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Autoreactive CD4+ T-cell clones to β_2-glycoprotein I in patients with antiphospholipid syndrome: preferential recognition of the major phospholipid-binding site

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Autoreactive CD4+ T cells to β_2-glycoprotein I (β_2GPI) that promote antiphospholipid antibody production were recently identified in patients with antiphospholipid syndrome (APS). To further examine antigen recognition profiles and T-cell helper activity in β_2GPI-reactive T cells, 14 CD4+ T-cell clones specific to β_2GPI were generated from 3 patients with APS by repeated stimulation of peripheral blood T cells with recombinant β_2GPI. At least 4 distinct T-cell epitopes were identified, but the majority of the β_2GPI-specific T-cell clones responded to a peptide encompassing amino acid residues 276 to 290 of β_2GPI (KVSFFCKNKEKKCSY; single-letter amino acid codes) that contains the major phospholipid-binding site in the context of the DRB4*0103 allele. Ten of 12 β_2GPI-specific T-cell clones were able to stimulate autologous peripheral blood B cells to promote anti-β_2GPI antibody production in the presence of recombinant β_2GPI. T-cell helper activity was exclusively found in T-cell clones capable of producing interleukin 6 (IL-6). In vitro anti-β_2GPI antibody production induced by T-cell clones was inhibited by anti-IL-6 or anti-CD40 ligand monoclonal antibody. In addition, exogenous IL-6 augmented anti-β_2GPI antibody production in cultures of the T-cell clone lacking IL-6 expression. These results indicate that β_2GPI-specific CD4+ T cells in patients with APS preferentially recognize the antigenic peptide containing the major phospholipid-binding site and have the capacity to stimulate B cells to produce anti-β_2GPI antibodies through IL-6 expression and CD40-CD40 ligand engagement. These findings are potentially useful for clarifying the pathogenesis of APS and for developing therapeutic strategies that suppress pathogenic antiphospholipid antibody production in these patients. (Blood. 2001;98:1889-1896)

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Introduction

Antiphospholipid syndrome (APS) is characterized by arterial and venous thrombosis as well as recurrent intrauterine fetal loss in association with antiphospholipid antibodies.[1] It is now widely accepted that β_2-glycoprotein I (β_2GPI) is the most common antigenic target for the antiphospholipid antibodies associated with the clinical features of APS.[2,3] Beta$_2$-GPI is a plasma glycoprotein that binds various kinds of negatively charged substances, including phospholipids (PLs), lipoproteins, activated platelets, and endothelial cells.[4-6] It possesses 5 complement control protein repeats (domains I-V), called "sushi" domains.[7] These domains have a homologous 60-amino acid repeating sequence with both inter- and intraregional disulfide bridges, and domain V has a 6-residue insertion and a 19-residue C-terminal extension. The major PL-binding site on β_2GPI has been identified as a highly positively charged amino acid sequence located at positions 281 to 288 in domain V.[8-10] Recent crystal structure analysis has revealed that β_2GPI adheres to anionic PLs via a large positively charged area formed by 14 basic amino acid residues in domain V, including the major PL-binding site.[11]

Autoantibodies to β_2GPI were shown to be pathogenic, based on the following observations in patients with APS and experimental animal models. First, the presence of serum anti-β_2GPI antibody is a major risk factor for arterial and venous thrombosis in patients with systemic lupus erythematosus (SLE).[12] Second, animals immunized with foreign β_2GPI develop a clinical manifestation of APS, including intrauterine fetal death and thrombocytopenia, along with the induction of anti-β_2GPI antibody production.[13,14] Finally, normal mice infused with anti-β_2GPI monoclonal antibodies (mAbs) or the IgG fraction of sera from patients with APS develop fetal resorption.[15,16] However, some reports failed to induce fetal death or resorption in similar immunization or passive transfer experiments.[17,18] These variable effects of anti-β_2GPI antibodies on murine pregnancy outcome could be explained by the antibody recognition of heterogeneous epitopes relevant or irrelevant to APS symptoms. Precise mechanisms for the thrombophilia caused by anti-β_2GPI antibodies remain unclear, but it is proposed that anti-β_2GPI antibodies bind to endothelial cell surfaces by recognizing the adhered β_2GPI and induce endothelial cell activation, resulting in the up-regulation of procoagulant and inflammatory processes.[19,20]

We have recently identified β_2GPI-reactive CD4+ T cells in the peripheral blood of patients with APS.[21] These β_2GPI-reactive T cells possess helper activity that induces the production of antibodies that specifically bind to β_2GPI immobilized on cardiolipin.

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Autoreactive T cells to β$_2$GPI have been presumed to recognize cryptic determinants, because they react with chemically reduced β$_2$GPI and recombinant β$_2$GPI fragments produced in bacteria, but not with β$_2$GPI in its native form. However, we were unable to analyze the T-cell epitopes on β$_2$GPI or the cytokines involved in the helper activity in bulk T-cell cultures. In this study, we have established β$_2$GPI-specific CD4$^+$ T-cell clones from the peripheral blood of patients with APS and investigated their antigen recognition profiles and helper activity involved in promoting anti-β$_2$GPI antibody production from B cells.

Patients, materials, and methods

Patients

Peripheral blood T cells from 3 Japanese patients with APS (O.M., E.Y., and K.S.) were analyzed in this study. All patients fulfilled the preliminary classification criteria for APS proposed by the International Workshop in Sapporo, Japan.[22] Primary APS was diagnosed in patients O.M. and K.S., and secondary APS accompanied by SLE was diagnosed in patient E.Y. At the time of blood examination, all patients were taking low-dose corticosteroids (< 10 mg/d) and aspirin. All samples were obtained after the patients gave their written informed consent, following Keio University Institutional Review Board guidelines.

