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Heparin and aspirin combination therapy restores T-cell phenotype in pregnant patients with antiphospholipid syndrome-related recurrent pregnancy loss

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ABSTRACT

Recurrent pregnancy loss (RPL) is the most common manifestation of anti-phospholipid syndrome (APS), and activated $CD4^+$ T cells are involved in its pathogenesis. Treatment with low-molecular weight heparin (LMWH) and aspirin combination improves pregnancy outcome, however, its mechanism of action is unclear. We investigated the effect of this therapy on Th1/Th2 cells in 89 patients with APS-RPL. The results showed that serum cytokine levels, T cell phenotypes, and transcription factors' gene expression levels representing Th1 responses were higher, whereas those representing Th2 responses were lower in patients with APS-RPL at the time of early pregnancy. This Th1-bias was reversed in patients who had live birth after receiving the combination therapy at the time of delivery. Patients with miscarriages continued to exhibit Th1-bias. In conclusion, these data support a role of Th1-bias in the pathogenesis of APS-RPL and suggest restoring T-cell phenotype as a new immunomodulatory mechanism of LMWH/aspirin combination.

1. Introduction

Recurrent pregnancy loss (RPL) is a major manifestation of anti-phospholipid syndrome (APS) [1]. Pathogenesis of APS-related RPL (APS-RPL) remains unclear. Thrombosis and hypercoagulable state were believed to mediate APS-RPL [2]. However, accumulating evidence suggests a pivotal role of immune dysfunction in the pathogenesis of APS-RPL [3,4]. For example, a recent study showed activated $CD4^+$ T cells in pregnant patients with lupus and/or anti-phospholipid antibodies [5]. Studies in patients and animal models also suggest a perturbed T-helper 1 (Th1)/Th2 cytokine homeostasis in APS [6,7]. Some studies have suggested a Th1-bias [8], while others have reported a shift to Th2 cytokines in APS [9]. However, the exact in vivo abnormalities in Th1/Th2 responses in APS-RPL are mostly unclear.

Numerous studies have shown that Th2 cytokines interleukin (IL)-4 and IL-10 are overexpressed, whereas the expression of Th1 cytokines IL-2 and interferon (IFN)-γ is reduced in the striatum and peripheral blood of normal pregnant women [10]. Thus, Th2-bias of the maternal immune response is generally considered to maintain pregnancy [11]. Such state of immune tolerance is perturbed in humans and animals with APS with a polarization towards a Th1 response [12]. This altered immune response is believed to damage the placental villi and embryonic tissues, affecting the development and implantation of fertilized eggs as well as the growth and development of embryos, consequently leading to adverse pregnancy outcomes including miscarriage and premature birth [13].

Combination therapy of LMWH and aspirin has been shown to improve pregnancy outcome in patients with APS-RPL and is considered to be the standard of care for APS-RPL [14]. Despite being the pillars of APS-RPL treatment, their precise mechanisms of action are not completely understood. This study evaluated the relationship between Th1/Th2 cells and APS-RPL. We further investigated the effect of

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combination therapy with LMWH and aspirin on Th1/Th2 cell profile in patients with APS-RPL. Our data suggest that the Th1/Th2 imbalance is restored in patients treated with LMWH/aspirin combination therapy.

2. Patients and methods

2.1. Patients

We enrolled a cohort of patients, who visited Peking University Shenzhen Hospital between August 2016 and November 2018, with a history of APS-RPL. All patients fulfilled the 2006 revised International Sapporo Classification of Antiphospholipid Syndrome (APS) [15]. Patients with uterine malformations, chromosomal abnormalities, endocrine factors, reproductive tract inflammation, and other autoimmune diseases, such as systemic lupus erythematosus (SLE), were excluded from this study. Patients using immunosuppressive therapies, such as glucocorticoids, within three months prior to enrollment were also excluded.

Eighty-nine APS-RPL patients who satisfied the above inclusion and exclusion criteria were consented to receive LMWH/aspirin combination therapy. All 89 had 3–6 previous pregnancies and 2–6 miscarriages. All but four participants had miscarriages within 12 weeks of pregnancy. Thirty-one age-matched healthy pregnant women who delivered in our hospital were used as a control group. All patients signed an informed consent form to participate in this study. Demographic and clinical features of patients are described in Table 1.

2.2. Therapeutic regimen

After confirming a positive pregnancy test (at about 5 weeks of pregnancy), patients were administered 5000 IU LMWH (Fragmin®Pharmacia & Upjohn) via subcutaneous injection once a day and 50–75 mg of aspirin (Beijing Taiyang pharmaceutical Co. LTD) orally once a day until 35–37 weeks of pregnancy or up to the time of delivery. All patients and healthy controls received oral folic acid supplementation as a standard of care. Live birth was used as an efficacy measurement.

