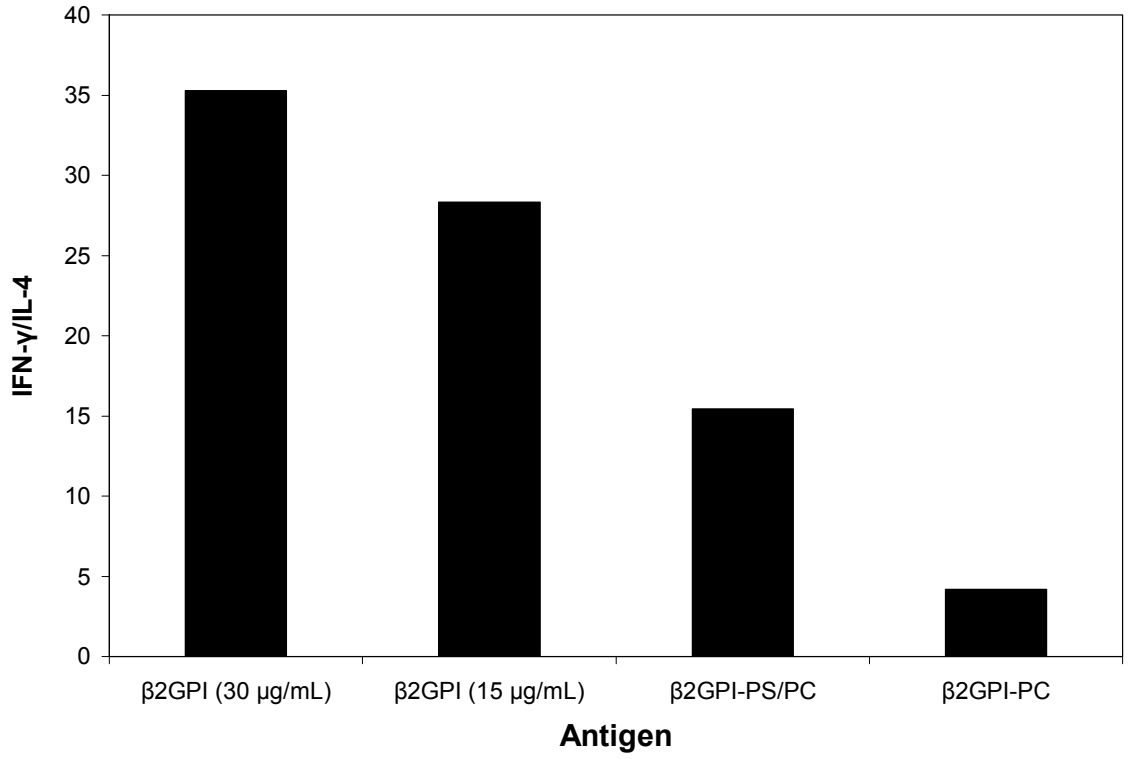


Figure 7. Ratio of IFN-γ to IL-4 production in T cells from mice immunized with β2GPI/LPS in response to native and PL-bound β2GPI. Each bar represents the ratio of the mean levels (pg/mL) of IFN-γ and IL-4 produced by splenic T cells from mice (n = 8 – 10) immunized with β2GPI/LPS. Mice with IL-4 levels <0.01 were excluded from analysis. Cytokine levels were detected by ELISA, as described for Figures 4 and 5 above.

CHAPTER IV. DISCUSSION AND CONCLUSION

The aim of this study was to characterize the β2GPI-reactive T cell response in a murine model of aPL production. We demonstrate here that T cells from mice immunized with β2GPI/LPS proliferate strongly, produce IL-2 and IFN-γ, and exhibit a T_H1 profile *in vitro* in response to native β2GPI. T cells from these mice also responded to PL-bound β2GPI, suggesting that a repertoire of T cells specific for cryptic epitopes may exist in these mice. We performed a kinetic study to evaluate the time of appearance of β2GPI-specific T cells in our model. T cell reactivity was detectable after four immunizations, but not after two. In contrast, a detectable antibody response was apparent after the first immunization (see Appendix, Figure 1). Thus, it appears that persistent immune activation is required to elicit a detectable (i.e., *in vitro*) β2GPI-specific T cell response in β2GPI/LPS-immunized mice.

The role of LPS as a potent stimulator of the immune system is evident in this study. LPS is a component of bacterial cell walls that stimulates the innate immune system, up-regulates expression of costimulatory molecules on APC, and results in the release of proinflammatory cytokines.[153-155] LPS has also been shown to disrupt T cell tolerance and promote memory T cell survival.[156,157] β2GPI, immunized in the presence of LPS, has been previously shown in our laboratory to induce aPL and other SLE-associated autoantibodies, as well as SLE-like disease, in nonautoimmune mice (Levine *et al*.).[100] In this model, LPS not only stimulated IgM anti-β2GPI and aCL responses (via polyclonal activation), but also IgG anti-β2GPI and aCL (presumably via upregulation of costimulatory molecules and induction of a β2GPI-specific T cell response). The present study makes use of this established murine model of aPL induction, and investigates the presence and nature of the β2GPI-reactive T cell response in this model. The immunization protocol in the present study differed somewhat from that of the published

model so that we could ensure survival of all of our mice. Co-immunization of β2GPI and LPS on the same day often leads to mortality of the mice for reasons that are as yet unknown. In our protocol, mice were injected with LPS 24 h after receiving β2GPI, rather than on the same day as in the original model.[100] Although this modified protocol resulted in high aPL production, the aPL IgG titers were lower and generally more variable (see Results, Figure 1) than those observed previously (Levine *et al.*)[100] and in a pilot study done prior to the major study (see Appendix, Figure 1). We have evidence in our laboratory that β2GPI interacts specifically with LPS and that this interaction may be important in eliciting a strong antibody response to β2GPI. This may explain why the aPL response in the current study was somewhat lower than that observed when mice received β2GPI and LPS within several hours of each other. Although co-immunization of LPS with β2GPI appears to elicit stronger antibody responses, the aPL responses observed here were extremely high (> 100,000 titer for anti-β2GPI). Furthermore, all of the immunized mice survived four immunizations with β2GPI and LPS using this modified protocol.

Immunization of mice with native β2GPI and LPS elicited a strong aPL response, increasing with each subsequent immunization. In contrast, LPS-immunized mice did not produce a significant aPL response. Although mean antibody titers in β2GPI-immunized mice appeared to decrease slightly after the fourth immunization in comparison to post-third immunization (see Results, Figure 1), it should be taken into account that these mice were bled seven days post-immunization, compared to twelve days post-immunization for the first and third timepoints. Antibody levels might be expected to differ on Days 7 and 12 post-immunization, thus explaining the apparent discrepancy between the antibody titers at these timepoints. Furthermore, the antibody titers of individual mice varied

considerably, making comparison between groups more difficult. However, aPL titers of individual mice did increase with each subsequent immunization (data not shown).

β2GPI-specific $CD4^+$ T cells have been identified in APS patients. The presence of these T cells, together with high levels of circulating IgG anti-β2GPI autoantibodies indicative of a T cell-dependent response, highly implicate T cells in the initiation and/or exacerbation of disease.[113,117] The precise role, however, of these T cells in the pathogenesis of APS has yet to be determined. To determine the existence and specificity of a T cell response to β2GPI in our model of aPL induction, we isolated splenic T cells from mice immunized with β2GPI/LPS to determine their reactivity to native and PL-bound β2GPI. LPS-immunized mice served as control mice and HSA served as a control antigen. After two immunizations, no detectable T cell response to β2GPI was observed in either β2GPI/LPS or LPS-immunized mice, even though both groups responded well and equivalently to stimulation by con A. After four immunizations, however, the majority of mice immunized with β2GPI/LPS responded strongly to native and PL-bound β2GPI, as assessed by proliferation, and IL-2 and IFN-γ production, while mice immunized with LPS showed no significant response. β2GPI/LPS-immunized mice with low or no response to β2GPI reacted similarly whether T cell activity was determined by proliferation or cytokine secretion. Intracellular cytokine staining was not performed and could provide a more sensitive means of detecting a β2GPI-specific T cell response. These results demonstrate that persistent stimulation (i.e., greater than two immunizations) of the immune system is required to elicit a detectable T cell response to β2GPI, *in vitro*.

The production of aPL was an expected outcome of β2GPI-specific T cell activity. Interestingly, significant titers (> 1/100) of circulating anti-β2GPI IgG antibodies were observed after the first immunization (data not shown), while measurable *in vitro* T cell

reactivity was observed only after the fourth. However, the levels of both anti-β2GPI and aCL became significantly higher (>100,000 and >1/5,000, respectively) following the second immunization. As anti-β2GPI IgG production requires β2GPI-specific T cell help,[117] activated β2GPI-reactive T cells must have been present at the time of the first appearance of anti-β2GPI IgG. These data suggest that the *in vitro* methods used to detect antibody production and T cell activity differ greatly in sensitivity. It must be recognized that the number of T cells activated early in the response is likely small and would be difficult to detect *in vitro*. In contrast, later in the response, significant proliferation of the relevant clone(s) would have occurred *in vivo* and the β2GPI-reactive T cells would have been numerous. It is, therefore, likely that β2GPI-specific T cells were insufficiently expanded to be detected *in vitro* after two immunizations, but sufficiently expanded after four immunizations. It is also probable that a stronger β2GPI-specific T cell response would have been observed if mice were immunized with β2GPI and LPS on the same day (as opposed to 24 h apart), given that higher aPL titers were observed under these conditions. Finally, it should be noted that splenic T cells were evaluated in these studies. As β2GPI is a plasma protein, activated β2GPI-specific T cells may also exist in the peripheral blood and would be of interest.