Genotyping for HLA class II alleles and β$_2$GPI

Genomic DNA was isolated from peripheral blood leukocytes using a standard phenol extraction procedure. The HLA-DRB1, DRB4, DRB5, DQB1, and DPB1 alleles were determined using polymerase-chain reaction (PCR) followed by analysis of restriction fragment length polymorphisms.[23,24] The polymorphism at position 247 of β$_2$GPI (^{247}V-^{247}L; single-letter amino acid codes) was determined by *Rsa*I digestion of the PCR-amplified DNA.[25]

Detection of anti-β$_2$GPI antibody

The IgG anti-β$_2$GPI antibody levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Yamasa, Choshi, Japan), in which cardiolipin-coated plates were incubated with purified human β$_2$GPI as a cofactor.[26] A cutoff value for serum samples was 3.5 U/mL, according to the manufacturer's recommendation.

β$_2$GPI preparations and synthetic peptides

Recombinant maltose-binding protein (MalBP) fusion proteins encoding β$_2$GPI amino acid sequences were prepared and used as antigens for T-cell stimulation.[21] These included GP-F encoding the entire amino acid sequence of β$_2$GPI (amino acid residues 1-326, reported by Matsuura et al[27]); GP1, encoding domains I and II (amino acid residues 1-133); GP2, encoding domains III and IV (amino acid residues 119-254); and GP3, encoding domains IV and V (amino acid residues 182-326). MalBP was also prepared and used as a control antigen. Human β$_2$GPI purified from pooled human plasma was provided by Yamasa, and native and reduced β$_2$GPI were prepared as described elsewhere.[21]

Fifteen 15-mer peptides with 9- or 10-residue overlaps encompassing the entire domain V of β$_2$GPI were synthesized using a solid-phase multiple synthesizer (Advanced Chemtech, Louisville, KY). The purity of all peptide preparations was more than 50%, as determined by high-performance liquid chromatography.

All β$_2$GPI preparations at a concentration of 20 μg/mL were confirmed not to interfere with T-cell proliferation induced by phytohemagglutinin (PHA).

Establishment of β$_2$GPI-specific T-cell clones

The β$_2$GPI-specific T-cell clones were generated according to a previously described method[28] with some modifications. Briefly, peripheral blood mononuclear cells (PBMNCs) were isolated from heparinized venous blood by Lymphoprep (Nycomed Pharma, Oslo, Norway) density-gradient centrifugation. The cells (2 × 10^6/well) were cultured in 24-well plates with GP-F (10 μg/mL) in RPMI 1640 supplemented with 8% autologous heat-inactivated plasma, 2 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 50 U/mL penicillin, and 50 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. On day 3, 30 U/mL interleukin (IL) 2 was added to the culture. Cells were restimulated with GP-F (10 μg/mL), IL-2 (100 U/mL), and 10^6 irradiated (40 Gy) autologous PBMNCs in fresh medium at day 10. Seven days after the second stimulation, T-cell blasts were cloned by limiting dilution at 0.3 cells/well using round-bottomed 96-well plates in the presence of GP-F, IL-2, and irradiated PBMNCs. After an additional stimulation, growth-positive wells were selected and the cells in these wells were expanded by a repeated stimulation with GP-F, IL-2, and autologous Epstein-Barr virus–transformed lymphoblastoid B-cell line cells irradiated at 100 Gy. The specificity of each T-cell clone was assessed by antigen-induced T-cell proliferation, and T-cell clones that proliferated in response to GP-F, but not to MalBP, were selected as β$_2$GPI-specific T-cell clones. T-cell clones were maintained in cultures by stimulation with GP-F, IL-2, and irradiated autologous B-cell line cells at 7- to 10-day intervals. The cell surface expression of CD4 and CD8 was examined by flow cytometry using fluorescein isothiocyanate–conjugated mAbs to CD4 and CD8 (Becton Dickinson, San Jose, CA), respectively.

T-cell proliferation assay

The antigen-specific proliferation of T-cell clones was determined principally as described previously.[28] The T-cell clones (2 × 10^4/well) were cultured with irradiated autologous B-cell line cells (2 × 10^4/well) and antigen, including GP-F, GP1, GP2, GP3, MalBP, native β$_2$GPI, and reduced β$_2$GPI (10 μg/mL) as well as tetanus toxoid (TT; List Biological Laboratories, Campbell, CA; 5 μg/mL). PHA (1 μg/mL) was also used to exclude nonspecific unresponsiveness. L cells transfected with the *DRA* gene and one of the following *DRB* genes, DRB1*1501 (LDR2B), *1502 (LARB1-1212), *0403 (B19), *0901 (L/KR1-3-A10), DRB4*0103 (L17.8), DRB5*0101 (LDR2A), and *0102 (AB5), were also used as antigen-presenting cells (APCs). These cell lines were distributed by the 11th International Histocompatibility Workshop.[29] L cells transfected with the *Neo^r* gene alone were used as a control. All transfectants were confirmed to express human HLA-DR molecules by flow cytometry before use in the assay. L cells were irradiated at 40 Gy and incubated with synthetic peptides (5 μg/mL unless indicated otherwise) for 2 hours before mixing with T-cell clones. After 60 hours of incubation with antigen, 0.5 μCi/well ^{3}H-thymidine was added to the cultures for 16 hours. The cells were then harvested and ^{3}H-thymidine incorporation was determined in a Top-Count microplate scintillation counter (Packard, Meriden, CT). All cultures were carried out in duplicate or triplicate, and all values represent the mean of duplicate or triplicate determinations.

The HLA class II restriction of individual T-cell clones was determined based on the inhibitory effects of anti-HLA-DR (L243; IgG2a), anti-HLA-DQ (1a3; IgG2a), and anti-HLA-DP (B7/21; IgG3) mAbs (1 μg/mL; Leinco Technologies, Ballwin, MO) on GP-F–induced T-cell proliferation.

Analysis of in vitro anti-β$_2$GPI antibody production

The helper activity of β$_2$GPI-specific T-cell clones was evaluated using an in vitro culture system in which β$_2$GPI-specific T-cell clones (3 × 10^5 cells) were cultured with autologous peripheral blood B cells (3 × 10^5 cells) in the presence or absence of antigen (GP-F, MalBP, or TT; 10 μg/mL) and pokeweed mitogen (1 μg/mL) for 10 days.[30] B cells were obtained from nonadherent PBMNCs by 2 purifications using nylon wool columns, followed by the depletion of contaminating T cells by incubation with anti-CD4 and anti-CD8 mAb-coupled magnetic beads (Dynal, Oslo, Norway).[30] Culture supernatants were then harvested and the anti-β$_2$GPI antibody levels were measured using an anti-β$_2$GPI ELISA. All samples were tested in duplicate, and results were expressed as the mean of the duplicate values minus the mean of the reference blank values.

