Published in final edited form as:

J Autoimmun. 2012 May ; 38(2-3): J209–J215. doi:10.1016/j.jaut.2011.11.009.

Anti-phospholipid induced murine fetal loss: novel protective effect of a peptide targeting the β 2glycoprotein I phospholipid-binding site. Implications for human fetal loss

Yeny Martinez de la Torre^{1,12}, Francesca Pregnolato², Fabio D'Amelio³, Claudia Grossi², Nicoletta DiSimone⁴, Fabio Pasqualini⁵, Manuela Nebuloni⁶, Pojen Chen⁷, Silvia Pierangeli⁸, Niccolò Bassani⁹, Federico Ambroggi⁹, Maria-Orietta Borghi^{2,3}, Annunciata Vecchi⁵, Massimo Locati^{1,10}, and Pier-Luigi Meroni^{2,3,11}

¹Istituto Clinico Humanitas, Rozzano, Italy

²Istituto Auxologico Italiano, Milan, Italy

³Department of Internal Medicine, University of Milan, Italy

⁴Catholic University of Sacred Heart, Rome, Italy

⁵Fondazione Humanitas per la Ricerca, Rozzano, Italy

⁶L. Sacco Department of Medical Science, University of Milan, Italy

⁷Department of Medicine, University of Los Angeles, CA -USA

⁸Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX - USA

⁹Department of Work Medicine, University of Milan, Italy

¹⁰Department of Translational Medicine, University of Milan, Italy

¹¹Istituto G. Pini, Milan, Italy

Abstract

β ₂ glycoprotein I (β 2GPI)-dependent anti-phospholipid antibodies (aPL) induce thrombosis and affect pregnancy. The CMV-derived synthetic peptide TIFI mimics the PL-binding site of β 2GPI and inhibits β 2GPI cell-binding *in vitro* and aPL-mediated thrombosis *in vivo*. Here we investigated the effect of TIFI on aPL-induced fetal loss in mice. TIFI inhibitory effect on *in vitro* aPL binding to human trophoblasts was evaluated by indirect immunofluorescence and ELISA. TIFI effect on aPL-induced fetal loss was investigated in pregnant C57BL/6 mice treated with aPL or normal IgG (NHS). Placenta/fetus weight and histology and RNA expression were analyzed. TIFI, but not the control peptide VITT, displayed a dose-dependent inhibition of aPL binding to trophoblasts *in vitro*. Injection of low doses of aPL at day 0 of pregnancy caused growth retardation and increased fetal loss rate, both significantly reduced by TIFI but not VITT.

© 2011 Elsevier Ltd. All rights reserved.

Corresponding author: Pier-Luigi Meroni, MD, Division of Rheumatology - Istituto G. Pini, Piazza Ferrari 1, 20122 Milan (Italy), Phone +39-02-58296746; Fax +39-02-58318176; pierluigi.meroni@unimi.it.

¹²Present address: Department Clinical Chemistry, School of Medicine University of Crete, Heraklion, Greece.

DISCLOSURE STATEMENT

The authors declare no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Consistent with observations in humans, histological analysis showed no evidence of inflammation in this model, as confirmed by the absence of an inflammatory signature in gene expression analysis, which in turn revealed a TIFI-dependent modulation of molecules involved in differentiation and development processes. These findings support the non-inflammatory pathogenic role of aPL and suggest innovative therapeutic approaches to aPL-dependent fetal loss.

Keywords

Anti-phospholipid syndrome; pregnancy complications; β 2 glycoprotein I; murine models; TIFI

INTRODUCTION

Anti-phospholipid antibodies (aPL) represent the most frequent acquired risk factor for a treatable cause of recurrent pregnancy loss and complications [1]. Such a clinical association is also supported by experimental models showing that passive transfer of aPL induces fetal loss and growth retardation in pregnant naive mice [2]. The pathogenic mechanisms are still matter of debate [2,3] as well as the treatment since the standard therapy (low dose aspirin and heparin) does not protect all the patients from recurrent miscarriages [4].