Of the existing literature on cellular immunity in APS patients, only one group has clearly demonstrated that patients possess T cells reactive to native β2GPI.[112] All other groups have demonstrated T cell reactivity to alternate forms of the protein, such as reduced,[112,117] recombinant (full-length),[117] PL-bound[149] and oxidized[158] β2GPI, but shown no response to native β2GPI. Here, we demonstrate T cell reactivity to the native form of β2GPI. However, it must be noted, that mice were immunized with heterologous (human) β2GPI and that T cell reactivity was determined in response to native human

β2GPI. We were unable to evaluate the T cell response to native murine β2GPI, as sufficient quantities of the purified protein could not be obtained for these experiments. Although human β2GPI is highly homologous to murine β2GPI, it differs sufficiently to elicit an immune response (76% identities, 87% positives [CAA7219042; CAA41113150; as performed by NCBI BLASTP V2.2.17151]). Future studies in our laboratory will investigate whether β2GPI/LPS-immunized mice possess T cells that recognize murine β2GPI and, if so, whether they recognize native or PL-bound β2GPI.

We found that β2GPI-specific T cells produced IFN-γ in response to native and PL-bound β2GPI, but little or no IL-4 and no IL-10. These results are consistent with a T_H1 profile. To date, little is known about whether APS is a T_H1- or a T_H2-mediated disease. Krause *et al*. showed that treatment of experimental APS with anti-idiotypic mAb to a human aCL led to an increase in IL-2 and IFN-γ producing cells and a decrease in IL-4 and IL-6 producing cells.[159] The increase of T_H1-producing cells and decrease of T_H2-producing cells after treatment suggests that the disease is T_H2-mediated. On the other hand, given the chronic inflammation in APS patients, one might expect β2GPI-reactive T cells to be of the T_H1 subset. Studies of T cells from APS patients have shown production of IFN-γ by β2GPI-reactive T cells.[112,117,149] Furthermore, a study by Karakantza *et al*. demonstrates that APS patients, but not healthy individuals, possess circulating T cells that are T_H1-polarized.[160] In our model, T_H1-polarization of β2GPI-reactive T cells is clearly evident from the individual cytokine results, as well as the ratio of IFN-γ to IL-4 (see Results, Figure 7). To ensure that this T_H1-polarization is not due to the T_H1-bias of the strain (C57BL/6) of mice used in these experiments, it will be necessary to evaluate this response in BALB/c mice, which exhibit a T_H2-biased phenotype.[161] It should be noted here that our murine model of aPL and SLE induction

was initially studied in both C57BL/6 and BALB/c mice, and both developed high levels of aPL and similar levels of SLE-related autoantibodies. However, aCL and SLE-related autoantibodies emerged earlier in C57BL/6 mice.[100] Prolonged and general inflammation is particularly damaging to tissues and once the cycle is initiated in APS, it is very difficult to break. Thus, isolating the triggers of inflammation and understanding how to maintain a balance between T_H1 and T_H2 is imperative to controlling disease. Further characterization of T cells in mice producing aPL may shed light on the nature of T cells required to produce these autoantibodies and the clinical manifestations associated with aPL.

To investigate whether binding of β2GPI to PL alters T cell recognition of this protein, T cell reactivity to β2GPI bound to PL vesicles was evaluated. β2GPI binds strongly to anionic PL such as PS and CL, but not neutral PL, such as PC. Moreover, binding of anionic PL to β2GPI can induce a conformational change in the protein itself, resulting in exposure of neoepitopes.[107,128-130] This conformational change may reveal sites that would now be accessible for antigen processing or antibody binding. Potential physiological sources of anionic PL include apoptotic cells,[103,126,127] endothelial cells,[55] oxLDL,[60] and activated platelets.[56,57] Specifically in APS, the exposure of anionic PL on the surface of apoptotic cells provides a physiological context for the binding of PL-binding proteins such as β2GPI, and apoptotic cells have been shown to act as both antigen and immunogen for aPL. Therefore, interaction of β2GPI with aPL or with anionic PL (e.g., apoptotic cells)[106] can result in the presentation of cryptic T cell epitopes. Modulation of antigen processing and presentation in this manner can lead to the activation of T cells specific for epitopes not observed in the nonautoimmune individual. These T cells can then provide help to other anti-β2GPI producing B cells, resulting in a

spread in the immune response within β2GPI (i.e., intramolecular epitope spread). In this manner, it is understandable how T cell reactivity to one epitope can easily shift to other epitopes during the course of an autoimmune disease such as APS. The evidence that β2GPI-specific T cells in APS patients recognize cryptic epitopes, suggests that events leading to exposure of these epitopes is an important factor in determining tolerance versus autoimmunity.

The PS/PC composition of the vesicles used in this study was similar to that of the plasma membrane of activated platelets (and, likely, apoptotic cells). In this study, T cells from β2GPI/LPS-immunized mice proliferated strongly and produced IL-2 and IFN-γ in response to PS/PC-bound β2GPI. In contrast, the same T cells exhibited a very low response to PC-bound β2GPI. Of note, a very high proportion (63%) of the β2GPI added bound to PS/PC vesicles, while only 35.6% bound to PC vesicles. It is also noteworthy that the T cell response to PS/PC-bound β2GPI (~4.7 μg/mL β2GPI) was greater than that to native β2GPI (15 μg/mL). In future, the use of homogeneously sized vesicles will add a level of control to this experiment, as it would ensure similar sizes of PS/PC and PC vesicles. Some β2GPI (2.7 μg/mL) was shown to bind to PC vesicles, but it is not clear whether this binding was specific. Unsaturated forms of PC (e.g., DOPC) have been shown to bind β2GPI in certain ELISAs.[162] Future studies will evaluate the antigenicity of β2GPI in the presence of saturated, as well as unsaturated, PC, and include a complete titration of soluble and PL-bound β2GPI. Moreover, given the recognition of PS/PC-bound β2GPI by β2GPI-reactive T cells, it would be interesting to evaluate whether these cells recognize β2GPI bound to potential physiological antigens, such as apoptotic cells.

It is unclear whether the T cells reactive with peptides presented from PS/PC-bound β2GPI recognized epitopes that differ from those presented by native β2GPI. The

lack of reactivity of β2GPI-reactive T cells with peptides derived from PC-bound β2GPI suggests that these T cells recognize epitopes presented by β2GPI bound to anionic, but not neutral, PL. However, this must be confirmed by further experiments in which equivalent amounts of β2GPI are presented on PC and PS/PC vesicles, as less β2GPI was bound to PC than to PS/PC vesicles (2.7 versus 4.7 μg/mL, respectively). Furthermore, the generation of T cells lines and clones would definitively address this question. Both APS patients and healthy individuals alike have been shown to possess T cells specific for the p276-290 peptide, which contains the PL-binding site of β2GPI.[117] These T cells are proposed to be specific for cryptic epitopes generated by the processing of PL-bound, but not soluble, β2GPI. It is further proposed that T cells reactive with native β2GPI have not been deleted from the T cell repertoire, but only become activated when exposed to PL-bound β2GPI. To examine whether similar T cells exist in the murine T cell repertoire, future studies in our laboratory will evaluate whether T cells from β2GPI/LPS-immunized mice are capable of recognizing murine β2GPI, either alone or bound to PS/PC vesicles. These experiments are now possible, as I have recently been able to establish a procedure for purifying native murine β2GPI (see Appendix).

The precise mechanism responsible for the induction of APS remains unknown. Binding of β2GPI to anionic PL has been shown to shield the PL-binding site from proteolytic attack, resulting in the generation of cryptic T cell epitopes that are not generated from processing of native β2GPI.[149] Presentation of these cryptic epitopes, in the context of MHC, would activate β2GPI-reactive T cells specific for these epitopes. This would, in turn, provide help to B cells specific for the relevant epitopes to produce anti-β2GPI IgG. Circulating anti-β2GPI can bind to β2GPI that is soluble or particle-bound, allowing for FcγRI-mediated macrophage uptake of lipid or cell-bound β2GPI.

This has been demonstrated for PS-bound β2GPI and has been shown to enhance presentation of the β2GPI to T cells.[163] Therefore, the generation of anti-β2GPI can provide a means for further uptake and presentation of β2GPI by macrophages, and possibly DCs, to β2GPI-specific T cells (Figure 1). These T cells would then be activated to produce proinflammatory cytokines and provide help to other anti-β2GPI-producing B cells. This cycle of T cell activation, antibody production and inflammation, once initiated would be very difficult to break. In this regard, it would be interesting to evaluate the influence of aPL of different specificities on the β2GPI-specific T cell response in our model. It should be noted, however, that binding of antibody to its cognate antigen can modulate antigen processing to enhance presentation of some T cell determinants and suppress others.[138] This would, thus, render evaluation of the effects of aPL on T cell activation very complex in an *in vivo* model.