The effects of blocking T- and B-cell interactions on in vitro anti-β$_2$GPI antibody production were determined by the addition of anti-HLA-DR,

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Table 1. Clinical and laboratory findings, HLA class II alleles, β_2GPI genotypes, and the number of β_2GPI-reactive CD4$^+$ T cell clones generated in patients with APS

Patient	Age/sex	Clinical findings		Anti-β_2GPI antibody* (U/mL)	HLA class II alleles					β_2GPI genotype at position 247†	Number of β_2GPI-specific T-cell clones
		Thrombosis	Pregnancy loss		DRB1	DRB4	DRB5	DQB1	DPB1		
O.M.	39/F	Stroke	+	> 125.0	*1501/*0403	*0103	*0101	*0602/*0302	*0201	^{247}L/^{247}L	4
E.Y.	38/F	DVT, PE	N/A	92.9	*0901	*0103	—	*0303	*0201	^{247}V/^{247}L	4
K.S.	38/F	Stroke	+	> 125.0	*1502/*0901	*0103	*0102	*0601/*0303	*0501/*0901	^{247}V/^{247}L	6

DVT indicates deep venous thrombosis of lower extremity; PE, pulmonary embolism; N/A, not applicable.

*Normal range < 3.5 U/mL.

†A valine (^{247}V) to leucine (^{247}L) amino acid dimorphism was determined by *Rsa*I digestion of the PCR-amplified DNA.

anti-HLA-DQ, anti-HLA-DP (1 μg/mL), anti-CD40 ligand (CD40L; 1 μg/mL; IgG1) (Ancell, Bayport, MN), anti-IL-6 (25 μg/mL); IgG1 or anti-interferon-γ (IFN-γ) mAb (25 μg/mL unless indicated otherwise; IgG2a) (Genzyme, Cambridge, MA). In some experiments, exogenous IL-4 or IL-6 (Life Technologies, Grand Island, NY) was added to the cultures. All mAbs and exogenous cytokines were added at the initiation of the cultures.

Cytokine production assay

The β_2GPI-specific T-cell clones were stimulated with PHA (1 μg/mL) and anti-CD3 mAb (OKT3; 30 ng/mL) for 48 hours. Culture supernatants were collected, and amounts of human IFN-γ, IL-4, IL-6, and IL-10 were measured in duplicate using ELISA kits (Biosource International, Camarillo, CA) according to the manufacturer's instruction.

Statistical analysis

Proliferation of T cells, anti-β_2GPI antibody, and cytokine data were summarized with means ± SDs. Statistical comparisons were performed using Student *t* tests for independent samples. A correlation between anti-β_2GPI antibody levels produced in vitro and the amounts of individual cytokines in supernatants was examined using a single regression model. *P* less than.05 was regarded as a significant difference.

Results

Establishment of β_2GPI-specific T-cell clones

A total of 14 T-cell clones specific to β_2GPI were generated from 3 patients who had typical clinical features of APS and a high level of serum anti-β_2GPI antibodies (Table 1). The clonality was confirmed in 7 lines based on the observation that each had only one type of functionally rearranged T-cell receptor β chain (data not shown). As shown in Figure 1, representative T-cell clones OM3, OM8, EY3, and KS7 proliferated in response to GP-F, but not to MalBP. Reduced β_2GPI induced a proliferative response in OM3, EY3, and KS7, but not in OM8. In addition, OM3 responded to GP1, OM8 and EY3 responded to GP3, and KS7 responded to both GP2 and GP3. Table 2 summarizes the surface phenotype, T-cell responses to various β_2GPI preparations, and HLA class II restriction for the 14 β_2GPI-specific T-cell clones. All T-cell clones had a CD4$^+$CD8$^-$ helper phenotype. None of the clones responded to native β_2GPI, but all except OM8 responded to reduced β_2GPI. Eleven T-cell clones generated from the 3 patients responded to GP3 (domains IV and V), but not to GP2 (domains III and IV). The β_2GPI-specific T-cell clones with this antigenic specificity recognized epitope(s) present within domain V in the context of HLA-DR. OM3 responded to GP1 (domains I and II) in an HLA-DP–restricted manner. KS7 and KS10 proliferated in response to both GP2 and GP3 in an HLA-DR–restricted manner and these T- cell clones appeared to recognize domain IV.

Determination of T-cell epitopes within domain V

Because the majority of the β_2GPI-specific CD4$^+$ T-cell clones recognized domain V, T-cell epitopes within domain V were further analyzed using 15 synthetic peptides spanning the entire domain. As shown in Figure 2, OM2 responded to p276 to 290 (KVSFFCKNKEKKCSY), which contains the major PL-binding site. T-cell proliferation induced by p276 to 290 at 5 μg/mL was weak, but was enhanced by higher peptide concentrations in a dose-dependent fashion (data not shown). In contrast, OM8 recognized another peptide, p247 to 261 (VPVKKATVVYQGERV). To identify HLA-DR molecules that present antigenic peptides to OM2 and OM8, a panel of L-cell transfectants expressing single HLA-DR molecules was used as APCs in the presence or absence of antigenic peptides (Figure 3). Patient O.M. possessed 4 different HLA-DRB alleles, including DRB1*1501, *0403, DRB4*0103, and DRB5*0101 (Table 1). OM2 responded to p276 to 290 presented by DRB4*0103$^+$ L cells, but not to p276 to 290 in the presence of L cells expressing DRB1*1501, *0403, or DRB5*0101. In contrast, OM8 responded to p247 to 261 presented by both DRB1*0403$^+$ L cells and DRB4*0103$^+$ L cells, indicating that OM8 was able to respond to p247 to 261 in the context of 2 different HLA-DR molecules. Because the clonality of OM8 was not confirmed, the possibility that OM8 consisted of 2 or more clones was not excluded. The specificity of these responses was confirmed by the specific inhibition of the peptide-induced T-cell proliferation by anti-HLA-DR mAb. The T-cell epitope peptide and HLA restriction were further examined in 5 additional domain V-reactive T-cell clones, OM7, EY3, EY8, KS3, and KS5, all of which were found to recognize p276 to 290 in the context of the

