

Recurrent miscarriage and autoimmunity

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Both organ-specific and systemic autoimmunity are associated with an increased prevalence of recurrent miscarriage (RM). The precise mechanism for this is unclear, as cross-reactivity between trophoblastic and maternal host autoantigens has not been demonstrated. In the antiphospholipid antibody syndrome, prothrombotic mechanisms are evident, along with direct inhibitory actions against trophoblastic activity. In many types of both systemic and organ-specific immunity, however, a disturbed T-helper cell profile is seen. This is also evident in women with RM. In both cases, reduced numbers of T regulatory cells have been reported. These are required to regulate excessive activity of the Th1 and the proinflammatory Th17 subsets that, when operating through excessive natural killer cell activity, may have antipregnancy effects. Checking for underlying autoimmunity is therefore a critical analysis in women with RM and future therapies will probably be aimed at correcting the deficiency of regulatory T cells or correcting excessive Th1 and Th17 function.

KEYWORDS: antiphospholipid antibody syndrome • autoimmunity • recurrent miscarriage • T-cell subsets • Th17 • Tregs

Recurrent miscarriage (RM), defined as three or more miscarriages, has a number of causes. After chromosomal and genetic abnormalities are excluded, autoimmunity and particularly that associated with antiphospholipid antibodies (APA) is generally the next most important. The mechanism by which general autoimmunity predisposes to RM is not clear but recent work on the dysregulation of various T-cell subsets and alteration of natural killer (NK) cell behavior may be of importance [1]. Regarding the RM associated with the APA syndrome (APAS), several mechanisms may be operative in addition to the well-known ability of these autoantibodies to promote placental vessel thrombosis. The distinction between autoimmunity and alloimmunity needs to be taken into consideration as there is controversial literature suggesting pathogenicity of anti-paternal cell antibodies in RM and the benefits of paternal cell immunization. In addition, while it is generally assumed that all autoimmunity is deleterious to pregnancy, there is a certain number of animal studies suggesting that this may not always be the case [2]. This article aims to summarize current thinking regarding the association between human maternal autoimmunity and RM, and expand on the immune disturbance that may be at the heart of both problems.

Organ-specific autoimmunity & RM

The increased incidence of RM in women with subclinical thyroid autoimmunity (TA) [3–6] has defied proper explanation. However, some investigators have found miscarriage rates to be comparable between healthy women with and without TA [7,8]. By contrast, Bellver *et al.* noted elevated thyroid peroxidase and/or thyroglobulin antibodies in women with only specific types of impaired reproduction, such as unexplained infertility and implantation failure but not RM [9]. More recently Toulis *et al.* have reported several important points in a meta-analysis of TA in euthyroid subfertile women undergoing *in vitro* fertilization [10]. Interestingly, while miscarriage was more frequent in the TA group, clinical pregnancy and live birth rate was little affected. Intriguingly, De Carolis *et al.* recorded a significantly lower rate of spontaneous pregnancy and live birth in women with anti-thyroid antibodies with and without simultaneous APA compared with women with APA alone [11]. However, Bussen *et al.* observed no correlation between anti-thyroid and anticardiolipin antibodies in women with RM, although the numbers in this study were small [4].

The prevalence of many organ-specific autoantibodies increase with age. This is particularly so with anti-thyroid peroxidase antibodies.

However, the presence of anti-thyroid peroxidase antibodies appears to confer a greater risk of RM than can be accounted for by the increased age of women with TA. Thus Iravani *et al.* recorded thyroid antibodies in 157 of 641 women with RM (24.5%) and in only 34 of 269 healthy women without RM (12.6%; $p < 0.001$) [6]. Importantly, however, the presence of thyroid antibodies among these women was independent of age. The prevalence of thyroid disease, arthritis and diabetes assessed by questionnaire has also been reported to be increased in the first- and second-degree relatives of women with RM [12]. This raises the possibility that genetic mechanisms may underlie the link between organ-specific immunity with RM.