The experiments described here demonstrate the presence of a β2GPI-reactive T cell response in β2GPI/LPS-immunized mice. In addition, they have established the methodology required to evaluate T cell reactivity to native and PL-bound β2GPI, and to purify native murine β2GPI (see Appendix). We show here, for the first time, the characterization of β2GPI-reactive T cells in a murine model of aPL induction. These data support the hypothesis that persistent activation of the immune system with β2GPI, in a proinflammatory context, activates β2GPI-specific T cells and that this persistent activation is necessary to observe a measurable T cell response *in vitro*. In contrast, induction of a measurable aPL response required less immune stimulation and was observed earlier than the β2GPI-reactive T cell response. In our model, LPS likely mimics the inflammatory context of APS, while human β2GPI may provide a heterologous antigen that enables a break in tolerance to endogenous β2GPI. Together,

they produce a physiologically relevant model of β2GPI-specific T cell activation and aPL induction. Future studies investigating the autoimmune nature of the β2GPI-specific response in this model will further our understanding of autoreactive T cells in APS, and will enable the development of new therapeutic strategies in this and other autoimmune diseases.

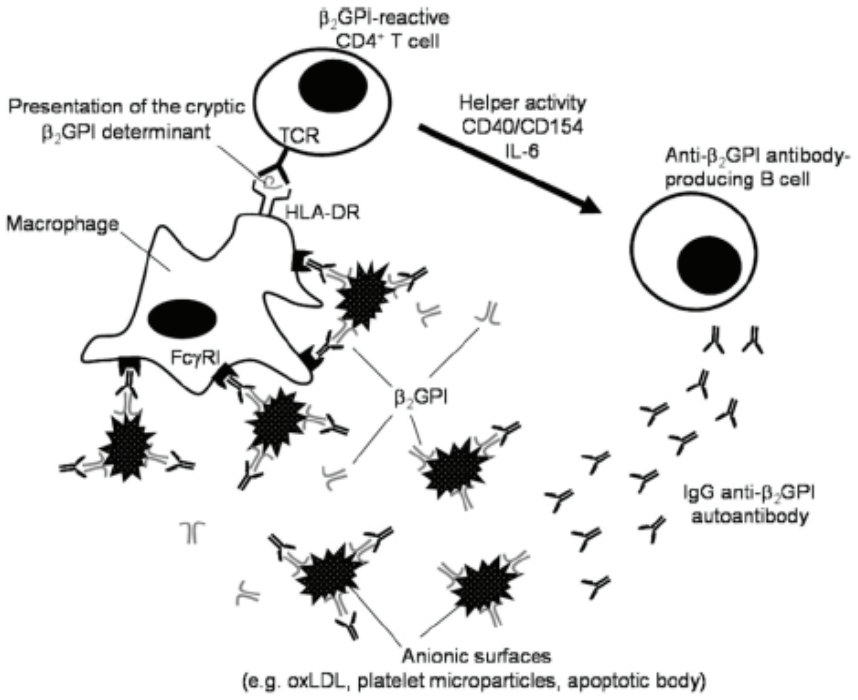


Figure 1. Schematic representation of the cycle of autoimmunity. Macrophages can present cryptic determinants of β2GPI to β2GPI-reactive T cells in the context of HLA class II molecules. These β2GPI-specific T cells can provide help to B cells to produce anti-β2GPI IgG. Anti-β2GPI autoantibodies can bind to β2GPI complexed with anionic PL, allowing PL-bound β2GPI to be internalized by macrophages via FCγRI-mediated endocytosis. This results in the continued cycle of T cell activation, inflammation caused by promotion of a T_H1-type helper T cell response, and autoantibody production.

CHAPTER V. BIBLIOGRAPHY

BIBLIOGRPAHY

1. Miyakis, S., M. D. Lockshin, T. Atsumi, D. W. Branch, R. L. Brey, R. Cervera, R. H. Derksen, P. G. DE Groot, T. Koike, P. L. Meroni, G. Reber, Y. Shoenfeld, A. Tincani, P. G. Vlachoyiannopoulos, and S. A. Krilis. 2006. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J. Thromb. Haemost.* 4:295.

2. Petri, M. 2000. Epidemiology of the antiphospholipid antibody syndrome. *J Autoimmun* 15:145.

3. Shoenfeld, Y., M. Blank, R. Cervera, J. Font, E. Raschi, and P. L. Meroni. 2006. Infectious origin of the antiphospholipid syndrome. *Ann. Rheum. Dis.* 65:2.

4. Domenico, S. G., G. Minisola, and M. Galeazzi. 2003. HLA class II alleles and genetic predisposition to the antiphospholipid syndrome. *Autoimmun Rev.* 2:387.

5. Wasserman, A., A. Neisser, and C. Bruck. 1906. Eine serodiagnostiche Reaktion bei Syphilis. *Deutsche Med Wochenschr*745.

6. Pangborn, M. 1941. A new serologically active phospholipid from beef heart. *Proc Soc Exp Biol Med* 48:484.

7. Schlame, M., D. Rua, and M. L. Greenberg. 2000. The biosynthesis and functional role of cardiolipin. *Prog. Lipid Res.* 39:257.

8. Castro, A. R., W. E. Morrill, W. A. Shaw, D. C. Gale, M. M. Park, L. A. Peregrino-Ferreira, M. L. Bazzo, and V. Pope. 2000. Use of synthetic cardiolipin and lecithin in the antigen used by the venereal disease research laboratory test for serodiagnosis of syphilis. *Clin Diagn Lab Immunol* 7:658.

9. Belisle, J. T., M. E. Brandt, J. D. Radolf, and M. V. Norgard. 1994. Fatty acids of Treponema pallidum and Borrelia burgdorferi lipoproteins. *J Bacteriol.* 176:2151.

10. HASERICK, J. R., and R. LONG. 1952. [Systemic lupus erythematosus preceded by false-positive serologic tests for syphilis: presentation of five cases.]. *Ann. Intern. Med* 37:559.

11. Harris, E. N., A. E. Gharavi, M. L. Boey, B. M. Patel, C. G. kworth-Young, S. Loizou, and G. R. Hughes. 1983. Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. *Lancet* 2:1211.

12. Tan, E. M., A. S. Cohen, J. F. Fries, A. T. Masi, D. J. McShane, N. F. Rothfield, J. G. Schaller, N. Talal, and R. J. Winchester. 1982. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* 25:1271.

13. Hughes, G. R. 1985. The anticardiolipin syndrome. *Clin Exp Rheumatol* 3:285.

14. Mackworth-Young, C. G., S. Loizou, and M. J. Walport. 1989. Primary antiphospholipid syndrome: features of patients with raised anticardiolipin antibodies and no other disorder. *Ann. Rheum. Dis.* 48:362.

15. Asherson, R. A., M. A. Khamashta, J. Ordi-Ros, R. H. Derksen, S. J. Machin, J. Barquinero, H. H. Outt, E. N. Harris, M. Vilardell-Torres, and G. R. Hughes. 1989. The "primary" antiphospholipid syndrome: major clinical and serological features. *Medicine (Baltimore)* 68:366.

16. Alarcon-Segovia, D., and J. Sanchez-Guerrero. 1989. Primary antiphospholipid syndrome. *J Rheumatol* 16:482.

17. Facer, C. A., and G. Agiostratidou. 1994. High levels of anti-phospholipid antibodies in uncomplicated and severe Plasmodium falciparum and in P. vivax malaria. *Clin. Exp Immunol.* 95:304.

18. Santiago, M. B., W. Cossermelli, M. F. Tuma, M. N. Pinto, and R. M. Oliveira. 1989. Anticardiolipin antibodies in patients with infectious diseases. *Clin. Rheumatol* 8:23.

19. de Larranaga, G. F., R. R. Forastiero, M. E. Martinuzzo, L. O. Carreras, G. Tsariktsian, M. M. Sturno, and B. S. Alonso. 2000. High prevalence of antiphospholipid antibodies in leprosy: evaluation of antigen reactivity. *Lupus* 9:594.

20. Ordi-Ros J, Villarreal J, Monegal F, Sauleda S, Esteban I, and Vilardell M. 2000. Anticardiolipin antibodies in patients with chronic hepatitis C virus infection: characterization in relation to antiphospholipid syndrome., pp. 241.

21. Maclean, C., P. J. Flegg, and D. C. Kilpatrick. 1990. Anti-cardiolipin antibodies and HIV infection. *Clin Exp Immunol* 81:263.

22. Koike, T., M. Sueishi, H. Funaki, H. Tomioka, and S. Yoshida. 1984. Anti-phospholipid antibodies and biological false positive serological test for syphilis in patients with systemic lupus erythematosus. *Clin. Exp Immunol.* 56:193.

23. McNeil, H. P., R. J. Simpson, C. N. Chesterman, and S. A. Krilis. 1990. 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* 87:4120.

24. Galli, M., P. Comfurius, C. Maassen, H. C. Hemker, M. H. de Baets, P. J. van Breda-Vriesman, T. Barbui, R. F. Zwaal, and E. M. Bevers. 1990. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. *Lancet* 335:1544.