Although intraplacental thrombosis with impairment of maternal-fetal blood exchange was thought to play a role, histopathological findings suggestive for thrombosis cannot be detected in the majority of miscarriage samples and placentas from anti-phospholipid syndrome (APS) women [2,5,6]. Accordingly, alternative mechanisms have been suggested. For example, passive infusion of large amounts of IgG aPL in naive mice after embryo implantation may induce fetal loss via placental inflammation [7–12]. Again, these experimental observations do not find correspondence in immunohistological analysis of abortive material or term placentas from APS women, which does not offer conclusive information on the pathogenic contribution of acute local inflammation and complement deposition [6,13–15]. Evidence has been collected for alternative aPL-mediated non-thrombotic/non-inflammatory mechanisms ending into a defective placentation [2,3,5,12,16]. Consistent with this, an inflammatory process does not appear to be involved in an alternative model of fetal resorption based on i.v. injection of smaller amount of human aPL into mice before implantation. This model is much closer to the human disease where the autoantibodies are present even before conception and at low levels [17,18]. As a whole these findings suggest that the pathogenesis of APS-associated pregnancy complications may be more heterogeneous than expected [2,3,5,19].

Irrespective to the underlying molecular mechanisms, the expression of β 2GPI at placental level is the cornerstone to explain the β 2GPI-aPL pathogenic role. β 2GPI may bind to placenta tissues via several cell receptors and a localized expression of the molecule was reported both in normal human placentas and at the embryo implantation sites in experimental models [2,20]. The synthetic peptide TIFI, spanning Thr(101)-Thr(120) of ULB0-HCMVA from human CMV, shares similarity with the β 2GPI PL-binding site. This peptide prevents aPL-mediated thrombosis *in vivo* and inhibits the *in vitro* binding of FITC-conjugated β 2GPI to human endothelial cells and murine monocytes [21]. Since aPL do not react with TIFI, the inhibitory effect was thought to result from its ability to compete with the β 2GPI PL-binding site and to displace the molecule from the cell surfaces, ultimately inhibiting aPL binding to the target tissues [21].

With this as background, we hypothesized that TIFI may be a novel therapeutic tool able to inhibit β 2GPI binding to trophoblast and to be protective against aPL-mediated placental

damage. In addition, the inhibitory effect may provide further insights on the nature of the involved non-thrombotic/non-inflammatory pathogenic mechanisms.

MATERIALS AND METHODS

2.1 Reagents

Polyclonal IgG were purified from the sera of 5 APS patients (aPL) diagnosed according to the Sidney criteria [1] and displaying medium-high titer of anti-cardiolipin and anti- β 2GPI antibodies as described [22] (Table 1 Supplemental File). Control IgG were from 5 aPL-negative blood donors (NHS). The final IgG concentration, their reactivity with cardiolipin or β 2GPI-coated plates, and the endotoxin contamination were evaluated as previously described [22]. The human IgG anti- β 2GPI monoclonal antibody (moAb) IS3 was obtained from an APS patient as described [23] and purified from the culture supernatants. A human moAb of irrelevant specificity was used as control. Human β 2GPI was purified from human serum and characterized as previously described [20,22]. Sequence for the TIFI peptide, spanning Thr101-Thr120 of the human CMV ULB0 protein, and the control peptide VITT, spanning Val51-Ile70 of the human CMV US27 protein, were obtained from Swiss Protein Database Designation. Both share structural similarity with the 15-aminoacid peptide called GDKV in the fifth domain of human β 2GPI, but display opposite effects *in vivo* [21,24].

2.2 Trophoblast cell cultures and binding assay

Placentas were obtained from healthy women immediately after uncomplicated vaginal delivery at ≥ 36 week gestation. Cytotrophoblast cells were isolated, cultured and characterized as described [25]. 95% of the cell preparations tested positive for anti-cytokeratin antibodies. Cytotrophoblasts at different times of culture were further assayed for the cytoplasmic presence of human chorionic gonadotrophin as a marker for syncytiotrophoblast. For binding assay, the trophoblast monolayer was washed three times with HBSS (Sigma Aldrich) and cultured in FBS-free medium to remove adherent β 2GPI. FBS-free medium trophoblast cells were then incubated for 1 h with exogenous human β 2GPI (5 μ g/ml). Polyclonal or monoclonal anti- β 2GPI antibodies (50 or 25 μ g/ml, respectively) were added to the wells in the presence or absence of serial concentrations of TIFI or VITT. After 2 h of incubation the antibody binding was detected as described [25]. The binding was also evaluated by indirect immunofluorescence in comparable experimental conditions using a FITC-labeled goat anti human IgG as a secondary antibody (Sigma Aldrich).