Interestingly, thyroid replacement therapy produced successful birth in 13 out of 16 euthyroid women with RM and anti-thyroid antibodies compared with five out of 11 euthyroid RM women with anti-thyroid antibodies treated with intravenous immunoglobulin (IVIg) therapy [13]. Of 15 women with mild thyroid dysfunction without anti-thyroid antibodies and treated with thyroid replacement therapy, 14 also delivered a live birth in this study. The mechanism for this benefit is unclear, although some subtle thyroid dysfunction has been considered to be present in those with thyroid antibodies even in the absence of overtly abnormal thyroid function tests. In women with infertility undergoing *in vitro* fertilization treatment, those with anti-thyroid antibodies receiving 50 µg of levothyroxine fared little better than those without these auto-antibodies [14]. However, the addition of prednisolone and acetyl salicylic acid significantly improved the live birth rate.

The benefits of IVIg therapy were also mentioned by Kiprof *et al.* in their uncontrolled study of 35 women with RM who had a number of different autoimmune and alloimmune factors but with a high frequency of anti-thyroid antibodies [15]. This group reported an 80% successful rate of delivery after the use of 200–250 mg/kg of IVIg given every 3 weeks for the first 8 months of pregnancy.

Systemic autoimmunity & RM

It is well known for women with systemic lupus erythematosus (SLE) to have an increased risk of RM. While this is mainly caused by the high frequency of APA in this condition, there is also an increased risk in women without demonstrable APA or the lupus anticoagulant. Interestingly, Ticconi *et al.* have recently reported that 50% of women with recurrent pregnancy loss have antinuclear antibody (ANA) at titers equal to or exceeding a one in 80 serum dilution compared with only 16% of healthy women [16]. In the past, Habara *et al.* have shown increased uterine artery pulsatile index in women with RM and ANA as compared with those without ANA and RM and healthy controls [17]. Thus, it is possible that ANA have the ability to alter blood flow to the developing embryo, fetus and child without placental vessel thrombosis.

Fetal wastage has been known to be increased in women with lupus having anti-Ro or anti-ribonucleoprotein (RNP) antibodies and particularly in black women with anti-Ro antibodies and SLE [18]. In women with anti-Ro antibodies as part of

SLE, Sjögren's syndrome or subacute lupus erythematosus, the increased prevalence of RM and infertility may, at least in part, be due to the ability of these antibodies to cause complete heart block in the developing fetal heart. This usually causes problems in the second and third trimester but problems have also been recorded in the first trimester. As such, RM was more frequent in women with anti-Ro antibodies but without lupus compared with anti-Ro-negative women with and without lupus and age-matched healthy women [19]. However, the precise mechanism of the increased miscarriage is unclear, although there is presently no evidence that anti-Ro antibodies are able to bind to trophoblastic antigens or impede implantation. Whether the detection of IgE anti-Ro antibodies is more predictive of RM, as suggested by Sekigawa *et al.* [20], is unclear.

Antiphospholipid antibodies syndrome & RM

Antiphospholipid antibodies have been shown to impair fertility by several mechanisms [21]. As such, several lines of *in vitro* work have shown impaired proliferation, human chorionic gonadotrophin (hCG) release and trophoblast invasiveness by human polyclonal and monoclonal APA [22]. More recent work has shown reduced adhesion molecule expression and increased apoptosis of trophoblast cells by the direct binding of these antibodies [23]. Whether complement activation contributes to the pathogenicity of APA remains unclear, although the evidence from complement-deficient mice [24] and the work of Girardi [25] appears compelling. In addition, Girardi *et al.* have shown increased decidual tissue factor (TF) expression in women with APAS and mice treated with APA [26]. Importantly, APA are also able to induce inflammation accompanied by complement activation and the generation of the C5a anaphylatoxin. The latter encourages neutrophil recruitment and activation as well as TF expression [27]. Acting via protease-activated receptor 2, TF is considered to be one of the main mediators of further neutrophil activation that then initiates trophoblast and fetal damage, often in the absence of fibrin deposition or thrombosis [28]. In support of these findings, mice treated with anti-TF monoclonal antibodies or genetically deficient in TF have significantly reduced miscarriage in response to APA [28]. APA are also detrimental to pregnancy progression and neonatal health. Thus, Chou *et al.* in their recent small study have observed increased rates of premature birth, intrauterine growth retardation, neonatal thrombocytopenia and intrauterine demise in women with APAS [29].