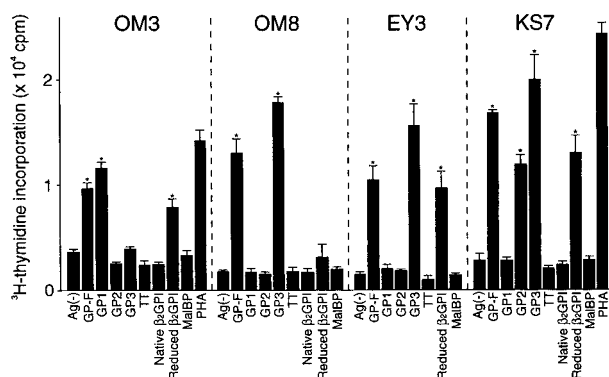

Figure 1. Proliferative responses of β_2GPI-specific CD4$^+$ T cell clones to various β_2GPI preparations. T-cell clones were cultured with autologous APCs for 3 days in medium alone or in medium supplemented with GP-F, GP1, GP2, GP3, native β_2GPI, reduced β_2GPI, MalBP (10 μg/mL), TT (5 μg/mL), or PHA (1 μg/mL), and antigen-induced T-cell proliferation was measured by ^{3}H-thymidine incorporation. Significant T-cell proliferation to recombinant β_2GPI or reduced β_2GPI in comparison with the respective controls is shown as an asterisk. A representative result of 3 independent experiments is shown.

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Table 2. Surface phenotype, T-cell proliferative responses to various β_2GPI preparations, predicted β_2GPI domain containing the epitope, and HLA class II restriction in 14 β_2GPI-specific CD4$^+$ T-cell clones

T-cell clones	Surface phenotype	Antigen-induced T-cell proliferative response					Predicted β_2GPI domain containing epitope	HLA class II restriction*
		Native β_2GPI	Reduced β_2GPI	GP1	GP2	GP3		
Patient O.M.								
OM2	CD4$^+$CD8$^-$	−	+	−	−	+	V	HLA-DR
OM3	CD4$^+$CD8$^-$	−	+	+	−	−	I/II	HLA-DP
OM7	CD4$^+$CD8$^-$	−	+	−	−	+	V	HLA-DR
OM8	CD4$^+$CD8$^-$	−	−	−	−	+	V	HLA-DR
Patient E.Y.								
EY3	CD4$^+$CD8$^-$	−	+	−	−	+	V	HLA-DR
EY8	CD4$^+$CD8$^-$	−	+	−	−	+	V	HLA-DR
EY9	CD4$^+$CD8$^-$	−	+	−	−	+	V	HLA-DR
EY12	CD4$^+$CD8$^-$	−	+	−	−	+	V	HLA-DR
Patient K.S.								
KS3	CD4$^+$CD8$^-$	−	+	−	−	+	V	HLA-DR
KS4	CD4$^+$CD8$^-$	−	+	−	−	+	V	HLA-DR
KS5	CD4$^+$CD8$^-$	−	+	−	−	+	V	HLA-DR
KS7	CD4$^+$CD8$^-$	−	+	−	+	+	IV	HLA-DR
KS8	CD4$^+$CD8$^-$	−	+	−	−	+	V	HLA-DR
KS10	CD4$^+$CD8$^-$	−	+	−	+	+	IV	HLA-DR

*Determined based on blocking experiments using anti-HLA-DR, anti-HLA-DQ, and anti-HLA-DP mAbs.

DRB4*0103 allele. Four T-cell clones responsive to domain V were not available because they died out before these assays were performed.

Helper activity and cytokine profiles in β_2GPI-specific CD4$^+$ T-cell clones

Anti-β_2GPI antibody levels were measured in the supernatants of cultures containing individual β_2GPI-specific CD4$^+$ T-cell clones and autologous B cells stimulated with GP-F, MalBP, or TT (Figure 4). The β_2GPI-specific T-cell clones OM2, OM3, and EY3 induced the production of anti-β_2GPI antibodies in response to GP-F, but anti-β_2GPI antibody synthesis was not observed in cultures without antigen or in cultures with irrelevant antigen. As summarized in Table 3, 10 of 12 β_2GPI-specific T-cell clones were able to promote anti-β_2GPI antibody production, whereas the remaining 2 (OM8 and EY9) lacked the helper activity. When the expression of cytokines, including IFN-γ, IL-4, IL-6, and IL-10, was examined in the 12 β_2GPI-specific T-cell clones, individual T-cell clones were

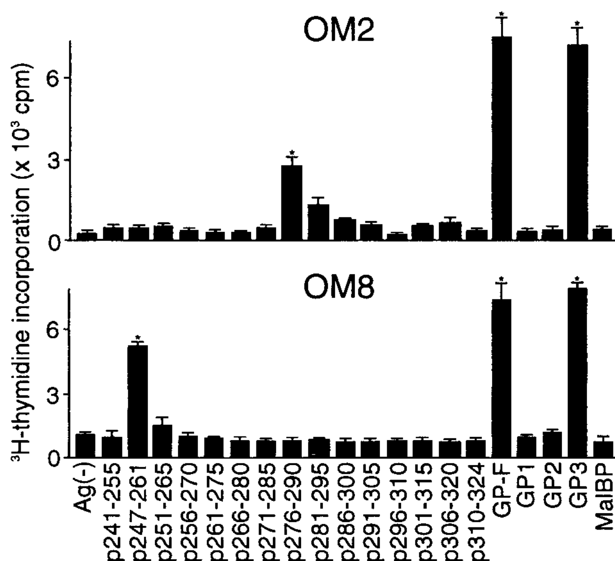

Figure 2. Proliferative responses of β_2GPI-specific CD4$^+$ T-cell clones to 15 synthetic peptides covering the entire domain V. The β_2GPI-specific CD4$^+$ T-cell clones OM2 and OM8 were cultured with autologous APCs for 3 days in medium alone or in medium supplemented with individual synthetic peptides (5 μg/mL), and peptide-induced T-cell proliferation was measured by ^{3}H-thymidine incorporation. GP-F, GP1, GP2, GP3, and MalBP (10 μg/mL) were also used as antigens for T-cell proliferation. Significant T-cell proliferation to domain V peptide or recombinant β_2GPI in comparison with the respective controls is shown as an asterisk. Similar results were obtained in 4 independent experiments.