2.3 Animals and experimental models

C57BL/6 mice (7–8 weeks old) from Charles River Italia were used in accordance with institutional guidelines in compliance with national and international law and policies [17]. The day of vaginal plug detection, day 0 of pregnancy, mice were infused i.v. with aPL (10–50–100 μ g/mouse/200 μ l PBS) or NHS. On days 0, 5, and 10 mice were treated i.p. with 40 μ g/mouse of TIFI or VITT in PBS or with PBS alone (200 μ l/mouse). On day 0 peptides were given 30 min before aPL or NHS injection (50 μ g/mouse). Mice were sacrificed on day 15, embryonic sacs removed and weighted, then opened and fetuses and placentas dissected and individually weighted. Reabsorbed fetuses were identified by their small size and necrotic or hemorrhagic appearance compared with normal embryos. Results are presented as the percentage of fetal loss - calculated as reported - and as weights [17,18].

2.4 Histology

Murine placentas were fixed in 10% buffered formalin for 24–48 h and paraffin embedded. Longitudinal sections of 3 μ m were stained with hematoxylin eosin and histological examination was performed in a blinded fashion by a pathologist.

2.5 Gene expression analysis

Total RNA was purified from homogenized murine placental tissues by TRIzol Reagent (Invitrogen), treated with DNase (Applied Biosystem), and assayed for quality by a BioPhotometer Plus (Eppendorf) and electrophoresis. Gene expression profile was analyzed by MouseWG-6 v2 Expression BeadChip kit (Illumina) for the evaluation of the whole mouse genome expression, according to manufacturer's protocol. Four different RNA samples were tested for mice treated with aPL and aPL+TIFI, while three samples were assayed for the NHS group. Array normalization was performed within the BeadStudio v.3 software using the Quantile method. Referring to the Chip version v2, probes mapping to the same gene according to the official Gene Symbol were averaged. Intensity data were filtered on a detection p-value > 0.1 on all samples, assuming that they reflected experimental noise and carried no relevant biological information, and log2 transformed. Differential expression analysis was performed within JMP Genomics 4.1 software, by fitting a gene-wise linear model using treatment group as only covariate and exploring relevant contrasts, evaluated using an empirical Bayes moderated t-test. Multiple tests were corrected with a 0.05 False Discovery Rate procedure. Gene Ontology (GO) enrichment was performed on differentially expressed genes to investigate the overrepresented GO terms with the platform EASE, available at <http://david.abcc.ncifcrf.gov/content.jsp?file=/ease/ease1.htm&type=1> [26]. We considered Biological Processes and Molecular Functions as GO pathways; maximal EASE score was 0.1, no GO clustering was applied. To confirm our results we used the GoStat platform, available at <http://gostat.wehi.edu.au> [27]. Minimal length of considered GO pathways was 3 and maximal p-value was 0.1. Benjamini False Discovery Testing was used for multiple testing corrections. All values were corrected as 29th of November 2010.

3. RESULTS

3.1 TIFI inhibits β 2GPI-dependent aPL binding to trophoblast cells

β 2GPI-dependent aPL showed background binding to trophoblast comparable to controls in FBS-free medium (data not shown). Addition of exogenous human β 2GPI restored monoclonal and polyclonal binding. TIFI inhibited the binding of β 2GPI-dependent aPL to trophoblast cells in a dose-dependent manner, while VITT was ineffective (Figure 1).

3.2 aPL-induced fetal loss in C57Bl/6 mice is recovered by TIFI treatment

In agreement with previous data (10–12), 50 μ g/mouse of aPL was the lowest dosage able to induce significant fetal loss (Figure 2) and was then used in this study. Under these experimental conditions, TIFI reverted aPL-induced fetal loss/growth retardation, while the control peptide VITT was ineffective (Figure 3).

3.3 Histology

Histological analysis of the placental tissues at day 15 of gestation revealed a scanty inflammatory infiltrate, with rare granulocytes and macrophages found within and around foci of necrosis localized in the placental maternal site (Figure 4).