25. Matsuura, E., Y. Igarashi, M. Fujimoto, K. Ichikawa, and T. Koike. 1990. Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. *Lancet* 336:177.

26. Hunt, J. E., H. P. McNeil, G. J. Morgan, R. M. Crameri, and S. A. Krilis. 1992. A phospholipid-beta 2-glycoprotein I complex is an antigen for anticardiolipin antibodies occurring in autoimmune disease but not with infection. *Lupus* 1:75.

27. Hojnik, M., B. Gilburd, L. Ziporen, M. Blank, Y. Tomer, M. A. Scheinberg, A. Tincani, B. Rozman, and Y. Shoenfeld. 1994. Anticardiolipin antibodies in infections are heterogenous in their dependency on beta 2-glycoprotein I: analysis of anticardiolipin antibodies in leprosy. *Lupus* 3:515.

28. Loizou, S., J. K. Cazabon, M. J. Walport, D. Tait, and A. K. So. 1997. Similarities of specificity and cofactor dependence in serum antiphospholipid antibodies from patients with human parvovirus B19 infection and from those with systemic lupus erythematosus. *Arthritis Rheum.* 40:103.

29. Fiallo, P., C. Travaglino, E. Nunzi, and P. P. Cardo. 1998. beta 2-Glycoprotein I-dependence of anticardiolipin antibodies in multibacillary leprosy patients. *Lepr. Rev.* 69:376.

30. Arvieux, J., B. Roussel, M. C. Jacob, and M. G. Colomb. 1991. Measurement of anti-phospholipid antibodies by ELISA using beta 2-glycoprotein I as an antigen. *J. Immunol. Methods* 143:223.

31. Ginsberg, J. S., P. S. Wells, P. Brill-Edwards, D. Donovan, K. Moffatt, M. Johnston, P. Stevens, and J. Hirsh. 1995. Antiphospholipid antibodies and venous thromboembolism. *Blood* 86:3685.

32. Simioni, P., P. Prandoni, E. Zanon, M. A. Saracino, A. Scudeller, S. Villalta, L. Scarano, B. Girolami, L. Benedetti, and A. Girolami. 1996. Deep venous thrombosis and lupus anticoagulant. A case-control study. *Thromb. Haemost.* 76:187.

33. Bevers, E. M., M. Galli, T. Barbui, P. Comfurius, and R. F. Zwaal. 1991. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. *Thromb. Haemost.* 66:629.

34. Oosting, J. D., R. H. Derksen, I. W. Bobbink, T. M. Hackeng, B. N. Bouma, and P. G. DE Groot. 1993. Antiphospholipid antibodies directed against a combination of phospholipids with prothrombin, protein C, or protein S: an explanation for their pathogenic mechanism? *Blood* 81:2618.

35. Greaves, M., H. Cohen, S. J. Machin, and I. Mackie. 2000. Guidelines on the investigation and management of the antiphospholipid syndrome. *Br J Haematol.* 109:704.

36. Derksen, R. H., P. Hasselaar, L. Blokzijl, F. H. Gmelig Meyling, and P. G. DE Groot. 1988. Coagulation screen is more specific than the anticardiolipin antibody ELISA in defining a thrombotic subset of lupus patients. *Ann. Rheum. Dis.* 47:364.

37. Nash, M. J., R. S. Camilleri, S. Kunka, I. J. Mackie, S. J. Machin, and H. Cohen. 2004. The anticardiolipin assay is required for sensitive screening for antiphospholipid antibodies. *J Thromb. Haemost.* 2:1077.

38. Nojima, J., H. Kuratsune, E. Suehisa, Y. Futsukaichi, H. Yamanishi, T. Machii, Y. Iwatani, and Y. Kanakura. 2001. Association between the prevalence of antibodies to beta(2)-glycoprotein I, prothrombin, protein C, protein S, and annexin V in patients with systemic lupus erythematosus and thrombotic and thrombocytopenic complications. *Clin Chem.* 47:1008.

39. Sugi, T., and J. A. McIntyre. 1996. Autoantibodies to kininogen-phosphatidylethanolamine complexes augment thrombin-induced platelet aggregation. *Thromb. Res.* 84:97.

40. Patthy, L. 1987. Detecting homology of distantly related proteins with consensus sequences. *J Mol. Biol* 198:567.

41. Schultze HE, Heidke K, and Haput H. 1961. Uber ein bisher ubekanntes niedermolekulars B2-Globulin des Humanserums., p. 719.

42. Steinkasserer, A., C. Estaller, E. H. Weiss, R. B. Sim, and A. J. Day. 1991. Complete nucleotide and deduced amino acid sequence of human beta 2-glycoprotein I. *Biochem. J* 277 (Pt 2):387.

43. Chamley, L. W., J. L. Allen, and P. M. Johnson. 1997. Synthesis of beta2 glycoprotein 1 by the human placenta. *Placenta* 18:403.

44. Averna, M., G. Paravizzini, G. Marino, E. Lanteri, G. Cavera, C. M. Barbagallo, S. Petralia, S. Cavallaro, G. Magro, S. Grasso, A. Notarbartolo, and S. Travali. 1997. Liver is not the unique site of synthesis of beta 2-glycoprotein I (apolipoprotein H): evidence for an intestinal localization. *Int. J Clin Lab Res.* 27:207.

45. Lee, N. S., H. B. Brewer, Jr., and J. C. Osborne, Jr. 1983. beta 2-Glycoprotein I. Molecular properties of an unusual apolipoprotein, apolipoprotein H. *J Biol Chem.* 258:4765.

46. Lozier, J., N. Takahashi, and F. W. Putnam. 1984. Complete amino acid sequence of human plasma beta 2-glycoprotein I. *Proc Natl. Acad. Sci. U. S. A* 81:3640.

47. Bouma, B., P. G. DE Groot, J. M. van den Elsen, R. B. Ravelli, A. Schouten, M. J. Simmelink, R. H. Derksen, J. Kroon, and P. Gros. 1999. Adhesion mechanism of human beta(2)-glycoprotein I to phospholipids based on its crystal structure. *EMBO J* 18:5166.

48. Schwarzenbacher, R., K. Zeth, K. Diederichs, A. Gries, G. M. Kostner, P. Laggner, and R. Prassl. 1999. Crystal structure of human beta2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. *EMBO J* 18:6228.

49. Hunt, J., and S. Krilis. 1994. The fifth domain of beta 2-glycoprotein I contains a phospholipid binding site (Cys281-Cys288) and a region recognized by anticardiolipin antibodies. *J Immunol* 152:653.

50. Sanghera, D. K., D. R. Wagenknecht, J. A. McIntyre, and M. I. Kamboh. 1997. Identification of structural mutations in the fifth domain of apolipoprotein H (beta 2-glycoprotein I) which affect phospholipid binding. *Hum. Mol. Genet.* 6:311.

51. Matsuura, E., J. Inagaki, H. Kasahara, D. Yamamoto, T. Atsumi, K. Kobayashi, K. Kaihara, D. Zhao, K. Ichikawa, A. Tsutsumi, T. Yasuda, D. A. Triplett, and T. Koike. 2000. Proteolytic cleavage of beta(2)-glycoprotein I: reduction of antigenicity and the structural relationship. *Int. Immunol* 12:1183.

52. Borensztajn, J., T. J. Kotlar, and S. C. Meredith. 1985. Fractionation of chylomicrons by heparin-sepharose chromatography. Characterization of two heparin-binding proteins. *J Biol Chem.* 260:13047.

53. Kroll, J., J. K. Larsen, H. Loft, M. Ezban, K. Wallevik, and M. Faber. 1976. DNA-binding proteins in Yoshida ascites tumor fluid. *Biochim. Biophys. Acta* 434:490.

54. Rojkjaer, R., D. A. Klaerke, and I. Schousboe. 1997. Characterization of the interaction between beta2-glycoprotein I and calmodulin, and identification of a binding sequence in beta2-glycoprotein I. *Biochim. Biophys. Acta* 1339:217.

55. Del, P. N., Y. H. Sheng, E. Raschi, D. A. Kandiah, A. Tincani, M. A. Khamashta, T. Atsumi, G. R. Hughes, K. Ichikawa, T. Koike, G. Balestrieri, S. A. Krilis, and P. L. Meroni. 1998. Human beta 2-glycoprotein I binds to endothelial cells through a cluster of lysine residues that are critical for anionic phospholipid binding and offers epitopes for anti-beta 2-glycoprotein I antibodies. *J Immunol* 160:5572.

56. Shi, W., B. H. Chong, and C. N. Chesterman. 1993. Beta 2-glycoprotein I is a requirement for anticardiolipin antibodies binding to activated platelets: differences with lupus anticoagulants. *Blood* 81:1255.

57. Bevers, E. M., M. P. Janssen, P. Comfurius, K. Balasubramanian, A. J. Schroit, R. F. Zwaal, and G. M. Willems. 2005. Quantitative determination of the binding of beta2-glycoprotein I and prothrombin to phosphatidylserine-exposing blood platelets. *Biochem. J* 386:271.

58. Nakaya, Y., E. J. Schaefer, and H. B. Brewer, Jr. 1980. Activation of human post heparin lipoprotein lipase by apolipoprotein H (beta 2-glycoprotein I). *Biochem. Biophys. Res. Commun.* 95:1168.