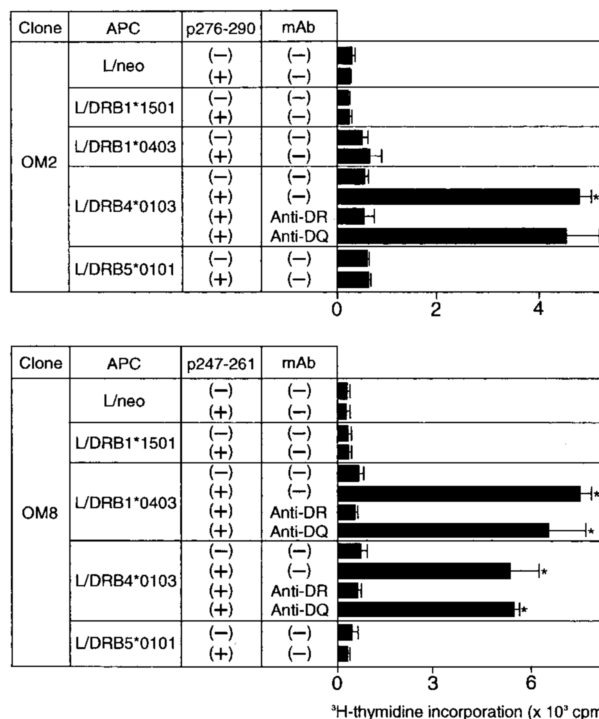

Figure 3. Peptide-induced proliferative responses of β_2GPI-specific CD4$^+$ T-cell clones incubated with L-cell transfectants expressing human HLA class II molecules. The β_2GPI-specific CD4$^+$ T-cell clones OM2 and OM8 were incubated with a series of L-cell transfectants in the presence or absence of p276 to 290 (10 μg/mL) and p247 to 261 (5 μg/mL), respectively. The peptide-induced T-cell proliferation was measured by ^{3}H-thymidine incorporation. In some experiments, T-cell clones were cultured with peptide-pulsed L cells in the presence of anti-HLA-DR or anti-HLA-DQ mAb (1 μg/mL). Significant T-cell proliferation in comparison with the control culture without antigenic peptide is shown as an asterisk. The results were similar in 2 independent experiments.

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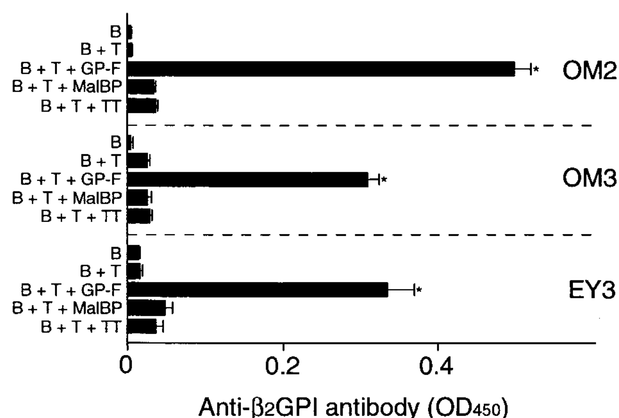

Figure 4. In vitro production of anti-β_2GPI antibodies in cultures of β_2GPI-specific CD4$^+$ T-cell clones and autologous B cells stimulated with GP-F. Peripheral blood B cells were cultured with β_2GPI-specific CD4$^+$ T-cell clones in the presence or absence of antigen (GP-F, MBP, or TT; 10 μg/mL) for 10 days. Anti-β_2GPI antibody levels in undiluted culture supernatants were measured by ELISA. Significant anti-β_2GPI antibody production in culture with antigen compared with the control culture without antigen is shown as an asterisk. A representative result of 3 independent experiments is shown.

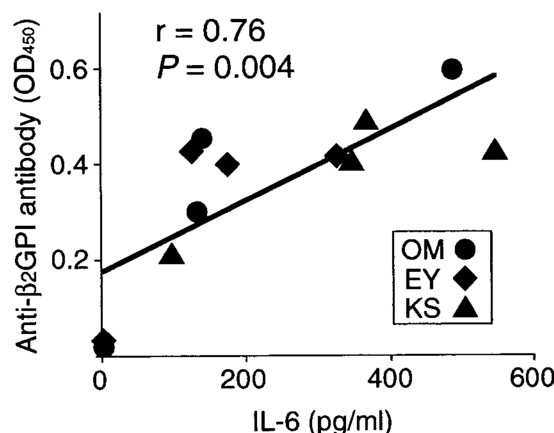

Figure 5. Correlation between anti-β_2GPI antibody levels produced in cultures and IL-6 expression in 12 β_2GPI-specific CD4$^+$ T cells. Anti-β_2GPI antibody levels produced in cultures of individual β_2GPI-specific T-cell clones plus autologous B cells are significantly correlated with amounts of IL-6 expressed on stimulation with PHA and anti-CD3 mAb ($r = 0.76$, $P = .004$).

found to express different sets of cytokines. Six β_2GPI-specific T-cell clones (OM8, EY3, EY8, EY9, KS4, and KS7) expressed IFN-γ, but expressed no or minimal amounts of IL-4. This pattern of cytokine expression is consistent with that of a Th1 subset. The remaining 6 clones expressed both IFN-γ and IL-4, a pattern of cytokine expression consistent with a Th0 phenotype. When the cytokine profiles of β_2GPI-specific T-cell clones were compared according to their ability to drive anti-β_2GPI antibody production, there was no correlation between the helper activity and the Th0/Th1 phenotype. Instead, it was evident that the 10 T-cell clones that provided help to B cells expressed IL-6, whereas the 2 clones lacking the helper activity did not express IL-6. Furthermore, the amounts of anti-β_2GPI antibody produced in the in vitro cultures were positively correlated with the IL-6 expression levels in β_2GPI-specific T-cell clones (Figure 5). In contrast, the levels of anti-β_2GPI antibody produced in vitro were not significantly correlated with the expression levels of IFN-γ, IL-4, or IL-10.