3.4 Gene expression profiling

A gene transcriptional profile analysis was performed on the placenta tissues affected by growth retardation only, excluding the aborted (necrotic) fetuses. Genes with low intensity

values ($p>0.1$) were filtered out, resulting in 19272 out of 31492 genes. According to the results of the probe-wise linear models, the comparison between aPL- and NHS-treated mice showed 426 differentially expressed genes, while 318 were found comparing aPL vs aPL +TIFI group and 671 comparing aPL+TIFI vs NHS-treated animals (Figure 5A). To gain insight on the potential biological significance of these genes, GO enrichment was performed to identify statistically overrepresented GO terms. Interestingly, comparing aPL or aPL+TIFI with NHS-treated pregnant mice we did not find any significant difference in GO categories related to immune response and inflammation. A relevant fraction of the genes differentially expressed in aPL vs NHS mice were comprised in the “metabolism” and “proliferation, signaling and behavior” categories, which also contained a significant number of genes differentially expressed between aPL and aPL+TIFI-treated mice (Figure 5B). In the comparison of transcripts expressed in aPL vs aPL+TIFI-treated mice, GO also identified an enrichment of genes belonging to the “differentiation and development” category linked to the GO terms epidermal differentiation, development, ectoderm development, histogenesis, and organogenesis. This category was not overrepresented in aPL versus NHS mice comparison (Figure 5B). When the 33 regulated genes included in this functional category were further investigated, an increased expression of 6 transcripts associated to response to stress conditions and deficient embryonic development (*ascl2*, *cdx2*, *hpcal4*, *ninj2*, *sri*, *tmp2*) was observed in aPL-treated-mice (Table 2 Supplemental file). Conversely, the 27 down-regulated molecules in aPL vs aPL+TIFI animals were involved in cell and tissue differentiation, vascular integrity and several developmental processes, including regulation of cell fate and patterning during embryogenesis (Table 2 Supplemental file). Finally, 9 genes of this functional category (*ascl2*, *cd86*, *cdx2*, *col18a1*, *evp*, *lpin1*, *ndrg2*, *prelp*, *upk2*) reached a statistical significance in their differential expression in the comparison between aPL and NHS-treated mice and showed similar modulation when comparing aPL vs aPL+TIFI groups, though GO enrichment did not identify overrepresented GO terms belonging to differentiation and development.

4. DISCUSSION

The *in vivo* pathogenic effect of aPL on pregnancy has been supported by several groups and by different animal models [2]. Recently, repeated *i.p.* injections of large amounts of human IgG with aPL activity (10 mg/mouse) to pregnant naive mice after embryo implantation were found to induce a strong complement-dependent inflammatory damage at the placental level that results in fetal resorption and growth retardation [7–11]. So, a local inflammation rather than a thrombotic damage was suggested to be responsible for the APS obstetric manifestations.

The use of large amounts of autoantibodies and their infusion just in the middle of pregnancy makes this model different from the obstetric APS in women. To better mimic human pathological conditions, a model based on the exposure to low dosage of aPL before implantation was established [17,18]. Our data show for the first time that smaller amounts of human aPL IgG (10–50 μ g/mouse) passively injected *i.v.* into mice before implantation are able to induce fetal resorptions and growth retardation without any histological evidence of placental inflammation. This finding is in line with the histology found in human placenta specimens and with the ability of aPL to mediate a noninflammatory damage to trophoblast as shown in *in vitro* experimental models [2,3,6]. As a whole these data do suggest that both inflammatory and non-inflammatory events unrelated to aPL procoagulant effect may play a role in different *in vivo* fetal loss models.

We hypothesize that one mechanism may be more emphasized than another one depending on the time of injection and on the amount of the injected IgG aPL.

Animals treated with a synthetic peptide sharing similarity with the β 2GPI PL-binding site are protected from the abortive effect of the autoantibodies, supporting at the same time their pathogenic role and the importance of β 2GPI as the antigenic target. Incubation with TIFI, but not with the control peptide VITT, inhibits in a dose dependent manner the in vitro aPL binding to trophoblast. As reported, TIFI is not recognized by aPL and the PL-binding site mediates the adhesion of β 2GPI to human trophoblasts suggesting that TIFI competes with β 2GPI for cell membrane binding, preventing its adhesion and ultimately its recognition by β 2GPI-dependent aPL [21,25].

