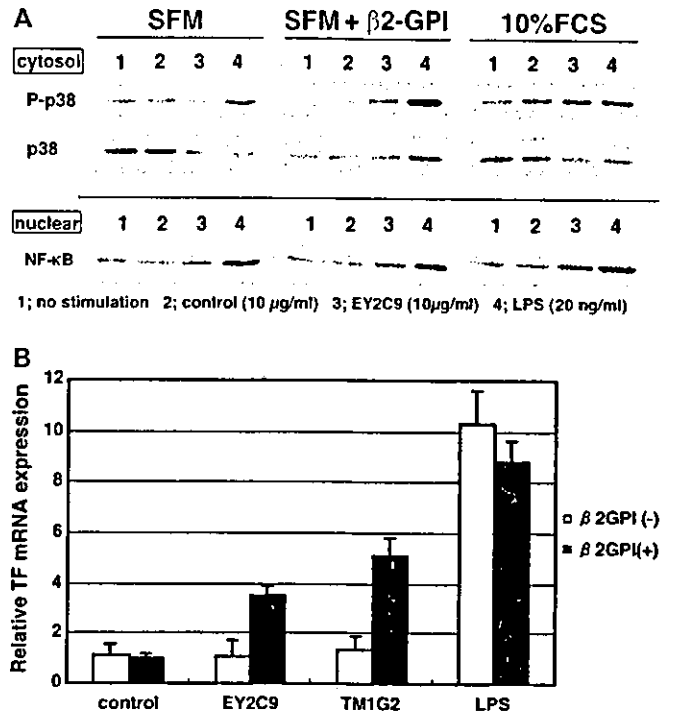


**Fig. 4.** Effect of p38 inhibitor on response to monoclonal IgM aCL/ $\beta_2$ GPI. **A.** RAW264.7 cells were treated with EY2C9 or control IgM antibody, in the presence or absence of SB203580 (200 nM) or SB202474 (200 nM) for 30 min. The phosphorylation of p38 was determined by western blotting. **B.** RAW264.7 cells were treated with EY2C9 or TM1G2 or control IgM antibody, in the presence or absence of SB203580 (200 nM) or SB202474 (200 nM) for 4 h. TF mRNA levels were determined using real-time PCR and demonstrated fold increase of stimulated/unstimulated cells. Each column represents the average  $\pm$  SD of three independent experiments.

Activation of p38 MAPK increases activities of pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ . Up-regulation of TNF- $\alpha$ , IL-1 $\beta$  and macrophage inflammatory protein 3 $\beta$  (MIP3 $\beta$ ) was also found in the present study (Figs 1C and 2B and C). Downstream of activated p38 MAPK, MAPKAPK-2/3 is a substrate for p38 that undergoes post-transcriptional regulation of TNF- $\alpha$ . p38 also activates transcriptional factors such as activating transcription factor-2, which forms a heterodimer with JUN family transcriptional factors and associates with the activator protein-1 (AP-1)-binding site. After LPS stimulation of dendritic cells, NH<sub>2</sub>-termini of histone H3 undergo structural alteration in a p38-dependent pathway, which results in enhancement of accessibility of the cryptic NF- $\kappa$ B-binding sites (56). The promoter region of the TF gene contains two AP-1-binding sites and one NF- $\kappa$ B-binding site, and these transcription factors are proven required for maximal induction of TF gene transcription. Moreover, p38 MAPK pathway has been implicated in the regulation of TF expression in monocytes, endothelial cells and smooth muscle cells (57–61).