59. Wurm, H., E. Beubler, E. Polz, A. Holasek, and G. Kostner. 1982. Studies on the possible function of beta 2-glycoprotein-I: influence in the triglyceride metabolism in the rat. *Metabolism* 31:484.

60. Hasunuma, Y., E. Matsuura, Z. Makita, T. Katahira, S. Nishi, and T. Koike. 1997. Involvement of beta 2-glycoprotein I and anticardiolipin antibodies in oxidatively modified low-density lipoprotein uptake by macrophages. *Clin Exp Immunol* 107:569.

61. Manfredi, A. A., P. Rovere, S. Heltai, G. Galati, G. Nebbia, A. Tincani, G. Balestrieri, and M. G. Sabbadini. 1998. Apoptotic cell clearance in systemic lupus erythematosus. II. Role of beta2-glycoprotein I. *Arthritis Rheum.* 41:215.

62. Balasubramanian, K., S. N. Maiti, and A. J. Schroit. 2005. Recruitment of beta-2-glycoprotein 1 to cell surfaces in extrinsic and intrinsic apoptosis. *Apoptosis.* 10:439.

63. Kaplan, A. P., and M. Silverberg. 1987. The coagulation-kinin pathway of human plasma. *Blood* 70:1.

64. Nimpf, J., H. Wurm, and G. M. Kostner. 1987. Beta 2-glycoprotein-I (apo-H) inhibits the release reaction of human platelets during ADP-induced aggregation. *Atherosclerosis* 63:109.

65. Ieko, M., K. Ichikawa, D. A. Triplett, E. Matsuura, T. Atsumi, K. Sawada, and T. Koike. 1999. Beta2-glycoprotein I is necessary to inhibit protein C activity by monoclonal anticardiolipin antibodies. *Arthritis Rheum.* 42:167.

66. Mori, T., H. Takeya, J. Nishioka, E. C. Gabazza, and K. Suzuki. 1996. beta 2-Glycoprotein I modulates the anticoagulant activity of activated protein C on the phospholipid surface. *Thromb. Haemost.* 75:49.

67. Sheng, Y., J. G. Hanly, S. W. Reddel, S. Kouts, J. Guerin, T. Koike, K. Ichikawa, A. Sturgess, and S. A. Krilis. 2001. Detection of 'antiphospholipid' antibodies: a single chromogenic assay of thrombin generation sensitively detects lupus anticoagulants, anticardiolipin antibodies, plus antibodies binding beta(2)-glycoprotein I and prothrombin. *Clin Exp Immunol* 124:502.

68. Shi, W., B. H. Chong, P. J. Hogg, and C. N. Chesterman. 1993. Anticardiolipin antibodies block the inhibition by beta 2-glycoprotein I of the factor Xa generating activity of platelets. *Thromb. Haemost.* 70:342.

69. Yasuda, S., A. Tsutsumi, H. Chiba, H. Yanai, Y. Miyoshi, R. Takeuchi, T. Horita, T. Atsumi, K. Ichikawa, E. Matsuura, and T. Koike. 2000. beta(2)-glycoprotein I

deficiency: prevalence, genetic background and effects on plasma lipoprotein metabolism and hemostasis. *Atherosclerosis* 152:337.

70. Bancsi, L. F., d. L. van, I, and R. M. Bertina. 1992. Beta 2-glycoprotein I deficiency and the risk of thrombosis. *Thromb. Haemost.* 67:649.

71. Sheng, Y., S. W. Reddel, H. Herzog, Y. X. Wang, T. Brighton, M. P. France, S. A. Robertson, and S. A. Krilis. 2001. Impaired thrombin generation in beta 2-glycoprotein I null mice. *J Biol Chem.* 276:13817.

72. Galli, M., G. Beretta, M. Daldossi, E. M. Bevers, and T. Barbui. 1997. Different anticoagulant and immunological properties of anti-prothrombin antibodies in patients with antiphospholipid antibodies. *Thromb. Haemost.* 77:486.

73. Chow, B. K., V. Ting, F. Tufaro, and R. T. MacGillivray. 1991. Characterization of a novel liver-specific enhancer in the human prothrombin gene. *J Biol Chem.* 266:18927.

74. Magnusson S. 2007. The Enzymes. Boyer PD, ed., pp. 277.

75. Shaw, E., J. C. Giddings, I. R. Peake, and A. L. Bloom. 1979. Synthesis of procoagulant factor VIII, factor VIII related antigen and other coagulation factors by the isolated perfused rat liver. *Br J Haematol.* 41:585.

76. Esmon, C. T., J. W. Suttie, and C. M. Jackson. 1975. The functional significance of vitamin K action. Difference in phospholipid binding between normal and abnormal prothrombin. *J Biol Chem.* 250:4095.

77. Matsuda, J., N. Saitoh, M. Gotoh, K. Kawasugi, K. Gohchi, and M. Tsukamoto. 1996. Phosphatidyl serine-dependent antiprothrombin antibody is exclusive to patients with lupus anticoagulant. *Br J Rheumatol* 35:589.

78. Atsumi, T., M. Ieko, M. L. Bertolaccini, K. Ichikawa, A. Tsutsumi, E. Matsuura, and T. Koike. 2000. Association of autoantibodies against the phosphatidylserine-prothrombin complex with manifestations of the antiphospholipid syndrome and with the presence of lupus anticoagulant. *Arthritis Rheum.* 43:1982.

79. Chu, J. L., J. Drappa, A. Parnassa, and K. B. Elkon. 1993. The defect in Fas mRNA expression in MRL/lpr mice is associated with insertion of the retrotransposon, ETn. *J Exp Med* 178:723.

80. Alderson, M. R., R. J. Armitage, E. Maraskovsky, T. W. Tough, E. Roux, K. Schooley, F. Ramsdell, and D. H. Lynch. 1993. Fas transduces activation signals in normal human T lymphocytes. *J Exp Med* 178:2231.

81. Gharavi, A. E., R. C. Mellors, and K. B. Elkon. 1989. IgG anti-cardiolipin antibodies in murine lupus. *Clin Exp Immunol* 78:233.

82. Aron, A. L., M. L. Cuellar, R. L. Brey, S. Mckeown, L. R. Espinoza, Y. Shoenfeld, and A. E. Gharavi. 1995. Early onset of autoimmunity in MRL/++ mice following immunization with beta 2 glycoprotein I. *Clin Exp Immunol* 101:78.

83. Hang, L. M., S. Izui, and F. J. Dixon. 1981. (NZW x BXSB)F1 hybrid. A model of acute lupus and coronary vascular disease with myocardial infarction. *J Exp Med* 154:216.

84. Hang, L., P. Stephens-Larson, J. P. Henry, and F. J. Dixon. 1983. The role of hypertension in the vascular disease and myocardial infarcts associated with murine systemic lupus erythematosus. *Arthritis Rheum.* 26:1340.

85. Mizutani, H., T. Furubayashi, A. Kuriu, H. Take, Y. Tomiyama, H. Yoshida, Y. Nakamura, M. Inaba, Y. Kurata, T. Yonezawa, and . 1990. Analyses of thrombocytopenia in idiopathic thrombocytopenic purpura-prone mice by platelet transfer experiments between (NZW x BXSB)F1 and normal mice. *Blood* 75:1809.

86. Hashimoto, Y., M. Kawamura, K. Ichikawa, T. Suzuki, T. Sumida, S. Yoshida, E. Matsuura, S. Ikehara, and T. Koike. 1992. Anticardiolipin antibodies in NZW x BXSB F1 mice. A model of antiphospholipid syndrome. *J Immunol* 149:1063.

87. Steinberg, R. T., M. L. Miller, and A. D. Steinberg. 1985. Effect of the BXSB Y chromosome accelerating gene on autoantibody production. *Clin Immunol Immunopathol.* 35:67.

88. Kotzin, B. L., and E. Palmer. 1987. The contribution of NZW genes to lupus-like disease in (NZB x NZW)F1 mice. *J Exp Med* 165:1237.

89. Kotzin, B. L., and E. Palmer. 1988. Genetic contributions to lupus-like disease in NZB/NZW mice. *Am. J Med* 85:29.

90. Vyse, T. J., S. J. Rozzo, C. G. Drake, V. B. Appel, M. Lemeur, S. Izui, E. Palmer, and B. L. Kotzin. 1998. Contributions of Ea(z) and Eb(z) MHC genes to lupus susceptibility in New Zealand mice. *J Immunol* 160:2757.

91. Vyse, T. J., R. K. Halterman, S. J. Rozzo, S. Izui, and B. L. Kotzin. 1999. Control of separate pathogenic autoantibody responses marks MHC gene contributions to murine lupus. *Proc Natl. Acad. Sci. U. S. A* 96:8098.

92. Adachi, Y., M. Inaba, K. Inaba, N. Nagata, Y. Kobayashi, and S. Ikehara. 1993. Functional analyses of B cells in (NZW x BXSB) F1 mice. *Autoimmunity* 15:99.

93. Branch, D. W., D. J. Dudley, M. D. Mitchell, K. A. Creighton, T. M. Abbott, E. H. Hammond, and R. A. Daynes. 1990. Immunoglobulin G fractions from patients with antiphospholipid antibodies cause fetal death in BALB/c mice: a model for autoimmune fetal loss. *Am. J Obstet. Gynecol.* 163:210.