2.3. Sample collection

20 mL peripheral venous blood was collected at about 5 weeks of pregnancy before starting the drug (baseline) and at 35–39 weeks of gestation or at the time of delivery or abortion after stopping the drug in APS-RPL patients (after treatment). The control blood samples were drawn from healthy pregnant women at about 5 weeks of pregnancy and at the time of delivery in our hospital.

Table 1
Demographic and main clinical features of APS-RPL patients.

Characteristic	APS-RPL (n = 89)	Control (n = 31)
Age, yr (mean ± SD)	30.69 ± 4.58	29.74 ± 5.02
Co-morbidities	No	No
Thrombotic events	No	No
Gravidities	3–6	1–3
Miscarriages	2–6	No
aPL profile		
aCL IgG/IgM (n, %)	35 (39.3)	Negative
Anti-β2GP1 IgG/IgM (n, %)	49 (55.1)	Negative
LA (n, %)	17 (19.1)	Negative
Single aPL positivity (aCL or anti-β2GPI) (n, %)	61 (68.5)	Negative
Double aPL positivity (aCL + anti-β2GPI) (n, %)	27 (30.3)	Negative
Triple positivity (aCL + anti-β2GP1 + LA) (n, %)	1 (1.1)	Negative
Treatment		
LMWH/aspirin combination	89 (100)	No
Folic acid	89 (100)	31 (100)

aCL, anti-cardiolipin; anti-β2-GPI, anti-β2 glycoprotein-I; aPL, antiphospholipid; LA, lupus anticoagulant; LDA, low-dose aspirin; RPL, recurrent pregnancy loss; SD, standard deviation.

2.4. PBMCs and serum

PBMCs were isolated using lymphocyte isolation solution (Sino-American Biotechnology Co., Ltd., Tianjin, China) and density centrifugation for flow cytometry and real-time qPCR. Isolated serum samples were stored at −80 °C for ELISA.

Reagents and Antibodies are listed in Supplemental material.

2.5. ELISA for serum Th1/Th2 cytokines measurement

Serum levels of Th1 cytokines, namely IL-2 and tumor necrosis factor (TNF)-α, and Th2 cytokines, namely IL-4 and IL-10, were measured using respective human cytokine ELISA kits obtained from R&D Systems Bio-Techne Co. (Minneapolis, MN). ELISA was performed in accordance with the manufacturer's instructions.

2.6. Analysis of Th1/Th2 cell subsets by flow cytometry

PBMCs were isolated and transferred to 12 wells plates, resuspended in 1 mL culture medium with 2 μL of Cell Activation Cocktail (PMA/ionomycin with Brefeldin A), then incubated at 37 °C in a CO_2 incubator for 6 h. Activated cells were harvested and collected in 96-well plates at a density of about 2×10^5 cells/well. 50 μL FITC-CD4 antibody and PerCP anti-human CD3 (1:200 dilution) were added, and plates incubated for 30 min at 4 °C in the dark. Cells were washed and fixed with 1% paraformaldehyde at room temperature in the dark. After fixation, cells were washed with permeabilization washing buffer (1×) and centrifuged at 2300 rpm for 5 min, then incubated with 50 μL APC-IFN-γ and PE-IL-4 (1:200 dilution) for 30 min at 4 °C in dark. Cells were acquired using Beckman Coulter Epics XL Flow Cytometer (SPW Industrial, CA) within 48 h, and data analyzed using FlowJo 10.5.3.

2.7. Analysis of Th1/Th2 cell-specific transcription factors expression by quantitative real-time PCR

Total RNA (0.5 μg) from freshly isolated PBMCs was assessed by qRT-PCR in accordance with the manufacturer's instructions. The cDNA products were used to detect the Th1/Th2 cell-specific gene mRNA expression under the following conditions: initial denaturation at 95 °C for 30 s, 40 cycles of 95 °C for 10 s and 65 °C transcription for 30 s. The relative expression level was calculated using *GAPDH* as internal reference gene and the Delta-Delta-CT method to calculate the relative quantity (RQ) as follows: $RQ = 2^{-\Delta\Delta ct}$ of T-bet and GATA3 mRNA.

Supplemental Table 1 shows the mRNA primer sequences of T-bet, GATA3, and GAPDH.

2.8. Statistical analysis

SPSS 24 software was used for statistical analysis in this study. Values are presented as the mean ± SD. Student's *t*-test was used to compare data between two groups. One-way ANOVA and Bonferroni *t*-test were used to compare data between three or more groups. $P < .05$ was considered significant.