There are numerous reports supporting a link between APA and RM and many others suggesting possible mechanisms. Placental vasculopathy and thrombosis are certainly well known to contribute significantly to RM [30]. While the precise mechanism of the thrombosis is unclear, proteomic analysis has shown a disturbance of annexin I, annexin II, protein disulfide isomerase, Nedd8, RhoA proteins and Hsp60 in the monocytes of APAS patients [31]. There is also a certain amount of evidence implicating a disruption of the anti-thrombotic annexin V shield by the binding of anionic phospholipids by APA [32]. More recently Di Simone *et al.* have used an *in vitro* Matrigel™

assay in mice inoculated with APA [33]. They observed that APA recognize and significantly reduce the number and total length of the tubules formed by human endometrial endothelial cells and also the ability of APA to decrease the formation of new vessels. Furthermore, APA also significantly reduced the production of VEGF and metalloproteinases as well as NF- κ B binding activity. Thus, APA impair several aspects of angiogenesis contributing to abnormal placentation and fetal loss in APAS-associated RM. In addition, APA may also induce inflammation, trophoblast activation and apoptosis. Thus Mulla *et al.* have shown APA to encourage the release of IL-8, IL-1 β and GRO- α via a Toll-like receptor–MyD88 pathway [34]. In high concentrations, these antibodies also induced caspase-mediated cell death. More recently, this group has also shown anti- β 2 glycoprotein 1 (β 2GP1) antibodies to reduce trophoblast migration involving a disturbance of IL-6 secretion via the STAT3 pathway [35].

Regarding testing for APA, there is debate as to whether extensive testing for the non-routine anionic phospholipids such as phosphatidic acid, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl inositol and phosphatidyl serine with and without β 2GP1 provide any useful information. Thus Tebo *et al.* found both IgM and IgG antibodies to these phospholipids to be no different between women with RM and healthy women without RM [36] and thus suggested that they had no clinical significance. Regarding the routine testing of anti- β 2GP1 antibodies in addition to APA, there is certainly evidence favoring its inclusion [37]. Further debate surrounds the issue of serial testing for APA. As can be imagined, such an approach is likely to lead to an increase in the frequency of positive results, as would be expected for any test. Not surprisingly, this was observed by Bao *et al.* in comparing women with RM having four or fewer tests for anti-cardiolipin and β 2GP1 antibodies compared with those had five or more tests [38].

While much of the medical literature on APA and RM has confirmed a clear relationship, this is not universally so. Thus, the presence of APA, as well as factors contributing to thrombophilia, were found to be no different in women with either two, three or a greater number of miscarriages by Jaslow *et al.* [39]. Jones *et al.* also observed a lack of association between anticardiolipin antibodies and β 2GP1 in women with APA or SLE and fetal loss [40]. There was, however, a weak association with the lupus anticoagulant and a stronger one with antibodies to factor XII. Other mechanisms have also been suggested. These include some sort of interaction with natural killer cells whose numbers were increased in RM women with APA [41]. Furthermore, at least in mice, experimental APAS was associated with an increase in the number of IL-2- and IFN- γ -secreting T cells and reduced numbers of IL-4- and IL-6-expressing T cells, suggestive of increased Th1-type immunity [42]. Interestingly, an increased risk of thrombosis was suggested in RM women with APA who had raised levels of CD27⁺ IgD⁺ naive B cells and activated CD8⁺ DR⁺ T cells [43]. Thus, it is likely that APA are both directly pathogenic and are also associated with other immune abnormalities that are harmful to pregnancy.