94. Olee, T., S. S. Pierangeli, H. H. Handley, D. T. Le, X. Wei, C. J. Lai, J. En, W. Novotny, E. N. Harris, V. L. Woods, Jr., and P. P. Chen. 1996. A monoclonal IgG anticardiolipin antibody from a patient with the antiphospholipid syndrome is thrombogenic in mice. *Proc Natl. Acad. Sci. U. S. A* 93:8606.

95. Bakimer, R., P. Fishman, M. Blank, B. Sredni, M. Djaldetti, and Y. Shoenfeld. 1992. Induction of primary antiphospholipid syndrome in mice by immunization with a human monoclonal anticardiolipin antibody (H-3). *J Clin Invest* 89:1558.

96. Yodfat, O., M. Blank, I. Krause, and Y. Shoenfeld. 1996. The pathogenic role of anti-phosphatidylserine antibodies: active immunization with the antibodies leads to the induction of antiphospholipid syndrome. *Clin Immunol Immunopathol.* 78:14.

97. Gharavi, A. E., S. S. Pierangeli, M. Colden-Stanfield, X. W. Liu, R. G. Espinola, and E. N. Harris. 1999. GDKV-induced antiphospholipid antibodies enhance thrombosis and activate endothelial cells in vivo and in vitro. *J Immunol* 163:2922.

98. Blank M, Faden D, A. Tincani, Kopolovic J, Goldberg I, Gilburd B, Allegri F, Balestrieri G, Valesini G, and Shoenfeld Y. 1994. Immunization with anticardiolipin cofactor (beta-2-glycoprotein I) induces experimental antiphospholipid syndrome in naive mice. *J Autoimmun* 7:441.

99. Tincani, A., B. Gilburd, M. bu-Shakra, M. Blank, F. Allegri, R. Ottaviani, M. Riboni, P. L. Meroni, G. Balestrieri, and Y. Shoenfeld. 2002. Immunization of naive BALB/c mice with human beta2-glycoprotein I breaks tolerance to the murine molecule. *Arthritis Rheum.* 46:1399.

100. Levine, J. S., R. Subang, S. H. Nasr, S. Fournier, G. Lajoie, J. Wither, and J. Rauch. 2006. Immunization with an apoptotic cell-binding protein recapitulates the nephritis and sequential autoantibody emergence of systemic lupus erythematosus. *J. Immunol.* 177:6504.

101. Pierangeli, S. S., J. H. Barker, D. Stikovac, D. Ackerman, G. Anderson, J. Barquinero, R. Acland, and E. N. Harris. 1994. Effect of human IgG antiphospholipid antibodies on an in vivo thrombosis model in mice. *Thromb. Haemost.* 71:670.

102. Meroni, P. L., M. O. Borghi, E. Raschi, D. Ventura, P. C. Sarzi Puttini, F. Atzeni, L. Lonati, G. Parati, A. Tincani, D. Mari, and F. Tedesco. 2004. Inflammatory response and the endothelium. *Thromb. Res.* 114:329.

103. Martin, S. J., C. P. Reutelingsperger, A. J. McGahon, J. A. Rader, R. C. van Schie, D. M. LaFace, and D. R. Green. 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 182:1545.

104. Fadok, V. A., C. A. de, D. L. Daleke, P. M. Henson, and D. L. Bratton. 2001. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J Biol Chem.* 276:1071.

105. Fadok, V. A., D. L. Bratton, A. Konowal, P. W. Freed, J. Y. Westcott, and P. M. Henson. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* 101:890.

106. Price, B. E., J. Rauch, M. A. Shia, M. T. Walsh, W. Lieberthal, H. M. Gilligan, T. O'Laughlin, J. S. Koh, and J. S. Levine. 1996. Anti-phospholipid autoantibodies bind to apoptotic, but not viable, thymocytes in a beta 2-glycoprotein I-dependent manner. *J Immunol* 157:2201.

107. Subang, R., J. S. Levine, A. S. Janoff, S. M. Davidson, T. F. Taraschi, T. Koike, S. R. Minchey, M. Whiteside, M. Tannenbaum, and J. Rauch. 2000. Phospholipid-bound beta 2-glycoprotein I induces the production of anti-phospholipid antibodies. *J. Autoimmun.* 15:21.

108. Gharavi, A. E., E. N. Harris, R. A. Asherson, and G. R. Hughes. 1987. Anticardiolipin antibodies: isotype distribution and phospholipid specificity. *Ann. Rheum. Dis.* 46:1.

109. Fanopoulos, D., M. R. Teodorescu, J. Varga, and M. Teodorescu. 1998. High frequency of abnormal levels of IgA anti-beta2-glycoprotein I antibodies in patients with systemic lupus erythematosus: relationship with antiphospholipid syndrome. *J Rheumatol* 25:675.

110. Blank, M., I. Krause, N. Lanir, P. Vardi, B. Gilburd, A. Tincani, Y. Tomer, and Y. Shoenfeld. 1995. Transfer of experimental antiphospholipid syndrome by bone marrow cell transplantation. The importance of the T cell. *Arthritis Rheum.* 38:115.

111. Tomer, Y., M. Blank, and Y. Shoenfeld. 1994. Suppression of experimental antiphospholipid syndrome and systemic lupus erythematosus in mice by anti-CD4 monoclonal antibodies. *Arthritis Rheum.* 37:1236.

112. Visvanathan, S., and H. P. McNeil. 1999. Cellular immunity to beta 2-glycoprotein-1 in patients with the antiphospholipid syndrome. *J Immunol* 162:6919.

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

114. Arunachalam, B., U. T. Phan, H. J. Geuze, and P. Cresswell. 2000. Enzymatic reduction of disulfide bonds in lysosomes: characterization of a gamma-

interferon-inducible lysosomal thiol reductase (GILT). *Proc Natl. Acad. Sci. U. S. A* 97:745.

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

116. Lehmann, P. V., T. Forsthuber, A. Miller, and E. E. Sercarz. 1992. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 358:155.

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

118. Wilson, W. A., E. Scopelitis, J. P. Michalski, S. S. Pierangeli, L. H. Silveira, R. C. Elston, and E. N. Harris. 1995. Familial anticardiolipin antibodies and C4 deficiency genotypes that coexist with MHC DQB1 risk factors. *J Rheumatol* 22:227.

119. Hashimoto, H., K. Yamanaka, Y. Tokano, N. Iida, Y. Takasaki, K. Kabasawa, and Y. Nishimura. 1998. HLA-DRB1 alleles and beta 2 glycoprotein I-dependent anticardiolipin antibodies in Japanese patients with systemic lupus erythematosus. *Clin Exp Rheumatol* 16:423.

120. Vargas-Alarcon, G., J. Granados, K. G. Ibanez de, J. cocer-Varela, and D. arcon-Segovia. 1995. Association of HLA-DR5 (DR11) with systemic sclerosis (scleroderma) in Mexican patients. *Clin Exp Rheumatol* 13:11.

121. Arnett, F. C., P. Thiagarajan, C. Ahn, and J. D. Reveille. 1999. Associations of anti-beta2-glycoprotein I autoantibodies with HLA class II alleles in three ethnic groups. *Arthritis Rheum.* 42:268.

122. Ito, H., S. Matsushita, Y. Tokano, H. Nishimura, Y. Tanaka, S. Fujisao, H. Mitsuya, H. Hashimoto, and Y. Nishimura. 2000. 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* 61:366.

123. D'Agnillo, P., J. S. Levine, R. Subang, and J. Rauch. 2003. Prothrombin binds to the surface of apoptotic, but not viable, cells and serves as a target of lupus anticoagulant autoantibodies. *J Immunol* 170:3408.

124. Levine, J. S., R. Subang, J. S. Koh, and J. Rauch. 1998. Induction of anti-phospholipid autoantibodies by beta2-glycoprotein I bound to apoptotic thymocytes. *J. Autoimmun.* 11:413.

125. Sorice, M., A. Circella, I. M. Cristea, T. Garofalo, R. L. Di, C. Alessandri, G. Valesini, and M. D. Esposti. 2004. Cardiolipin and its metabolites move from

mitochondria to other cellular membranes during death receptor-mediated apoptosis. *Cell Death. Differ.* 11:1133.

126. Sorice, M., A. Circella, R. Misasi, V. Pittoni, T. Garofalo, A. Cirelli, A. Pavan, G. M. Pontieri, and G. Valesini. 2000. Cardiolipin on the surface of apoptotic cells as a possible trigger for antiphospholipids antibodies. *Clin Exp Immunol* 122:277.

127. Alessandri, C., M. Sorice, M. Bombardieri, P. Conigliaro, A. Longo, T. Garofalo, V. Manganelli, F. Conti, M. D. Esposti, and G. Valesini. 2006. Antiphospholipid reactivity against cardiolipin metabolites occurring during endothelial cell apoptosis. *Arthritis Res. Ther.* 8:R180.

128. Wagenknecht, D. R., and J. A. McIntyre. 1993. Changes in beta 2-glycoprotein I antigenicity induced by phospholipid binding. *Thromb. Haemost.* 69:361.