**Fig. 5.**  $\beta_2$ GPI dependency of monoclonal aCL/ $\beta_2$ GPI stimulation. **A.** Serum-free medium-adapted RAW264.7 were treated with EY2C9 or control IgM antibody, in the absence/presence of  $\beta_2$ GPI (50  $\mu$ g ml<sup>-1</sup>) or FCS (10%) for 30 min. The phosphorylation of p38 and nuclear/cytoplasmic localization of NF- $\kappa$ B were determined by western blotting. 1, no stimulation. 2, control (10  $\mu$ g ml<sup>-1</sup>). 3, EY2C9 (10  $\mu$ g ml<sup>-1</sup>). 4, LPS (20 ng ml<sup>-1</sup>). **B.** RAW264.7 were treated with EY2C9, TM1G2 or control IgM antibody, in the absence/presence of  $\beta_2$ GPI (50  $\mu$ g ml<sup>-1</sup>) or FCS (10%) for 4 h. TF mRNA levels were determined using real-time PCR and demonstrated fold increase of stimulated/unstimulated cells. Each column represents the average  $\pm$  SD of three separate experiments.

In the present study, we have shown that stimulation by monoclonal aCL/ $\beta_2$ GPI induced phosphorylation of p38, locational shift of NF- $\kappa$ B into the nucleus and up-regulation in TF expression. TF expression induced by aCL/ $\beta_2$ GPI occurred only in the presence of  $\beta_2$ GPI, suggesting that perturbation of monocytes by aCL/ $\beta_2$ GPI is initiated by interaction between the cell and the auto-antibody-bound  $\beta_2$ GPI. It remains to be determined how aCL/ $\beta_2$ GPI bind  $\beta_2$ GPI on the cell surface and how signal transduction events occur upstream of p38. Using endothelial cells, Raschi *et al.* (62) reported that the dominant negative construct of TRAF6 and that of myeloid differentiation protein 88 (MyD88) abrogated the NF- $\kappa$ B activation induced by monoclonal aCL/ $\beta_2$ GPI as well as that induced by IL-1 or LPS. They proposed that aCL/ $\beta_2$ GPI react with  $\beta_2$ GPI likely associated to a member of the Toll-like receptor (TLR)/IL-1 receptor family. The results of our study are compatible with this report; firstly, in our cDNA array experiment, the expression levels of the members of the MyD88 signaling pathway such as interleukin-1 receptor associated kinase 1 (IRAK-1), TRAF6 and I $\kappa$ B kinase  $\alpha$ ,  $\beta$  and  $\gamma$  were up-regulated after treatment with monoclonal aCL/ $\beta_2$ GPI (Fig. 1) and, secondly, TRAF6 activates MEK3 and MEK6 which are the kinases upstream of p38 and JNK via activation of a MAPKKK called transforming growth factor- $\beta$ -activated kinase. The different

behaviors in p38, ERK and JUN activation between aPL and IL-1/TLR suggest that the signal/receptor structure of aCL/ $\beta_2$ GPI should be different from that of IL-1/TLR.

Several inhibitors for p38 have been developed and investigated in animal models of inflammatory diseases, and recently some of these inhibitors, such as BIRB796 or RWJ67657, are under clinical trials. Administration of SB203580 was beneficial in a murine model of endotoxin-induced shock and collagen-induced arthritis in mice. BIRB796 inhibited endothelial activation after administration of LPS in humans (63, 64), and RWJ67657 inhibited TNF- $\alpha$ , IL-8 and IL-6 in humans without significant adverse effects (65).

Strategies are focused on preventing the induction of pro-coagulant substances by aPL. New lipophilic statins, such as fluvastatin and simvastatin, can inhibit endothelial cell activation induced by aCL/ $\beta_2$ GPI (66, 67) and also the TF up-regulation on endothelial cells can be inhibited by these drugs, providing an additional therapeutic tool for treatment of thrombosis in APS. Recently, dilazep, an anti-platelet agent, was reported to reduce TF induction by aPL *in vitro* (48). In addition, our findings give a clue to establish more specific treatments by down-regulating the p38 MAPK pathway, presumably contributing to better management of the affected patients.