Autoimmunity to proteins involved in the coagulation system

The frequent observation of thrombosis in the placentas of women suffering RM in the absence of the APAS suggests that procoagulation or impaired fibrinolysis may be evident in some APA-negative women with RM. Interestingly, initial reports had also suggested impaired fibrinolysis in those with APAS. Arnold *et al.* observed an increased frequency of anti-annexin V antibodies in RM with APA but without any overlap in women with anti- β 2GP1 antibodies [44]. In 2002, however, Makino observed not only an increased frequency of anti-phosphatidyl ethanolamine but also anti-annexin V antibodies among 1500 women with RM seen over a 20-year period [45]. Bizzaro *et al.* then reported anti-annexin V antibodies as the only frequent procoagulant autoantibody in women with idiopathic RM [46]. However, these antibodies, as well as those to β 2GP1 and prothrombin, were also seen not infrequently in women with SLE. Subsequently, Zammit *et al.* confirmed raised IgG anti-annexin V but not IgG or IgM anti- β 2GP1 antibodies in 172 Tunisian women with more than three consecutive idiopathic miscarriages compared with 173 matched control women [47]. Schoenfeld *et al.* have also observed an increased frequency of anti-prothrombin and APA in women with recurrent pregnancy loss with an odds ratios of 5.4 (95% CI: 2.4–12.5) and 4.89 (95% CI: 1.2–22.2), respectively [8].

In 2007 Bu *et al.* reported antibodies to tissue-type plasminogen activator and plasminogen in women with RM [48]. While these were more frequent in women with APA, they were nevertheless still evident in women with RM without APA. However, as with other reports in this area, it may be argued that APA to untested phospholipids may have been present in the former group. Thus it is possible that these autoantibodies represent autoimmunity additional to an APAS rather than a unique type of autoimmunity.

Anti-endothelial cell & anti-trophoblast antibodies in RM

Autoantibodies able to bind to endothelial cells, fix complement and, theoretically at least, disrupt blood flow to the trophoblast/early embryo are also evident in women with RM [49]. In a subsequent report, anti-endothelial cell antibodies were shown to reduce the adherence of trophoblast cells to endothelial cells in an inhibition ELISA [50]. However, such autoantibodies are by no means universally present in RM and their pathogenicity awaits definition. Interestingly, anti-endothelial cell antibodies are present more frequently in those with APA and it is quite possible that at least some of the adverse effects overlap. By contrast, antibodies to trophoblast cells are more frequently evident and have been shown to be cytotoxic for the target cells. Cross-reactivity between organ-specific antibodies and those to trophoblastic antigens is presently considered to be unlikely. In regard to anti-elastin antibodies [51], their exact role in RM awaits clarification.

T-cell subsets in pregnancy

T regulatory cells (Tregs) are identified by their CD4⁺ CD25⁺ phenotype as well as the presence of the intracellular transcription factor forkhead box P3 (FoxP3) [52]. They are important in regulating the activity of both the Th1 subset, which is considered harmful

to the trophoblast and embryo, and the pregnancy-supportive Th2 subset [53]. The exact manner of interaction between Tregs and the newly described Th17 cells has yet to be defined. However, Th17 cells have recently been found to be raised in women with RM [54] and thus may be harmful to the early pregnancy. There is some evidence that Tregs may convert to Th17 cells in certain situations [55] and it is important to realize that formation of both cells from naive CD4⁺ T cells is dependent on the presence of TGF- β . In the presence of the proinflammatory cytokine IL-6, Th17 cells are encouraged, while the relative absence of this cytokine encourages the development of Tregs [56]. Thus the presence of ongoing inflammation encourages the formation of Th17 cells [57]. Of interest, Vacca *et al.* have recently confirmed the importance of CTLA-4-mediated NK cell interaction with decidual CD14⁺ myelomonocytic cells, which encourages the formation of Tregs via the release of TGF- β [58]. It is therefore possible that abnormal uterine NK cell activity and decidual inflammation mediated in part by subclinical infection, APA and autoimmunity may encourage miscarriage via impaired Treg formation [1].