We speculate that the protective effect of TIFI may offer information on the biological events involved in the non-inflammatory defective placentation observed in our model. For the first time, gene expression profiling shows that modulation of transcripts involved in histogenesis and organogenesis seems to play a key role in mediating aPL dependent fetal loss and growth retardation. These results are in line with the histological analysis and further stress the fact that our fetal loss model is not related to inflammation.

In conclusion, TIFI may affect β 2GPI tissue expression at the placental level inhibiting the binding of β 2GPI-dependent aPL and ultimately the trophoblast modulation mediated by the autoantibodies.

We recently showed that exogenous labeled β 2GPI can be detected by in vivo optical imaging on EC of uterine vessels only in non pregnant mice and at the embryo implantation sites in pregnant ones, further supporting the idea that the molecule displays a specific tropism for the reproductive tissues [20]. Hence, the use of molecules interfering with the tissue expression of the target antigen(s) for aPL may represent innovative therapeutic approach.

Standard treatment of aPL-associated miscarriages includes low dose aspirin and heparin but their use is mainly empirical and based on the initial assumption that thrombotic events play the major role [28,29]. Because of the finding that thrombosis cannot explain all the aPL-mediated complications, the anti-inflammatory (anticomplement) activity of heparin was then advocated [30]. However, the lack of a clear evidence for a complement-dependent inflammatory signature at the placental levels makes such explanation questionable. Moreover, the anti-inflammatory effect of corticosteroids is not associated to a significant clinical advantage, stressing again the clash between the inflammatory hypothesis and the clinical data [30,32]. Understanding the pathogenesis of aPL-associated miscarriages is pivotal to improve the pregnancy outcome, since there is a significant proportion of women unresponsive to the standard treatment with low dose aspirin and heparin [2,29,33,34].

We suggest that the pathogenesis of aPL-associated miscarriages is more heterogeneous but that a pivotal role is played by antibodies reacting with β 2GPI overexpressed on placenta tissues. Hence, new therapeutic tools able to downregulate the β 2GPI presence on placental tissues may be useful, in particular for the treatment of the resistant cases.

5. CONCLUSIONS

Our alternative experimental model mimics human APS since small amounts of human aPL IgG are injected before implantation inducing fetal resorptions and growth retardation without any histological evidence of placental inflammation. Our findings underline the heterogeneity of the pathogenic mechanisms involved in aPL-mediated fetal loss. In this regard, different experimental models may emphasize the various mechanisms depending on the characteristics of the model itself. These mechanisms are not mutually exclusive and may play a role together or in different combination at different times of the pregnancy process. Such a variety of pathogenic mechanisms fits well with the heterogeneity of the

clinical manifestations of the obstetric APS, spanning from early to late miscarriages or pre-eclampsia.

The evidence of a central role of β 2GPI and the related autoantibodies is becoming more and more clear in aPL-mediated obstetric manifestations. Molecules interfering with β 2GPI expression at placental level may be crucial in protecting the pregnancy from the aPL damaging effect. This is the case for heparin, which was recently reported to be able to displace β 2GPI from trophoblast monolayers in vitro making them no more attacked by β 2GPI-dependent aPL [35]. In line with this finding, we found that a synthetic peptide targeted to β 2GPI PL-binding site inhibits the adhesion of the molecule to trophoblast cell membrane in vitro. Such a mechanism may explain the protective effect of TIFI in our in vivo animal model, offering a new therapeutic approach characterized by a theoretically safer profile (i.e. reduced side effects).

Alternative therapies for obstetric APS are needed because of the evidence that a significant proportion of APS women is still suffering from miscarriages in spite of the standard therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by: MIUR research project n. 20083MNL5X 2008-10; PRIN and FIRB projects; Italian Association for Cancer Research; Regione Lombardia (LIIN project); Fondazione Cariplo; Ricerca Corrente, Istituto Auxologico Italiano; NIH-NIAMS R0-1 grant 5R01AR05674502 (SP).