3. Results

3.1. Therapeutic effect of LMWH and aspirin combination therapy

Of 120 human subjects we enrolled, none had other comorbidities or thrombotic events. Before this pregnancy, all 89 patients with APS-RPL had 3–6 gravidities and 2–6 miscarriages. Anti-β2 glycoprotein I

Table 2
Outcomes of pregancy in patients with APS-RPL.

Outcomes	APS-RPL (n = 89)	Control (n = 31)
Live birth (n, %)	72 (80.9)	30 (96.8)
Full-term delivery (n, %)	67 (75.3)	30 (96.8)
Premature delivery (n, %)	5 (5.6)	–
Pregnancy loss	17 (9.1)	1 (3.2)
Early miscarriage (< 10 weeks of gestation) (n, %)	14 (5.7)	1 (3.2)
Intrauterine death (> 10 weeks of gestation) (n, %)	3 (3.4)	–

antibody (anti-β2GPI) was present in 55% patients, anti-cardiolipin antibody (aCL) in 39%, and lupus anticoagulant (LA) in 19% (Table 1). None of 31 healthy controls had history of adverse pregnancy outcome (s) and anti-phospholipids.

Of the 89 patients receiving combination therapy, 72 patients (80.9%) had live births, including 67 full-term and 5 premature births. The remaining 17 patients had miscarriages, including early miscarriages in 14 patients at 8–10 weeks of pregnancy and late miscarriages in 3 patients at 23–27 weeks of pregnancy. Only one case of early abortion occurred in the control group. No placental abruption, stillbirth, or neonatal malformations were detected. Table 2 shows the outcomes of pregnacy in all patients.

3.2. Effect of LMWH and aspirin combination therapy on serum cytokines levels

At 5 weeks of gestation (baseline), serum levels of IL-2 and TNF-α were significantly higher in patients with APS-RPL than in healthy pregnant women, whereas serum levels of IL-10 and IL-4 were significantly lower in APS-RPL patients than in the healthy group (Fig. 1). These cytokine differences were no more seen at the time of delivery between healthy control women and in women with APS-RPL who had a successful pregnancy outcome (live birth [LB] group). However, women with APS-RPL who had an adverse pregnancy outcome (non-response [NR] group) continued to have significantly elevated IL-2 and TNF-α and significantly reduced IL-10 and IL-4 as compared to healthy control group and to women with APS-RPL who had a live birth. These data demonstrate a link between Th1 polarization and adverse pregnancy outcome in APS-RPL. Furthermore, the successful pregnancy outcome after LMWH/aspirin therapy correlates with the restoration of Th1-Th2 imbalance.

3.3. Effect of LMWH and aspirin combination therapy on Th1/Th2 cell subsets

We determined the profile of Th1/Th2 cell subsets in APS-RPL patients before and after treatment and at equivalent time points in healthy pregnant women. At the baseline, $CD4^+IFN\text{-}\gamma^+$ cells, designated Th1 subset, were significantly increased, while $CD4^+IL\text{-}4^+$ cells, designated Th2, were significantly reduced in APS-RPL compared to healthy control pregnant women (Fig. 2A, C, D, F). However, at the delivery time after combination therapy, the percentages of $CD4^+IFN\text{-}\gamma^+$ T cells in the live-birth group were similar to those in healthy controls, but significantly lower than that in the non-responder group (Fig. 2B, C). The percentages of $CD4^+IL\text{-}4^+$ T cells in the live-birth group were also similar to those in healthy controls, but significantly higher than that in the non-responder group (Fig. 2E, F).

3.4. Effect of LMWH and aspirin combination therapy on mRNA expression of Th1/Th2 cell-specific transcription factors

To provide further evidence of the increased Th1/Th2 ratio in patients with APS- RPL, we performed qPCR to detect mRNA expression of Th1 cell-specific transcription factor T-bet and Th2 cell-specific gene GATA3 (Fig. 3). At the baseline (5 weeks gestation), patients with APS-RPL had a significantly elevated expression of T-bet, but significantly reduced expression of GATA3 mRNA as compared with the healthy control group. At the time of delivery, both T-bet and GATA3 expression levels were restored to healthy control levels in patients who had live-birth after the combination therapy. Patients who did not respond to treatment and had miscarriages continued to have elevated T-bet and reduced GATA3 as compared to the live birth group and healthy controls.