Role of IL-6 on in vitro anti-β_2GPI antibody production

To further examine the role of T-cell–derived IL-6 in the helper activity, the effects of anti-IL-6 and anti-IFN-γ mAbs on in vitro anti-β_2GPI antibody production were examined in cultures of β_2GPI-specific T-cell clones OM2 or KS3 with autologous B cells and GP-F (Figure 6). The effects of anti-HLA class II and anti-CD40L mAbs on the anti-β_2GPI antibody production were also tested. Anti-IL-6 mAb inhibited the anti-β_2GPI antibody production, but anti-IFN-γ mAb had no effect. Anti-β_2GPI antibody production was also blocked by anti-HLA-DR or anti-CD40L mAb. Similar results were obtained when β_2GPI-specific T-cell clones OM7 and EY3 were tested.

To examine whether the inability of some β_2GPI-specific T-cell clones to provide help to B cells could be due to the lack of endogenous IL-6 in cultures, the effect of exogenous IL-6 on in vitro anti-β_2GPI antibody production was examined using β_2GPI-specific T-cell clone EY9 that lacked the helper activity (Figure 7). Because EY9 lacked the expression of IL-4 in addition to IL-6, the

Table 3. Ability to induce in vitro anti-β_2GPI antibody production from autologous B cells and cytokine profiles in 12 β_2GPI-specific CD4$^+$ T-cell clones

T-cell clones	Anti-β_2GPI antibody levels in supernatants (OD_{450})*	Cytokine levels (pg/mL)†			
		IFN-γ	IL-4	IL-6	IL-10
Patient O.M.					
OM2	0.590 ± 0.022	880 ± 18	> 500	487 ± 27	525 ± 30
OM3	0.297 ± 0.014	1248 ± 22	191 ± 10	132 ± 7	< 1
OM7	0.450 ± 0.034	280 ± 11	305 ± 9	138 ± 5	720 ± 18
OM8‡	0.016 ± 0.002	560 ± 15	< 1	< 1	< 1
Patient E.Y.					
EY3	0.422 ± 0.034	380 ± 10	< 1	126 ± 16	316 ± 24
EY8	0.412 ± 0.024	468 ± 9	< 1	326 ± 25	461 ± 13
EY9‡	0.023 ± 0.003	746 ± 18	7 ± 1	< 1	262 ± 11
EY12	0.396 ± 0.031	1632 ± 39	> 500	175 ± 10	41 ± 5
Patient K.S.					
KS3	0.400 ± 0.028	490 ± 7	> 500	343 ± 25	371 ± 17
KS4	0.418 ± 0.041	1214 ± 32	13 ± 7	546 ± 5	74 ± 11
KS5	0.486 ± 0.020	824 ± 21	286 ± 16	366 ± 13	626 ± 22
KS7	0.209 ± 0.012	653 ± 29	< 1	97 ± 4	759 ± 30

*IgG anti-β_2GPI antibody levels were measured in supernatants of β_2GPI-specific T-cell clones cultured with autologous B cells in the presence of GP-F. Results are shown in mean ± SD. IgG anti-β_2GPI antibody levels in cultures of B cells alone were 0.011 ± 0.001 (O.M.), 0.012 ± 0.002 (E.Y.), and 0.001 ± 0.001 (K.S.).

†Individual cytokine levels were measured in culture supernatants of β_2GPI-specific T-cell clones stimulated with anti-CD3 mAb and PHA. Results are shown in mean ± SD.

‡T-cell clones lacking helper activity.

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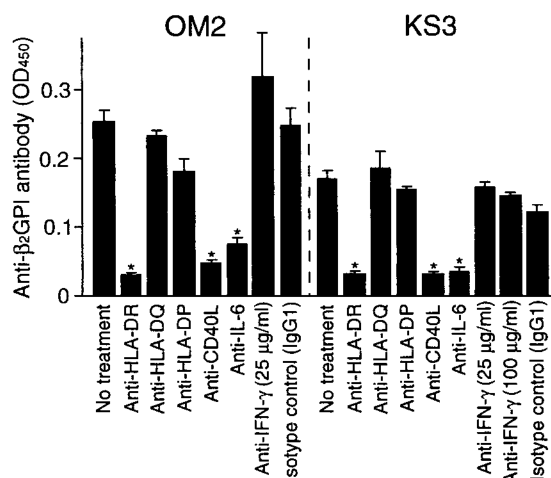

Figure 6. Effects of anti-HLA class II, anti-CD40L, anti-IL-6, and anti-IFN-γ mAbs on in vitro anti-β_2GPI antibody production. Autologous peripheral blood B cells were cultured with β_2GPI-specific $CD4^+$ T-cell clones (OM2 and KS3) and GP-F (10 μg/mL) in the presence of anti-HLA-DR, anti-HLA-DQ, anti-HLA-DP, anti-CD40L (1 μg/mL), anti-IL-6, or anti-IFN-γ (25 μg/mL unless indicated otherwise) mAbs for 10 days. Anti-β_2GPI antibody levels in undiluted culture supernatants were measured by ELISA. Significant inhibition of anti-β_2GPI antibody production by anti-HLA class II or anticytokine mAbs in comparison with the control culture with isotype control mAb is shown as an asterisk. Similar results were obtained in 3 independent experiments.

possible involvement of IL-4 in this process was also tested. B cells alone did not produce anti-β_2GPI antibody when stimulated with IL-4 or IL-6. IL-6 did induce anti-β_2GPI antibody production when it was added to the culture of B cells and EY9, but exogenous IL-4 had no effect. The IL-6–induced anti-β_2GPI antibody production was completely abolished when the cells were cultured with anti-HLA-DR or anti-CD40L mAb.