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#### Abbreviations

aCL	anti-cardiolipin antibodies
aCL/ $\beta_2$ GPI	$\beta_2$ GPI-dependent anti-cardiolipin antibodies
AP-1	activator protein-1
aPL	anti-phospholipid antibodies
APS	anti-phospholipid syndrome
FVIIa	activated factor VII
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
$\beta_2$ GPI	$\beta_2$ Glycoprotein I
MAPK	mitogen-activated protein kinase
MAPKAPK	MAPK-activated protein kinase
MyD88	myeloid differentiation protein 88
NF- $\kappa$ B	nuclear factor $\kappa$ B
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TLR	Toll-like receptor
TNF- $\alpha$	tumor necrosis factor-alpha
TRAF6	TNF receptor-associated factor 6

#### References

- Atsumi, T., Matsuura, E. and Koike, T. 2004. Immunology of anti-phospholipid antibodies and cofactors. In Lahita, R., ed., *Systemic Lupus Erythematosus*, 4th edn. Academic Press, San Diego, CA.
- Harris, E., Gharavi, A. and Boey, M. 1983. Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. *Lancet* 11:1211.
- Hughes, G. 1983. Thrombosis, abortion, cerebral disease and lupus anticoagulant. *Br. Med. J.* 287:1088.
- Harris, E. N., Gharavi, A., Patel, S. and Hughes, G. 1987. Evaluation of the anti-cardiolipin antibody test: report of an international workshop held 4 April 1986. *Clin. Exp. Immunol.* 68:215.
- Koike, T., Sueishi, M., Funaki, H., Tomioka, H. and Yoshida, S. 1984. Antiphospholipid antibodies and biological false positive serological test for syphilis in patients with systemic lupus erythematosus. *Clin. Exp. Immunol.* 56:193.
- McNeil, H. P., Simpson, R. J., Chesterman, C. N. and Krilis, S. A. 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. USA* 87:4120.
- Galli, M., Comfurius, P., Maassen, C. *et al.* 1990. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. *Lancet* 335:1544.
- Matsuura, E., Igarashi, Y., Fujimoto, M., Ichikawa, K. and Koike, T. 1990. Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. *Lancet* 336:177.
- Matsuura, E., Igarashi, Y., Yasuda, T., Triplett, D. A. and Koike, T. 1994. Anticardiolipin antibodies recognize beta 2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. *J. Exp. Med.* 179:457.
- Igarashi, M., Matsuura, E., Igarashi, Y. *et al.* 1996. Human beta2-glycoprotein I as an anticardiolipin cofactor determined using mutants expressed by a baculovirus system. *Blood* 87:3262.
- Matsuura, E., Igarashi, Y., Fujimoto, M. *et al.* 1992. Heterogeneity of anticardiolipin antibodies defined by the anticardiolipin cofactor. *J. Immunol.* 148:3885.
- Hunt, J. and Krilis, S. 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.