4. Discussion

T helper cells have been implicated in the induction and regulation of experimental APS-RPL [16,17]. However, data on different Th subsets in patients with APS-RPL are scarce [18,19]. In the present study, we measured serum levels of cytokines and enumerated the number of circulating Th1 and Th2 cells during the time of early pregnancy and the time of delivery or abortion in patients with APS-RPL and at equivalent timepoints in healthy pregnant women. More importantly, we performed a long term follow-up to investigate the dynamic changes in the balance between Th1 and Th2 cells before and after LMWH/aspirin combination therapy. We found that serum cytokine levels, T cell phenotypes, and transcription factors' gene expression levels representing Th1 responses were higher, whereas those representing Th2 responses were lower in patients with APS-RPL. This Th1-Th2 imbalance was corrected in patients who were successfully treated with LMWH/aspirin combination. To the best of our knowledge, this is the first documentation of direct effects of LMWH/aspirin combination therapy on Th responses in vivo in humans.

The pathogenic role of such Th1/Th2 perturbation in APS-RPL remains to be elucidated. In vitro studies have suggested a synergistic effect of TNF–α and IFN–γ on inhibiting the growth of embryo [20], and on induction of trophoblast apoptosis [21]. IL-2, along with TNF-α, may induce the proliferation of decidual natural killer (NK) cells and promote their differentiation into cytotoxic lymphokine-activated killer cells [22]. On the other hand, in vivo experiments have shown that serum from CBA/J x DBA/2 mice, which undergo spontaneous resorption of the placenta, contains a high level of Th1 cytokines, e.g., IL-2, TNF-α, and IFN-γ; while serum from CBA/J x DBA/2 mice with normal pregnancies contains Th2 cytokines, e.g., IL-4 and IL-10 [23]. Thus, it is possible to speculate that circulating IL-2 and TNF-α in APS-RPL patients in our study might contribute to the above effects, namely trophoblast apoptosis and NK cell differentiation, which might promote RPL in APS patients, whereas higher levels of IL-4 and IL-10 in normal pregnant women and in successfully treated APS-RPL patients might contribute to the maintenance of normal pregnancy.

Mechanisms underlying Th1 cell polarization in APS-RPL are unclear. Animal studies have shown that high doses of β2GPI can induce Th cell clonal differentiation into Th1 cells, which may then lead to miscarriage [8]. Oxidative post-translational modifications of β2-GPI have also been shown to stimulate dendritic cells and prime naive T lymphocytes, inducing Th1 polarization [24]. Furthermore, β2-GPI-specific $CD4^+$ T cell has been considered as a potential therapeutic target to selectively suppress pathogenic aPL production in APS patients [25,26]. In future, we will determine whether increased anti-ß2GPI antibodies and increased circulating Th1 cells in our patients correlate with increased ß2GPI-reactive Th1 cells. We will further determine whether LMWH/aspirin treatment modulates the activation and differentiation of ß2GPI-reactive T cells from Th1 to Th2.

Th1 and Th2 cell differentiation is regulated by their transcription factors. T-bet, a major regulator of Th1 cells, plays a decisive role in the differentiation of Th1 cells [27,28]. GATA3 is a critical transcription factor that selectively induces the differentiation from naïve Th cells into Th2 cells [29]. Studies have shown that the relationship between T-