References

1. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost*. 2006; 4:295–306. [PubMed: 16420554]
2. Meroni PL, Borghi MO, Raschi E, Tedesco F. Pathogenesis of the antiphospholipid syndrome (APS): understanding the antibodies. *Nat Rev Rheumatol*. 2011; 7:330–9.
3. Meroni PL, Tedesco F, Locati M, Vecchi A, Di Simone N, Acaia B, et al. Anti-phospholipid antibody mediated fetal loss: still an open question from a pathogenic point of view. *Lupus*. 2010; 19:453–6. [PubMed: 20353987]
4. Danza A, Ruiz-Irastorza G, Khamashta M. Antiphospholipid syndrome in obstetrics. *Best Pract Res Clin Obstet Gynaecol*. 2011 Nov 10. [Epub ahead of print].
5. Meroni PL, Gerosa M, Raschi E, Scurati S, Grossi C, Borghi MO. Updating on the pathogenic mechanisms of the antiphospholipid antibodies-associated pregnancy loss. *Clin Rev Allergy Immunol*. 2008; 34:332–7. [PubMed: 18175073]
6. Park, AL. Placental pathology in antiphospholipid syndrome. In: Khamashta, MA., editor. *Hughes' syndrome*. London: Springer-Verlag; 2006. p. 362-74.
7. Holers VM, Girardi G, Mo L, Guthridge JM, Molina H, Pierangeli SS, et al. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. *J Exp Med*. 2002; 195:211–20. [PubMed: 11805148]
8. Berman J, Girardi G, Salmon JE. TNF-alpha is a critical effector and a target for therapy in antiphospholipid antibody-induced pregnancy loss. *J Immunol*. 2005; 174:485–90. [PubMed: 15611274]
9. Redecha P, Franzke CW, Ruf W, Mackman N, Girardi G. Neutrophil activation by the tissue factor/ Factor VIIa/ PAR2 axis mediates fetal death in a mouse model of antiphospholipid syndrome. *J Clin Invest*. 2008; 118:3453–61. [PubMed: 18802482]