- Roubey, R. and Hoffman, M. 1997. From antiphospholipid syndrome to antibody-mediated thrombosis. *Lancet* 350:1491.
- Ieko, M., Ichikawa, K., Triplett, D. A. *et al.* 1999. Beta2-glycoprotein I is necessary to inhibit protein C activity by monoclonal anticardiolipin antibodies. *Arthritis Rheum.* 42:167.
- Takeuchi, R., Atsumi, T., Ieko, M., Amasaki, Y., Ichikawa, K. and Koike, T. 2002. Suppressed intrinsic fibrinolytic activity by monoclonal anti-beta-2 glycoprotein I autoantibodies: possible mechanism for thrombosis in patients with antiphospholipid syndrome. *Br. J. Haematol.* 119:781.
- Shi, W., Iverson, G., Qi, J. *et al.* 2004. Beta 2-glycoprotein I binds factor XI and inhibits its activation by thrombin and factor XIIIa: loss of inhibition by clipped beta 2-glycoprotein I. *Proc. Natl. Acad. Sci. USA* 101:3939.
- Schousboe, I. and Rasmussen, M. S. 1995. Synchronized inhibition of the phospholipid mediated autoactivation of factor XII in plasma by beta 2-glycoprotein I and anti-beta 2-glycoprotein I. *Thromb. Haemostasis* 73:798.
- Shi, W., Chong, B., Hogg, P. and Chesterman, C. N. 1993. Anticardiolipin antibodies block the inhibition by beta 2-glycoprotein I of the factor Xa generating activity of platelets. *Thromb. Haemostasis* 70:342.
- Nimpf, J., Bevers, E. M., Bomans, P. *et al.* 1986. Prothrombinase activity of human platelets is inhibited by beta 2-glycoprotein-I. *Biochim. Biophys. Acta* 884:142.
- Takeuchi, R., Atsumi, T., Ieko, M. *et al.* 2000. Coagulation and fibrinolytic activities in 2 siblings with beta(2)-glycoprotein I deficiency. *Blood* 96:1594.
- Yasuda, S., Tsutsumi, A., Chiba, H. *et al.* 2000. Beta(2)-glycoprotein I deficiency: prevalence, genetic background and effects on plasma lipoprotein metabolism and hemostasis. *Atherosclerosis* 152:337.
- Amengual, O., Atsumi, T., Khamashta, M. A. and Hughes, G. R. 1998. The role of the tissue factor pathway in the hypercoagulable state in patients with the antiphospholipid syndrome. *Thromb. Haemostasis* 79:276.
- Reverter, J. C., Tassies, D., Font, J. *et al.* 1998. Effects of human monoclonal anticardiolipin antibodies on platelet function and on tissue factor expression on monocytes. *Arthritis Rheum.* 41:1420.
- Del Papa, N., Guidali, L., Spatola, L. *et al.* 1995. Relationship between anti-phospholipid and anti-endothelial cell antibodies III: beta 2 glycoprotein I mediates the antibody binding to endothelial membranes and induces the expression of adhesion molecules. *Clin. Exp. Rheumatol.* 13:179.
- Simantov, R., LaSala, J. M., Lo, S. K. *et al.* 1995. Activation of cultured vascular endothelial cells by antiphospholipid antibodies. *J. Clin. Investig.* 96:2211.