Discussion

This is the first report describing T-cell epitopes recognized by β_2GPI-specific $CD4^+$ T cells capable of inducing anti-β_2GPI antibody production in patients with APS. Our results obtained from 14 β_2GPI-specific $CD4^+$ T-cell clones generated from 3 patients with APS can be summarized as follows: (1) an immunodominant epitope on β_2GPI is located at p276 to 290, which corresponds to the major PL-binding site, although other minor determinants are also recognized by β_2GPI-specific T-cell clones; (2) T-cell recognition of p276 to 290 is restricted by DRB4*0103 (DR53); (3) the majority of β_2GPI-specific T-cell clones have the helper activity promoting anti-β_2GPI antibody production from B cells; and (4) both T cell-derived IL-6 and CD40-CD40L engagement are necessary for the anti-β_2GPI antibody production from B cells.

The β_2GPI-specific T-cell clones that recognized p276 to 290 in the context of DRB4*0103 were frequently generated from all 3 patients examined and had strong helper activity inducing anti-β_2GPI antibody production. These findings strongly suggest that autoreactive $CD4^+$ T cells carrying this antigenic specificity are primarily involved in anti-β_2GPI antibody production in many patients with APS. On the other hand, β_2GPI-specific T-cell clones responsive to the epitopes in domains I/II, IV, and p247 to 261 in domain V were also generated from patients with APS, indicating heterogeneous antigenic specificities in β_2GPI-reactive T cells in APS. T-cell clones responsive to the epitopes other than p276 to 290 may be of the minor repertoire or present only in a small number of APS patients because they were generated from a single patient in this study.

Recently, Ito and coworkers analyzed the T-cell epitopes on β_2GPI using β_2GPI-specific $CD4^+$ T-cell lines established from patients with APS and healthy individuals, by the repeated stimulation of peripheral blood T cells with a mixture of 40 synthetic peptides covering the entire amino acid sequence of β_2GPI.[31] They identified 4 distinct epitopes, including p64 to 83, p154 to 174, p226 to 246, and p244 to 264. The majority of the β_2GPI-reactive T-cell lines responded to p244 to 264, which was also recognized in this study by β_2GPI-specific T-cell clone OM8. However, Ito and coworkers did not generate T-cell lines that were reactive with the peptide containing the major PL-binding site. The reason for this difference in the epitope recognition is unclear, but it is possible that the antigenic peptide containing the major PL-binding site was not efficiently presented to T cells in the context of the HLA-DR molecule in their cultures, because synthetic peptides containing the major PL-binding site have been shown to bind negatively charged PLs,[8,32] which are thought to be expressed in abundance on the surfaces of apoptotic cells in cultures. In fact, T-cell clones reactive to p276 to 290 required a much higher peptide concentration to respond in the proliferation assay, compared with the β_2GPI-specific T-cell clone OM8 response to p247 to 261. In contrast, in our study, β_2GPI-specific T-cell clones were established in response to the peptides naturally processed from recombinant β_2GPI.

It is interesting to note that p276 to 290 includes the epitopes recognized by anti-β_2GPI antibodies in the sera from some patients with APS[33] and by anti-β_2GPI mAbs derived from a patient with APS.[34] Therefore, both T- and B-cell epitopes are located in the vicinity of and included in p276 to 290, although the major B-cell epitopes on β_2GPI are located in domain I[35] or IV[36] or both. In addition, Gharavi and colleagues reported that the immunization of normal mice with a peptide encompassing amino acid residues 274 to 288, which contains the major PL-binding site of human β_2GPI, conjugated to bovine serum albumin induced the production of anti-β_2GPI antibodies possessing thrombotic properties.[37,38] Because the amino acid sequence of this region is highly conserved between human and mouse, an immunodominant region on β_2GPI that induces the production of pathogenic anti-β_2GPI antibodies may be shared by patients with APS and experimental mouse models for APS.

This study demonstrates that DR53 is a restricting element in

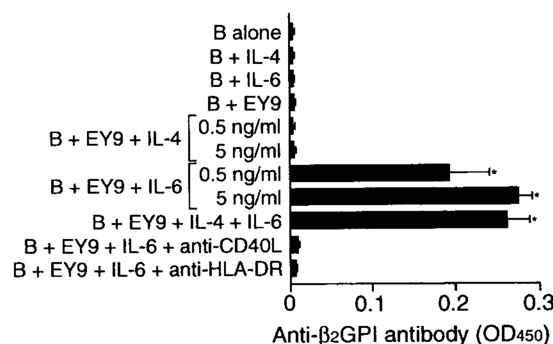

Figure 7. Effect of exogenous IL-4 and IL-6 on in vitro anti-β_2GPI antibody production. Peripheral blood B cells from patient E.Y. were cultured with or without β_2GPI-specific $CD4^+$ T-cell clone EY9 in the presence of GP-F (10 μg/mL) plus exogenous IL-4, IL-6, or both IL-4 and IL-6 (5 ng/mL unless indicated otherwise) for 10 days. In some experiments, anti-HLA-DR (1 μg/mL) or anti-CD40L (1 μg/mL) mAb was added to the cultures supplemented with IL-6 as indicated. Anti-β_2GPI antibody levels in undiluted culture supernatants were measured by ELISA. Significant anti-β_2GPI antibody production in culture with exogenous cytokine compared with the control culture without cytokine is shown as an asterisk. The results were similar in 2 independent experiments.

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the presentation of the immunodominant p276 to 290 to β_2GPI-specific T cells. This finding may explain the previously reported associations of anti-β_2GPI antibodies with DR4, DR7, and DR9 haplotypes,[39-41] all of which are in linkage disequilibrium with DR53. Moreover, $CD4^+$ T-cell responses to β_2GPI in bulk PBMNC cultures are associated with the presence of DR53 in patients with APS and healthy individuals.[21] The DR53-specific peptide-binding motif is composed of a positively charged residue at positions 1 and 9, and a hydrophobic residue at positions 4 and 10.[42] For the binding of p276 to 290 to the DR53 molecule, ^{276}K, ^{279}F, and ^{284}K are likely to be the 1st, 4th, and 9th DR anchors, respectively, whereas the 10th anchor is absent.