10. Girardi G. Role of tissue factor in the maternal immunological attack of the embryo in the antiphospholipid syndrome. *Clin Rev Allergy Immunol.* 2010; 39:160–5. [PubMed: 19921475]
11. Girardi G, Prohászka Z, Bulla R, Tedesco F, Scherjon S. Complement activation in animal and human pregnancies as a model for immunological recognition. *Mol Immunol.*
12. Francis J, Rai R, Sebire NJ, El-Gaddal S, Fernandes MS, Jindal P, et al. Impaired expression of endometrial differentiation markers and complement regulatory proteins in patients with recurrent pregnancy loss associated with antiphospholipid syndrome. *Mol Hum Reprod.* 2006; 12:435–42. 2011. 48, 1621–30. [PubMed: 16735457]
13. Shamoni JM, Salmon JE, Hyjek E, Baergen RN. Excessive complement activation is associated with placental injury in patients with antiphospholipid antibodies. *Am J Obstet Gynecol.* 2007; 196:167.e1–5. [PubMed: 17306667]
14. Cavazzana I, Manuela N, Irene C, Barbara A, Sara S, Orietta BM, et al. Complement activation in anti-phospholipid syndrome: a clue for an inflammatory process? *J Autoimmun.* 2007; 28:160–4. [PubMed: 17419007]
15. Gerosa M, De Angelis V, Trespidi L, Acaia B, Cetin I, Calabrese S, et al. Complement involvement in antiphospholipid antibody-mediated placental damage: prospective study in APS pregnant women. *Ann Rheum Dis.* 2009; 68(S3):210.
16. Borghi MO, Raschi E, Brogini V, Grossi C, D'Amelio F, Chen P, et al. Antiphospholipid antibodies reactivity with human decidual cells: an additional mechanism of pregnancy complications in APS and a potential target for innovative therapeutic intervention. *Ann Rheum Dis.* 2009; 68:109.
17. Martinez de la Torre Y, Buracchi C, Borroni EM, Dupor J, Bonecchi R, Nebuloni M, et al. Protection against inflammation- and autoantibody-caused fetal loss by the chemokine decoy receptor D6. *Proc Natl Acad Sci USA.* 2007; 104:2319–24. [PubMed: 17283337]
18. Martinez de la Torre Y, Raschi E, Borghi M, Di Simone N, Nebuloni M, Vecchi A, et al. Pregnant naive mice are protected from aPL-induced foetal loss by the injection of a synthetic peptide (TIFI) mimicking the β 2GPI PL-binding site. *Arthritis Rheum.* 2008; 58:S404.
19. Poindron V, Berat R, Knapp AM, Toti F, Zobairi F, Korganow AS, et al. Evidence for heterogeneity of the obstetric antiphospholipid syndrome: thrombosis can be critical for antiphospholipid-induced pregnancy loss. *J Thromb Haemost.* 2011; 9:1937–47. [PubMed: 21848640]
20. Agostinis C, Biffi S, Garrovo C, Durigutto P, Lorenzon A, Bek A, et al. In vivo distribution of β 2 glycoprotein I under various pathophysiological conditions [published online ahead of print Jul 26, 2011]. *Blood.* 2011; 118:4231–8. [PubMed: 21791419]
21. Ostertag MV, Liu X, Henderson V, Pierangeli SS. A peptide that mimics the Vth region of beta-2-glycoprotein I reverses antiphospholipid-mediated thrombosis in mice. *Lupus.* 2006; 15:358–65. [PubMed: 16830882]
22. Fischetti F, Durigutto P, Pellis V, Debeus A, Macor P, Bulla R, et al. Thrombus formation induced by antibodies to β 2-glycoprotein I is complement dependent and requires a priming factor. *Blood.* 2005; 106:2340–6. [PubMed: 15956288]
23. Zhu M, Olee T, Le DT, Roubey RA, Hahn BH, Woods VL Jr, et al. Characterization of IgG monoclonal anti-cardiolipin/anti β 2GPI antibodies from two patients with the anti-phospholipid syndrome reveals three species of antibodies. *Br J Haematol.* 1999; 105:102–9. [PubMed: 10233371]
24. Gharavi AE, Pierangeli SS, Espinola RG, Liu X, Colden-Stanfield M, Harris NE. Antiphospholipid Antibodies Induced in Mice by Immunization With a Cytomegalovirus-Derived Peptide Cause Thrombosis and Activation of Endothelial Cells In Vivo. *Arthritis Rheum.* 2002; 46:545–52. [PubMed: 11840458]
25. Di Simone N, Raschi E, Testoni C, Castellani R, D'Asta M, Shi T, et al. Pathogenic role of anti-beta 2 glycoprotein I antibodies in antiphospholipid-associated fetal loss: characterisation of beta 2 glycoprotein I binding to trophoblast cells and functional effects of anti-beta 2 glycoprotein I antibodies in vitro. *Ann Rheum Dis.* 2005; 64:462–7. [PubMed: 15256379]
26. Hosack DA, Dennis G Jr, Sherman BT, Lane HC, Lempicki RA. Identifying biological themes within lists of genes with EASE. *Genome Biol.* 2003; 4:R70. [PubMed: 14519205]

27. Beissbarth T, Speed TP. GStat: find statistically overrepresented Gene Ontologies within a group of genes. *Bioinformatics*. 2004; 20:1464–5. [PubMed: 14962934]
28. Branch W. Obstetric Task Force. Report of the Obstetric APS Task Force: 13th International Congress on Antiphospholipid Antibodies, 13th April 2010. *Lupus*. 2011; 20:158–64. [PubMed: 21303832]
29. Hoppe B, Burmester GR, Dörner T. Heparin or aspirin or both in the treatment of recurrent abortions in women with antiphospholipid antibody (syndrome). *Curr Opin Rheumatol*. 2011; 23:299–304. [PubMed: 21346577]
30. Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody- induced fetal loss by inhibiting complement activation. *Nat Med*. 2004; 10:1222–6. [PubMed: 15489858]
31. Cowchock FS, Reece EA, Balaban D, Branch DW, Plouffe L. Repeated fetal losses associated with antiphospholipid antibodies: a collaborative randomized trial comparing prednisone with low-dose heparin treatment. *Am J Obstet Gynecol*. 1992; 166:1318–23. [PubMed: 1595785]
32. Bramham K, Thomas M, Nelson-Piercy C, Khamashta M, Hunt BJ. First-trimester low-dose prednisolone in refractory antiphospholipid antibody-related pregnancy loss. *Blood*. 2011; 117:6948–51. [PubMed: 21527518]
33. Ziakas PD, Pavlou M, Voulgarelis M. Heparin treatment in antiphospholipid syndrome with recurrent pregnancy loss: a systematic review and meta-analysis. *Obstet Gynecol*. 2010; 115:1256–62. [PubMed: 20502298]
34. Ruffatti A, Tonello M, Visentin MS, Bontadi A, Hoxha A, De Carolis S, et al. Risk factors for pregnancy failure in patients with anti-phospholipid syndrome treated with conventional therapies: a multicentre, case-control study. *Rheumatology*. 2011; 50:1684–9. [PubMed: 21652586]
35. Di Simone N, Marana R, Castellani R, Di Nicuolo F, D'Alessio MC, Raschi E, et al. Decreased expression of heparin-binding epidermal growth factor-like growth factor as a newly identified pathogenic mechanism of antiphospholipid-mediated defective placentation. *Arthritis Rheum*. 2010; 62:1504–12. [PubMed: 20131286]