- 26 Pierangeli, S. S., Colden-Stanfield, M., Liu, X., Barker, J. H., Anderson, G. L. and Harris, E. N. 1999. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. *Circulation* 99:1997.
- 27 Atsumi, T., Khamashta, M. A., Haworth, R. S. *et al.* 1998. Arterial disease and thrombosis in the antiphospholipid syndrome: a pathogenic role for endothelin 1. *Arthritis Rheum.* 41:800.
- 28 Nemerson, Y. 1988. Tissue factor and hemostasis. *Blood* 71:1.
- 29 Nemerson, Y. 1992. The tissue factor pathway of blood coagulation. *Semin. Hematol.* 29:170.
- 30 Mann, K. 1999. Biochemistry and physiology of blood coagulation. *Thromb. Haemostasis* 82:165.
- 31 Mackman, N. 1995. Regulation of the tissue factor gene. *FASEB J.* 9:883.
- 32 Bevilacqua, M., Pober, J., Majeau, G., Fiers, W., Cotran, R. and Gimbrone, M., Jr. 1986. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proc. Natl. Acad. Sci. USA* 83:4533.
- 33 Conway, E., Bach, R., Rosenberg, R. and Konigsberg, W. 1989. Tumor necrosis factor enhances expression of tissue factor mRNA in endothelial cells. *Thromb. Res.* 53:231.
- 34 Nawroth, P., Handley, D., Esmon, C. and Stern, D. 1986. Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity. *Proc. Natl. Acad. Sci. USA* 83:3460.
- 35 Atsumi, T., Khamashta, M. A., Amengual, O. and Hughes, G. R. 1997. Up-regulated tissue factor expression in antiphospholipid syndrome. *Thromb. Haemostasis* 77:222.
- 36 Kornberg, A., Blank, M., Kaufman, S. and Shoenfeld, Y. 1994. Induction of tissue factor-like activity in monocytes by anti-cardiolipin antibodies. *J. Immunol.* 153:1328.
- 37 Ginsberg, J., Demers, C., Brill-Edwards, P. *et al.* 1993. Increased thrombin generation and activity in patients with systemic lupus erythematosus and anticardiolipin antibodies: evidence for a pro-thrombotic state. *Blood* 81:2958.
- 38 Oosting, J., Derksen, R., Blokzijl, L., Sixma, J. and de Groot, P. G. 1992. Antiphospholipid antibody positive sera enhance endothelial cell procoagulant activity: studies in a thrombosis model. *Thromb. Haemostasis* 68:278.
- 39 Branch, D. and Rodgers, G. 1993. Induction of endothelial cell tissue factor activity by sera from patients with antiphospholipid syndrome: a possible mechanism of thrombosis. *Am. J. Obstet. Gynecol.* 168:206.
- 40 Schved, J., Gris, J., Ollivier, V., Wautier, J., Tobelem, G. and Caen, J. 1992. Procoagulant activity of endotoxin or tumor necrosis factor activated monocytes is enhanced by IgG from patients with lupus anticoagulant. *Am. J. Hematol.* 41:92.
- 41 Martini, F., Farsi, A., Gori, A. M. *et al.* 1996. Antiphospholipid antibodies (aPL) increase the potential monocyte procoagulant activity in patients with systemic lupus erythematosus. *Lupus* 5:206.
- 42 Cuadrado, M. J., Lopez-Pedraza, C., Khamashta, M. A. *et al.* 1997. Thrombosis in primary antiphospholipid syndrome: a pivotal role for monocyte tissue factor expression. *Arthritis Rheum.* 40:834.
- 43 Cuadrado, M. J., Dobado-Berrios, P. M., Lopez-Pedraza, C. *et al.* 1998. Variability of soluble tissue factor in primary antiphospholipid syndrome. *Thromb. Haemostasis* 80:712.
- 44 Ames, P. R., Pyke, S., Iannaccone, L. and Brancaccio, V. 1995. Antiphospholipid antibodies, haemostatic variables and thrombosis—a survey of 144 patients. *Thromb. Haemostasis* 73:768.
- 45 Ichikawa, K., Khamashta, M. A., Koike, T., Matsuura, E. and Hughes, G. R. 1994. Beta 2-glycoprotein I reactivity of monoclonal anticardiolipin antibodies from patients with the antiphospholipid syndrome. *Arthritis Rheum.* 37:1453.
- 46 Koike, T., Ichikawa, K., Atsumi, T., Kasahara, H. and Matsuura, E. 2000. Beta 2-glycoprotein I-anti-beta 2-glycoprotein I interaction. *J. Autoimmun.* 15:97.
- 47 Nagasako, T., Sugiyama, T., Mizushima, T., Miura, Y., Kato, M. and Asaka, M. 2003. Up-regulated Smad5 mediates apoptosis of gastric epithelial cells induced by *Helicobacter pylori* infection. *J. Biol. Chem.* 278:4821.