Tregs & pregnancy

The importance of Tregs in inducing tolerance at the fetal–maternal interface is clearly important in the continuation of a viable pregnancy [59]. This is deduced from several lines of evidence. Initial work showed an accumulation of these cells within the decidua of pregnant mice. Subsequently, significant numbers of Tregs were reported in early human pregnancy decidua by Sasaki *et al.* [60]. Importantly, these cells produced a marked inhibition of autologous T-cell proliferation by anti-CD3 stimulation and reduced the proliferation of autologous CD4⁺CD25⁻ T cells in a dose-dependent fashion. Tilburgs *et al.* then reported increased Tregs in the decidua basalis and parietalis relative to the peripheral blood [61]. Thus, cells that have the ability to downregulate embryo-harmful Th1 cells appear to be present in the postfertilization endometrium. More recently, the importance of hCG in attracting Tregs to the fetal–maternal interface has been reported [62]. As mentioned previously, APA have been shown to inhibit hCG production. Therefore, it is quite possible that these autoantibodies not only impair trophoblast invasiveness and angiogenesis but may also augment T-cell dysfunction in RM by reducing the recruitment of Tregs.

In regard to RM, the importance of Treg function is suggested by several recent reports. Thus, the proportion of Tregs has been found to be significantly lower in decidual specimens from women suffering a spontaneous compared with an induced abortion. This was also observed by Heikkinen *et al.* who reported 14% of the decidual CD4⁺ T cells to have the Treg CD4⁺CD25⁺ phenotype and also expressed intracellular CTLA-4 [63]. Using FoxP3 as a marker of Treg function, Jasper *et al.* observed a twofold reduction in the endometrial expression of this transcription factor in women with primary infertility while that of Tbet (Th1) and GATA3 (Th2) expression was no different [64]. More recently, Yang *et al.* have reported significantly reduced CD4⁺CD25⁺ Tregs in both the decidua and the peripheral blood of women prone to RM [65]. In addition, Xia *et al.* (abstract only) and Jin *et al.*

have also recorded similar findings [66,67]. Whether the peripheral blood Tregs migrate to the decidua as subsequently suggested by Tilburgs *et al.* requires confirmation [68]. Mechanistically, it is interesting that the decidual cells were more responsive to IL-2 and IFN- γ in the miscarriage group, suggesting increased Th1-type immunity arising from the impaired Treg function.

Impaired Treg function & autoimmunity

Taken as a whole, the presence of reduced Treg function suggests a relative increase in Th17 and Th1 activity. These are associated with inflammation and autoimmunity. Together, the changes in the activity of these cells have a deleterious effect on early pregnancy, most probably mediated by increased uterine NK cells [1]. From a clinical standpoint, it is interesting that impaired Treg function has been described in several organ-specific and systemic autoimmune diseases. These include Type 1 diabetes, multiple sclerosis [69] and SLE [70,71]. Interestingly, Tregs have been shown to directly suppress autoantibody-producing B cells in patients with SLE via a contact-dependent process [72]. However, Treg activity is clearly evident in the IPEX syndrome (immunodeficiency, polyendocrinopathy, enteropathy and X-linked) in which defects of the FoxP3 factor lead to marked deficiency in Treg numbers and function [73].

Autoimmunity, RM & impaired Treg function

Current evidence suggests that women with even minor degrees of autoimmunity have an increased frequency of RM. It is possible that this relates to the reduced Treg function evident in autoimmunity. The diminished regulation of the deleterious effects of Th1 and Th17 cells by reduced Treg function then represents the mechanism by which these autoantibodies are associated with RM. In APAS, direct trophoblast and placental pathogenicity is important; however, the possibility remains that women with anti-thyroid antibodies, ANA and other organ antibodies may have APA directed to untested anionic phospholipids, endothelial cells or proteins involved in coagulation. Stimulating Treg activity may, in the long term, represent a possible means of reducing RM [74].