Research Highlights

- β 2GPI-dependent aPL induce fetal loss/growth retardation in naïve pregnant mice
- Histogenesis/organogenesis inhibition & not inflammation is involved in our model
- The synthetic peptide TIFI competes with β 2GPI binding and protects pregnant mice
- Inhibition of β 2GPI tissue expression represents an innovative therapeutic approach

Figure 1.

Human anti- β 2GPI antibody binding to trophoblast cells

(A to C) Trophoblast monolayers were exposed to IS3 (25 μ g/ml; panels B and C) or irrelevant moAb (panel A) in the absence (panel B) or in the presence of TIFI (20 μ g/ml) (panel C) and exogenous β 2GPI (5 μ g/ml) (panel B and C). The moAb binding was revealed by FITC-labeled goat anti human IgG and evaluated by fluorescence microscopy. (D) Trophoblast cell cultures were incubated with aPL (50 μ g/ml), β 2GPI (5 μ g/ml) and serial concentrations of TIFI or VITT. The aPL binding was revealed by alkaline phosphatase-labeled goat anti-human IgG and expressed as mean optical density (ODx10⁻³) units $\pm$ SEM. *p<0.01 vs VITT-exposed cells by Student t test.

Figure 2. Dose-response of aPL-induced fetal damage

Effect of increasing amounts of aPL on fetal loss (panel A) and fetus weight (panel B) at day 15 of pregnancy. Results are reported as mean $\pm$ SEM of the total number of fetuses from 3 mice/group. * $p < 0.05$ and ** $p < 0.01$ vs PBS-treated mice by Fisher exact test (panel A) and Student t test (panel B).

Figure 3. TIFI effect on aPL-induced fetal damage

TIFI or VITT (40 μ g/mouse on day 0, 5 and 10) effect on fetal loss (panel A), weight of embryonic sacs (panel B), fetuses (panel C) and placentas (panel D). Mice were treated with: PBS (C; 8 mice), NHS (11 animals), aPL (8 animals), aPL+TIFI (11 animals) or aPL+VITT (4 animals). Numbers under columns are total number of events evaluated. In panel A results are reported as percentage of resorbed fetuses and statistical significance is calculated by Fisher exact test. In panels B to D results are reported as mg, mean $\pm$ SEM, and statistical significance is calculated by ANOVA with Tukey post-test. * $p < 0.05$ and ** $p < 0.01$ vs PBS-treated mice.

Figure 4. Histological analysis of the placentas

Hematoxylin-eosin staining (OM: 10x) of placenta sections at day 15 of pregnancy from animals treated with aPL (panel A), aPL+TIFI (panel B), aPL+VITT (panel C), or NHS (panel D). In all groups no signs of overt inflammation are evident in the labyrinth (L) and the junctional zone (jz), and a few leukocytes and focal areas of mild necrosis (**) are detected in the maternal site of placenta (decidua basalis, db).

Figure 5. Gene expression analysis

Venn diagram representing number of genes differentially expressed comparing aPL vs NHS, aPL vs aPL+TIFI and aPL+TIFI vs NHS treated mice (panel A). Over-represented biological processes related to differentially expressed genes identified by GO categories enrichment in aPL vs NHS (filled columns) and aPL vs aPL+TIFI (open columns) comparisons (panel B).