- 48 Zhou, H., Wolberg, A. S. and Roubey, R. A. 2004. Characterization of monocyte tissue factor activity induced by IgG antiphospholipid antibodies and inhibition by diltiazep. *Blood* 29:29.
- 49 Del Papa, N., Guidali, L., Sala, A. *et al.* 1997. Endothelial cells as target for antiphospholipid antibodies. Human polyclonal and monoclonal anti-beta 2-glycoprotein I antibodies react in vitro with endothelial cells through adherent beta 2-glycoprotein I and induce endothelial activation. *Arthritis Rheum.* 40:551.
- 50 Del Papa, N., Sheng, Y. H., Raschi, E. *et al.* 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.
- 51 Del Papa, N., Meroni, P. L., Barcellini, W. *et al.* 1992. Antibodies to endothelial cells in primary vasculitides mediate in vitro endothelial cytotoxicity in the presence of normal peripheral blood mononuclear cells. *Clin. Immunol. Immunopathol.* 63:267.
- 52 Dunoyer-Geindre, S., de Moerloose, P., Galve-de Rochemonteix, B., Reber, G. and Kruihof, E. K. 2002. NF-kappaB is an essential intermediate in the activation of endothelial cells by anti-beta(2)-glycoprotein I antibodies. *Thromb. Haemostasis* 88:851.
- 53 Ono, K. and Han, J. 2000. The p38 signal transduction pathway: activation and function. *Cell. Signalling* 12:1.
- 54 Akira, S., Takeda, K. and Kaisho, T. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2:675.
- 55 Baud, V. and Karin, M. 2001. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol.* 11:372.
- 56 Saccani, S., Pantano, S. and Natoli, G. 2002. p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. *Nat. Immunol.* 3:69.
- 57 McGilvray, I. D., Tsai, V., Marshall, J. C., Dackiw, A. P. and Rotstein, O. D. 2002. Monocyte adhesion and transmigration induce tissue factor expression: role of the mitogen-activated protein kinases. *Shock* 18:51.
- 58 Ohsawa, M., Koyama, T., Nara, N. and Hirose, S. 2003. Induction of tissue factor expression in human monocytic cells by protease inhibitors through activating activator protein-1 (AP-1) with phosphorylation of Jun-N-terminal kinase and p38. *Thromb. Res.* 112:313.
- 59 Blum, S., Issbrucker, K., Willuweit, A. *et al.* 2001. An inhibitory role of the phosphatidylinositol 3-kinase-signaling pathway in vascular endothelial growth factor-induced tissue factor expression. *J. Biol. Chem.* 276:33428.
- 60 Eto, M., Kozai, T., Cosentino, F., Joch, H. and Luscher, T. F. 2002. Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways. *Circulation* 105:1756.
- 61 Herkert, O., Diebold, I., Brandes, R. P., Hess, J., Busse, R. and Gortlach, A. 2002. NADPH oxidase mediates tissue factor-dependent surface procoagulant activity by thrombin in human vascular smooth muscle cells. *Circulation* 105:2030.
- 62 Raschi, E., Testoni, C., Bosisio, D. *et al.* 2003. Role of the MyD88 transduction signaling pathway in endothelial activation by antiphospholipid antibodies. *Blood* 101:3495.
- 63 Pargellis, C., Tong, L., Churchill, L. *et al.* 2002. Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. *Nat. Struct. Biol.* 9:268.
- 64 Branger, J., van den Blink, B., Weijer, S. *et al.* 2003. Inhibition of coagulation, fibrinolysis, and endothelial cell activation by a p38 mitogen-activated protein kinase inhibitor during human endotoxemia. *Blood* 101:4446.
- 65 Parasrampur, D. A., de Boer, P., Desai-Krieger, D., Chow, A. T. and Jones, C. R. 2003. Single-dose pharmacokinetics and pharmacodynamics of RWJ 67657, a specific p38 mitogen-activated protein kinase inhibitor: a first-in-human study. *J. Clin. Pharmacol.* 43:406.
- 66 Meroni, P. L., Raschi, E., Testoni, C. *et al.* 2001. Statins prevent endothelial cell activation induced by antiphospholipid (anti-beta2-glycoprotein I) antibodies: effect on the proadhesive and proinflammatory phenotype. *Arthritis Rheum.* 44:2870.
- 67 Ferrara, D., Liu, X., Espinola, R. *et al.* 2003. Inhibition of the thrombogenic and inflammatory properties of antiphospholipid antibodies by fluvastatin in an in vivo animal model. *Arthritis Rheum.* 48:3272.