129. Hamdan, R., S. N. Maiti, and A. J. Schroit. 2007. Interaction of beta2-glycoprotein 1 with phosphatidylserine-containing membranes: ligand-dependent conformational alterations initiate bivalent binding. *Biochemistry* 46:10612.

130. Borchman, D., E. N. Harris, S. S. Pierangeli, and O. P. Lamba. 1995. Interactions and molecular structure of cardiolipin and beta 2-glycoprotein 1 (beta 2-GP1). *Clin Exp Immunol* 102:373.

131. Matsuura, E., Y. Igarashi, T. Yasuda, D. A. Triplett, and T. Koike. 1994. Anticardiolipin antibodies recognize beta 2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. *J Exp Med* 179:457.

132. Koike, T., A. Tsutsumi, K. Ichikawa, and E. Matsuura. 1995. Antigenic specificity of the "anticardiolipin" antibodies. *Blood* 85:2277.

133. Ichikawa, K., M. A. Khamashta, T. Koike, E. Matsuura, and G. R. Hughes. 1994. beta 2-Glycoprotein I reactivity of monoclonal anticardiolipin antibodies from patients with the antiphospholipid syndrome. *Arthritis Rheum.* 37:1453.

134. Pengo, V., A. Biasiolo, and M. G. Fior. 1995. Autoimmune antiphospholipid antibodies are directed against a cryptic epitope expressed when beta 2-glycoprotein I is bound to a suitable surface. *Thromb. Haemost.* 73:29.

135. Roubey, R. A., R. A. Eisenberg, M. F. Harper, and J. B. Winfield. 1995. "Anticardiolipin" autoantibodies recognize beta 2-glycoprotein I in the absence of phospholipid. Importance of Ag density and bivalent binding. *J Immunol* 154:954.

136. Viard, J. P., Z. Amoura, and J. F. Bach. 1992. Association of anti-beta 2 glycoprotein I antibodies with lupus-type circulating anticoagulant and thrombosis in systemic lupus erythematosus. *Am. J Med* 93:181.

137. Keeling, D. M., A. J. Wilson, I. J. Mackie, S. J. Machin, and D. A. Isenberg. 1992. Some 'antiphospholipid antibodies' bind to beta 2-glycoprotein I in the absence of phospholipid. *Br J Haematol.* 82:571.

138. Simitsek, P. D., D. G. Campbell, A. Lanzavecchia, N. Fairweather, and C. Watts. 1995. Modulation of antigen processing by bound antibodies can boost or suppress class II major histocompatibility complex presentation of different T cell determinants. *J Exp Med* 181:1957.

139. Yang, C. D., S. L. Chen, N. Shen, M. Qi, and F. Xu. 1998. Detection of anti-recombinant beta 2-glycoprotein 1 and anti-recombinant beta 2-glycoprotein 1 fifth-domain antibodies in sera from patients with systemic lupus erythematosus. *Rheumatol Int.* 18:5.

140. George, J., B. Gilburd, M. Hojnik, Y. Levy, P. Langevitz, E. Matsuura, T. Koike, and Y. Shoenfeld. 1998. Target recognition of beta2-glycoprotein I (beta2GPI)-dependent anticardiolipin antibodies: evidence for involvement of the fourth domain of beta2GPI in antibody binding. *J Immunol* 160:3917.

141. de, L. B., R. H. Derksen, R. T. Urbanus, and P. G. DE Groot. 2005. IgG antibodies that recognize epitope Gly40-Arg43 in domain I of beta 2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. *Blood* 105:1540.

142. de, L. B., R. H. Derksen, L. M. van, M. T. Pennings, and P. G. DE Groot. 2006. Pathogenic anti-beta2-glycoprotein I antibodies recognize domain I of beta2-glycoprotein I only after a conformational change. *Blood* 107:1916.

143. Igarashi, M., E. Matsuura, Y. Igarashi, H. Nagae, K. Ichikawa, D. A. Triplett, and T. Koike. 1996. Human beta2-glycoprotein I as an anticardiolipin cofactor determined using mutants expressed by a baculovirus system. *Blood* 87:3262.

144. Iverson, G. M., E. J. Victoria, and D. M. Marquis. 1998. Anti-beta2 glycoprotein I (beta2GPI) autoantibodies recognize an epitope on the first domain of beta2GPI. *Proc Natl. Acad. Sci. U. S. A* 95:15542.

145. McNeeley, P. A., J. S. Dlott, R. A. Furie, R. M. Jack, T. L. Ortel, D. A. Triplett, E. J. Victoria, and M. D. Linnik. 2001. Beta2-glycoprotein I-dependent anticardiolipin antibodies preferentially bind the amino terminal domain of beta2-glycoprotein I. *Thromb. Haemost.* 86:590.

146. Iverson, G. M., S. Reddel, E. J. Victoria, K. A. Cockerill, Y. X. Wang, M. A. Marti-Renom, A. Sali, D. M. Marquis, S. A. Krilis, and M. D. Linnik. 2002. Use of single point mutations in domain I of beta 2-glycoprotein I to determine fine antigenic specificity of antiphospholipid autoantibodies. *J Immunol* 169:7097.

147. Koike, T., K. Ichikawa, H. Kasahara, T. Atsumi, A. Tsutsumi, and E. Matsuura. 1998. Epitopes on beta2-GPI recognized by anticardiolipin antibodies. *Lupus* 7 Suppl 2:S14-S17.

148. Iverson, G. M., E. Matsuura, E. J. Victoria, K. A. Cockerill, and M. D. Linnik. 2002. The orientation of beta2GPI on the plate is important for the binding of anti-beta2GPI autoantibodies by ELISA. *J Autoimmun* 18:289.

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

150. Okkels, H., T. E. Rasmussen, D. K. Sanghera, M. I. Kamboh, and T. Kristensen. 1999. Structure of the human beta2-glycoprotein I (apolipoprotein H) gene. *Eur. J Biochem.* 259:435.

151. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J Mol. Biol* 215:403.

152. BARTLETT, G. R. 1959. Phosphorus assay in column chromatography. *J Biol Chem.* 234:466.

153. Khoruts, A., A. Mondino, K. A. Pape, S. L. Reiner, and M. K. Jenkins. 1998. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J Exp Med* 187:225.

154. Vella, A. T., J. E. McCormack, P. S. Linsley, J. W. Kappler, and P. Marrack. 1995. Lipopolysaccharide interferes with the induction of peripheral T cell death. *Immunity.* 2:261.

155. Liu, Y., and C. A. Janeway, Jr. 1991. Microbial induction of co-stimulatory activity for CD4 T-cell growth. *Int. Immunol* 3:323.

156. Parks, D. E., S. M. Walker, and W. O. Weigle. 1981. Bacterial lipopolysaccharide (endotoxin) interferes with the induction of tolerance and primes thymus-derived lymphocytes. *J Immunol* 126:938.

157. Maxwell, J. R., R. J. Rossi, S. J. McSorley, and A. T. Vella. 2004. T cell clonal conditioning: a phase occurring early after antigen presentation but before clonal expansion is impacted by Toll-like receptor stimulation. *J Immunol* 172:248.

158. Buttari, B., E. Profumo, V. Mattei, A. Siracusano, E. Ortona, P. Margutti, B. Salvati, M. Sorice, and R. Rigano. 2005. Oxidized beta2-glycoprotein I induces human dendritic cell maturation and promotes a T helper type 1 response. *Blood* 106:3880.

159. Krause, I., M. Blank, Y. Levi, T. Koike, V. Barak, and Y. Shoenfeld. 1999. Anti-idiotype immunomodulation of experimental anti-phospholipid syndrome via effect on Th1/Th2 expression. *Clin Exp Immunol* 117:190.

160. Karakantza, M., G. L. Theodorou, N. Meimaris, A. Mouzaki, E. John, A. P. Andonopoulos, and A. Maniatis. 2004. Type 1 and type 2 cytokine-producing CD4+ and CD8+ T cells in primary antiphospholipid syndrome. *Ann. Hematol.* 83:704.

161. Hsieh, C. S., S. E. Macatonia, A. O'Garra, and K. M. Murphy. 1995. T cell genetic background determines default T helper phenotype development in vitro. *J Exp Med* 181:713.

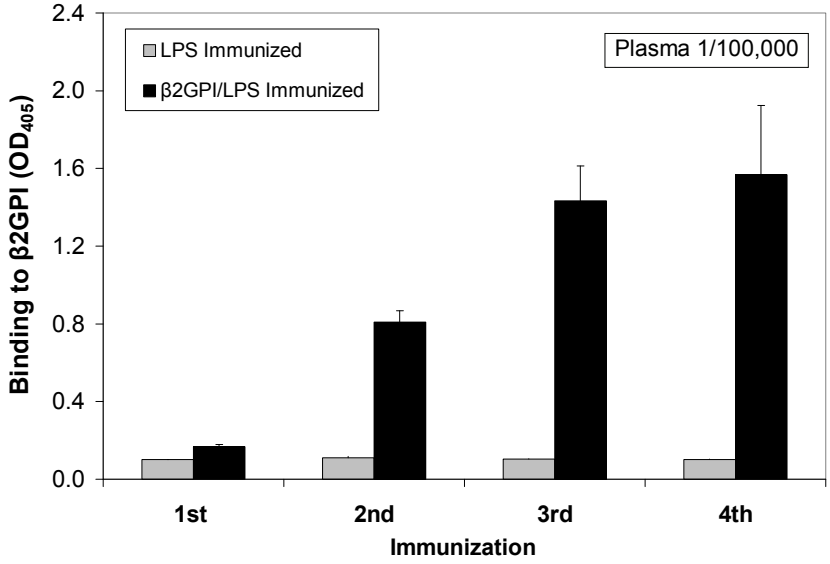
162. Horkko, S., E. Miller, D. W. Branch, W. Palinski, and J. L. Witztum. 1997. The epitopes for some antiphospholipid antibodies are adducts of oxidized phospholipid and beta2 glycoprotein 1 (and other proteins). *Proc Natl. Acad. Sci. U. S. A* 94:10356.