At least 4 different allelic polymorphisms have been reported in the *β_2GPI* gene, and these residues include position 88 in domain II and positions 247, 306, and 316 in domain V.[25,43] A valine-leucine dimorphism at position 247 (^{247}V-^{247}L) was included in the epitope peptide recognized by the β_2GPI-specific T-cell clone OM8. Analysis of the β_2GPI genotype at position 247 revealed that patient OM had a *β_2GPI* gene that was homozygous for ^{247}L. Because the recombinant β_2GPI fragments and synthetic peptides used to stimulate T cells had a valine residue at position 247, it is possible that OM8 was generated due to alloreactivity. In this regard, OM8 had a typical Th1 phenotype and lacked helper activity. However, it is also possible that OM8 is an autoreactive T cell to β_2GPI with ^{247}L and that it cross-reacts with β_2GPI containing ^{247}V, because valine and leucine residues have similar aliphatic and hydrophobic characteristics.

All β_2GPI-specific $CD4^+$ T-cell clones produced IFN-γ and had a Th1- or Th0-like cytokine expression profile, but our results indicated that IFN-γ was not involved in the B-cell activation leading to anti-β_2GPI antibody production. Visvanathan and colleagues have recently reported that Th1 cells responsive to β_2GPI activate monocytes to produce tissue factor through IFN-γ synthesis[44,45]; therefore, the β_2GPI-specific T-cell clones generated in this study could mediate the production of tissue factor from monocytes. However, in the reports by Visvanathan and coworkers, T cell-dependent monocyte activation was induced by β_2GPI in its native form, which stimulated none of our β_2GPI-specific T-cell clones. Although the helper activity inducing anti-β_2GPI antibody production in $CD4^+$ T cells reactive with native β_2GPI was not examined in their reports, it is possible that anti-β_2GPI antibody synthesis from B cells and tissue factor production from monocytes are mediated by a different population of β_2GPI-reactive $CD4^+$ T cells.

The mechanisms for the T- and B-cell collaboration regulating the production of anti-β_2GPI antibodies were examined using an in vitro assay system consisting of β_2GPI-specific $CD4^+$ T-cell clones and autologous B cells. Our results identified IL-6 as a major B cell-activating factor produced by β_2GPI-specific $CD4^+$ T cells that promotes anti-β_2GPI antibody production, because (1) IL-6 expression was detected exclusively in the β_2GPI-specific T-cell clones capable of driving anti-β_2GPI antibody production; (2) the levels of anti-β_2GPI antibody produced in vitro were positively correlated with the expression levels of IL-6; (3) neutralization of IL-6 by anti-IL-6 mAb inhibited the in vitro anti-β_2GPI antibody production; and (4) exogenous IL-6 augmented the helper function of β_2GPI-specific T-cell clone lacking IL-6 expression. A primary role of T-cell–derived IL-6 in autoantibody production is analogous to findings in our previous study on the antitopoisomerase I antibody response in patients with scleroderma.[30] However, the effect of IL-6 required the CD40-CD40L engagement, because IL-6 alone did not induce anti-β_2GPI antibody production and anti-CD40L mAb almost completely blocked anti-β_2GPI antibody production induced by IL-6. Therefore, $CD4^+$ T cell-dependent B-cell activation depends on 2 types of stimuli: CD40-CD40L engagement and T-cell–derived IL-6.

The β_2GPI-specific $CD4^+$ T-cell clones responded to reduced β_2GPI and recombinant β_2GPI fragments produced in bacteria, but none of them responded to native β_2GPI. Taken together with the fact that β_2GPI-reactive T cells are present in some healthy individuals,[21,32] it is likely that the epitopes recognized by β_2GPI-specific T cells are cryptic determinants that are not produced from native β_2GPI under normal circumstances. Lehmann and coworkers proposed that a pathogenic autoreactive T-cell response is induced by the de novo presentation of a previously cryptic self-determinant under special conditions.[46] Cryptic self-peptides can be revealed due to factors that affect normal antigen processing such as structural modification of self-antigens due to an unusual cleavage event or the formation of a complex with ligands.[47,48] Such modifications are thought to mask or unmask cleavage sites for proteases and reductases in endosomes, resulting in the expression of cryptic self-peptides. The factors that induce the expression of cryptic determinants on β_2GPI that result in the activation of β_2GPI-reactive T cells in patients with APS are unknown, but several lines of evidence suggest that cryptic epitopes are revealed when β_2GPI is complexed with anionic surfaces. For example, PL-bound β_2GPI, but not PL or β_2GPI alone, induces a high level of anti-β_2GPI antibodies and lupus anticoagulant activity in normal mice without adjuvant.[49] Moreover, immunization of β_2GPI-bound apoptotic cells into normal mice induces the production of pathogenic anti-β_2GPI antibodies.[50] The major PL-binding site recognized by the majority of β_2GPI-specific T-cell clones has a positively charged sequence located on a surface-exposed turn[11] and is presumed to be easily accessed by proteases during antigen processing. Therefore, β_2GPI binding to anionic surfaces, which protects the major PL-binding site from protease attack, could induce the appearance of the previously cryptic peptide containing the PL-binding site. Further studies examining the conditions that reveal the cryptic epitope within p276 to 290 could provide a clue to the pathogenesis of the induction of the anti-β_2GPI antibody response in patients with APS.

In addition, this study provides novel information that is potentially useful in developing therapeutic strategies that suppress pathogenic anti-β_2GPI antibody production in patients with APS. It has been shown that manipulation of autoreactive $CD4^+$ T-cell responses can be achieved by altered peptide ligands[51] or induction of regulatory T cells,[52] when immunodominant T-cell epitope and its restricting element are already known. Furthermore, IL-6 and CD40L that mediate the T-cell helper function inducing anti-β_2GPI antibody production are candidate targets for biologic agents. In fact, humanized antibodies to IL-6 receptor and those to CD40L are already manufactured and used in clinical trials in several autoimmune diseases.[53,54] Further studies should be done to evaluate the effectiveness of these potential therapies for patients with APS who are resistant to anticoagulation.

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