Regarding the current treatment of RM in women with APAS, comparison of retrospective fertility and pregnancy outcome with audit of outcome after the combined use of aspirin and low-molecular-weight heparin has suggested benefit [75]. This was also evident in the prevention of future miscarriage in women with APAS [76] and presently remains the standard therapy. However, the treatment appears to provide little benefit for women with idiopathic miscarriage without APA [77]. Interestingly, Ziakas *et al.* in their systemic review and meta-analysis, record unfractionated heparin to be superior to low-molecular-weight heparin in reducing miscarriage in women with APAS [78]. However, the value of heparin and aspirin treatment in the management of women with APAS has recently been challenged [79,80]. In this regard, it is interesting that heparin, in conventional dosage, was shown to be incapable of downregulating trophoblast IL-6 secretion and STAT3 activity [35]. In support of its use, heparin may reduce miscarriage in women with APAS by inhibition of APA binding to heparin-binding EGF, which is important in

blastocyst implantation [81]. In regard to low-dose aspirin, the principal mechanism of action in women with APA relates to its inhibition of platelet activation and reduced thrombus formation. Whether it improves reproductive outcome in APAS remains incompletely answered [82].

Expert commentary

Systemic autoimmunity and the APAS are very likely to be linked to RM by both the direct pathogenicity of the respective auto-antibodies for trophoblastic or fetal cells and the promotion of thrombosis. However, an etiological/associated impairment of Treg function and in the consequently increased Th1 and Th17 activity may also be operative. By contrast, it is likely that organ-specific autoimmunity may be linked to RM simply as a reflection of the underlying impaired Treg function and heightened Th1/Th17 function. Reducing miscarriage in systemic autoimmunity and APAS therefore requires efforts in both reducing coagulation and restoring Treg function. In organ-specific autoimmunity, therapy should be aimed at the latter by creating a decidual environment where naive T cells are encouraged to form Tregs rather than Th17 cells. It will be interesting to see how some of the newer ideas for targeting Tregs [83] will translate into benefit for women with RM.

Five-year view

Over the next few years, the role of the different T-cell subsets in encouraging or inhibiting the development of autoimmunity will be dissected. The precise role of endometrial

infection/inflammation, age, female hormones, stress and the HLA system in altering the balance of antipregnancy Th17/Th1 activity and pro-pregnancy Tregs/Th2 immunity will hopefully be clarified. How this disturbance translates into RM via the action of uterine NK cells will also become clearer. Therapeutically, agents geared to suppressing endometrial inflammation and limiting the IL-6 production that promotes autoimmunity via a reduction of Treg function will be tested. Anti-cytokine therapies that limit the action of specific sets of cytokines are likely to be investigated in animal models with a view to encouraging Treg and Th2 function and reducing Th17 and Th1 activity. With greater understanding of the different mechanisms contributing to the RM in APAS, newer therapies will explore means of reducing the production of APA by autoreactive B cells. There will also be refinement of existing therapies aimed at inhibiting thrombosis. The greatest challenge will be preventing these therapies from crossing the placenta to cause damage or permanent alteration of the developing fetal immune system.

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Key issues

- Both organ-specific and systemic autoimmunity may be associated with recurrent miscarriage (RM).
- Impaired T regulatory cell function is evident in many types of autoimmune disease and appears to be critical to maintaining an optimal balance of Th1, Th2 and Th17 function; this balance is vital for a successful pregnancy.
- Autoimmunity to proteins of the coagulation cascade and endothelial cells may also be important in RM via prothrombotic mechanisms and a reduction of fetal nutrition involving compromised placental blood flow.
- Placental vasculopathy is probably important in late-stage RM associated with the antiphospholipid antibodies syndrome (APAS).
- Several mechanisms are involved in the early-stage RM of APAS. These include reduced proliferation, human chorionic gonadotrophin production and invasiveness of trophoblast cells, as well as increased apoptosis of these cells and impaired angiogenesis.
- APAS is also associated with premature delivery, intra-uterine growth retardation and neonatal complications.
- There is uncertainty as to the minimal panel of anionic phospholipids that need to be included in an assessment for APAS in women with RM. Clarity is also needed for whether testing for anti-anti- β_2 glycoprotein 1 antibodies and the lupus anticoagulant provides any additional information.
- There is still controversy as to the optimal management of women with RM and APAS, although a low dose of aspirin and low-molecular-weight heparin is most commonly used.

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