163. Yamaguchi, Y., N. Seta, J. Kaburaki, K. Kobayashi, E. Matsuura, and M. Kuwana. 2007. Excessive exposure to anionic surfaces maintains autoantibody response to {beta}2-Glycoprotein I in patients with antiphospholipid syndrome. *Blood*.

CHAPTER VI. APPENDIX

Induction of an Anti-β2GPI and aCL Response

a)



b)

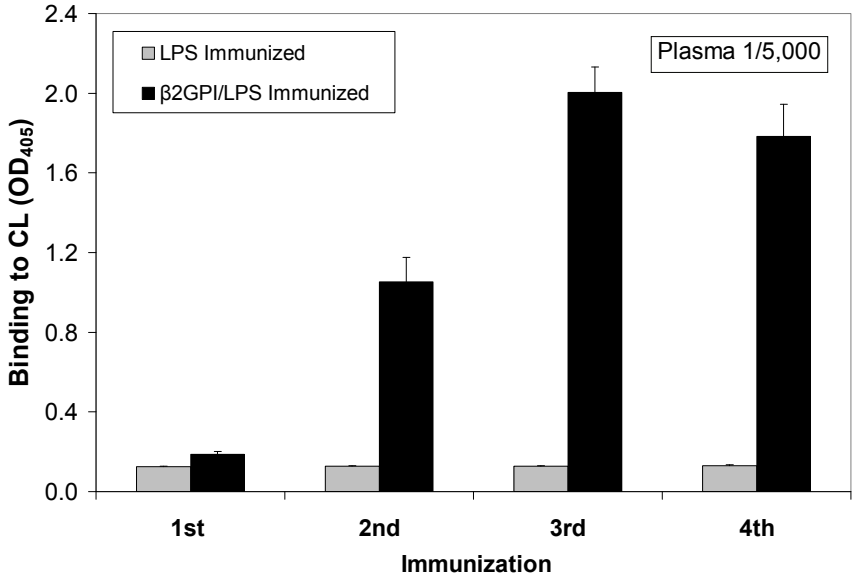


Figure 1. Induction of anti-β2GPI and aCL IgG responses in mice immunized with β2GPI and LPS on the same day. Plasma dilutions (as indicated) from mice immunized with LPS or β2GPI/LPS were evaluated by ELISA for IgG antibodies to (a) β2GPI or (b) CL. Mice immunized with β2GPI in the presence of LPS produced significantly elevated titers of anti-β2GPI ($p < 0.0009$) and aCL ($p < 0.004$) IgG, compared to mice immunized with LPS alone, after the first immunization. The data represent the mean OD_{405} ± SEM for each group of mice ($n = 5 – 8$ mice/group).

Purification and Detection of Native Murine β2GPI

Methods

Purification of Native Murine β2GPI

Normal mouse serum was treated with 1.2% perchloric acid. Briefly, the appropriate volume of 70% (v/v) perchloric acid was added dropwise to the serum with constant stirring on ice for 15 min, and the solution was centrifuged at 15 300 x *g* for 30 min at 4ºC. The supernatant was neutralized with 2M Na_2CO_3 (pH 8.0) and dialyzed 6 times against 20 mM Tris buffer (pH 8.0), with six changes of dialysis buffer. Following dialysis, the supernatant was applied to a High Q column (Bio-Rad, Hercules, CA). Bound protein was eluted from the column using a stepwise gradient of 0.1 M, 0.2 M, 0.25 M, 0.3 M, and 0.35 M NaCl in 20 mM Tris buffer, pH 8.0. Murine β2GPI activity was detected by ELISA (see below). Column fractions containing both high protein (Micro BCA Protein Assay Kit, Pierce, Rockford, IL) and β2GPI content were pooled and dialyzed against two changes of 20 mM Tris buffer, pH 8.0. The β2GPI-containing fraction was then applied to a HiTrap Heparin column (Amersham Pharmacia Biotech, Uppsala, Sweden). Bound protein was eluted from the column using a linear gradient of NaCl (0 – 1.0 M) in 20 mM Tris buffer, pH 8.0. Fractions containing both high protein and β2GPI content were pooled and dialyzed against two changes of 20 mM Tris-HCl buffer, containing 150 mM NaCl, pH 8.0. The dialyzed material was then applied to a Protein A Sepharose column (Amersham Pharmacia Biotech) to remove any contaminating IgG, and the effluent was collected and assayed for protein content using the MicroBCA Protein Assay kit. CL-binding activity was determined using the aCL ELISA (see Materials and Methods in Chapter II). Protein purity was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10%

polyacrylamide gel run under reducing conditions and stained with silver nitrate (Figure 2).

Detection of Native Murine β2GPI

Antibody reactivity to murine β2GPI was detected on Immulon-2 plates (Dynatech Laboratories, Chantilly, VA) coated with CL at 90 μg/mL (50 μL/well) in 10 mM PBS, pH 7.3 for 16 h at 37ºC. Plates were blocked with PBS containing 0.3% gelatin for 2 h at 4ºC and washed three times with 10 mM TBS, pH 7.4. Column fractions, and other test samples, were titrated and diluted in PBS containing 0.3% gelatin. Thirty μL of test sample were mixed with 100 μL of purified rabbit anti-mouse β2GPI IgG (10 μg/mL). This mixture was then added to the CL-coated wells (50 μL/well) in duplicate and incubated for 3 h at 25ºC. The plates were washed three times with TBS, and bound antibody was detected by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) diluted 1/2000 in PBS containing 0.4% BSA (75 μL/well) for 16 h at 4ºC. Plates were washed three times with TBS and developed with *p*-nitrophenol phosphate for approximately 30 min at 37ºC and the OD at 405 nm was read using an ELISA reader (model EL800 Universal Microplate Reader, Bio-Tek Instruments, Winooski, VT).

Results

Figure 2 shows the purity of native murine β2GPI as assessed by SDS-PAGE. Purified murine β2GPI (Lane 4) exhibited three major bands: one stronger band at a molecular weight of approximately 55 kDa and two weaker bands between 25 – 30 kDa. A minor

band at approximately 50 kDa was also observed. In contrast, commercial purified human β2GPI (Lane 2) showed a single band at approximately 52 kDa, while purified murine IgG (Lane 3) showed the expected heavy and light chain bands at approximately 50 kDa and 20 – 25 kDa, respectively. This data indicates that the purified murine β2GPI contains a major band slightly higher in molecular weight than, but consistent with, the known molecular weight of murine β2GPI; however, it also contains contaminating protein(s) with reduced molecular weights of between 25 – 30 kDa. At present, it is unclear what these contaminating proteins are. There is also a very light band at approximately 50 kDa, which could represent either residual murine IgG that has not been removed by Protein A-Sepharose adsorption in the purification protocol or a breakdown product of murine β2GPI. Western blot analysis using rabbit anti-murine IgG should help to differentiate between these two possibilities, and will be done when more purified murine β2GPI becomes available.

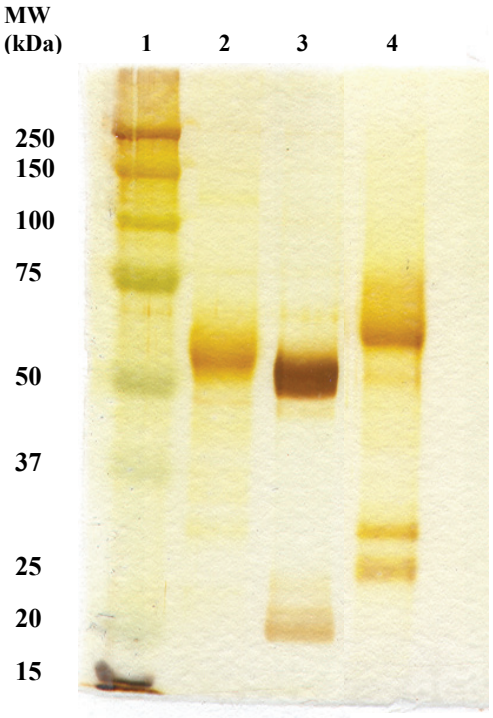


Figure 2. SDS-PAGE of purified native murine β2GPI. Purified native murine β2GPI (Lane 4) was analyzed by SDS polyacrylamide gel electrophoresis under reducing conditions and the gel was stained with silver nitrate. Commercial purified human β2GPI (Lane 2) and murine IgG (Lane 3) are shown for comparison. Molecular weight (MW) standards are shown in Lane 1.