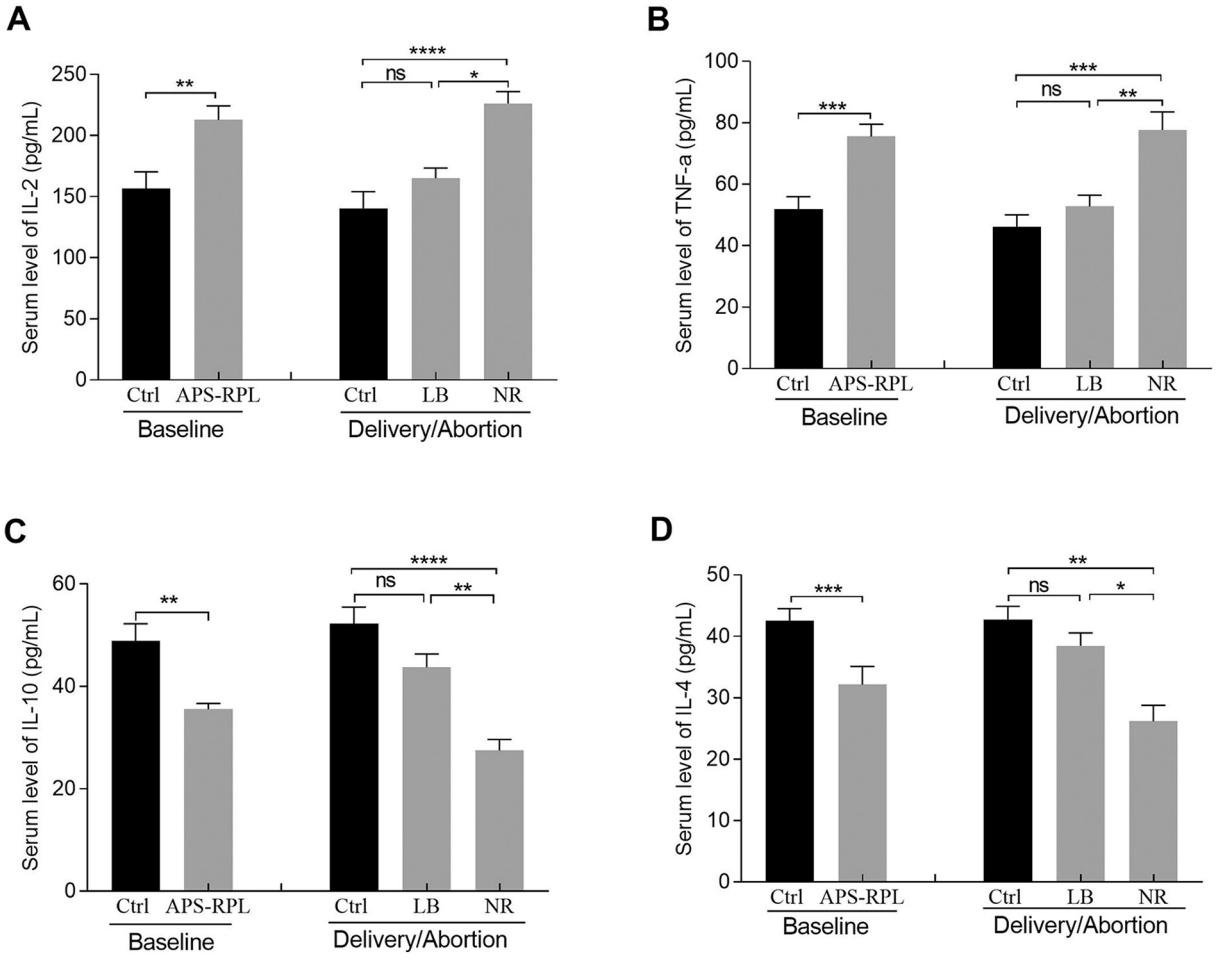

Fig. 1. Serum Levels of Th1/Th2 Cytokines in Pregnant Women. 240 serum samples from pregnant patients with APS-RPL before and after treatment with LMWH/aspirin combination therapy and at equivalent time points in healthy pregnant women were assayed for IL-2 (A), TNF-α (B), IL-10 (C) and IL-4 (D) by ELISA. Data are presented as the mean ± SD. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$, Bonferroni *t*-test ($n = 31$ healthy pregnant women at the baseline and at delivery; 89 APS-RPL patients before starting treatment, 72 patients at delivery who responded favorably to LMWH/aspirin combination therapy [LB], and 17 at abortion who did not respond to therapy [NR]). Ctrl, control; LB, live birth; NR, no response, ns = not significant.

bet and GATA3 mRNA expression is characterized by mutual regulation and antagonism, which effectively regulates the balance of Th1 and Th2 cell differentiation, thus affecting the incidence and the development of diseases [30,31]. Moreover, the expression level of T-bet mRNA was upregulated, while GATA 3 mRNA was downregulated in RPL patients compared with the healthy subjects [32]. Consistent with this report, we found increased T-bet and reduced GATA3 mRNA expression in APS-RPL patients. It remains to be determined how LMWH/aspirin therapy led to normalized T-bet and GATA3 expression in these patients.

Numerous studies have demonstrated that untreated APS-RPL have a 90% incidence of recurrent miscarriage [33,34]. Current guidelines recommend the use of LMWH/aspirin combination therapy in APS patients to reduce the risk of RPL, which is better than monotherapy [14,35–37]. A few previous studies have explored mechanisms underlying the effects of these drugs in APS. For example, aspirin may act via aspirin-triggered lipoxin that prevents antiphospholipid antibody effects on human trophoblast migration and endothelial cell interactions [38]. Heparin has been suggested to prevent antiphospholipid antibody-induced fetal loss by inhibiting complement activation [39]. A recent study also examined the effects of LMWH on the polarization and cytokine profile of Th cells in vitro [40]. They found reduced Th2 cytokines and increased Th1 cytokines upon adding LMWH to cultures [40]. We did not find a previous study on LMWH/aspirin effects on Th responses in vivo. In the present study, APS-RPL patients, who were treated with LMWH/aspirin combination and had live births, had increased Th2 and decreased Th1 cytokines, cells, and transcription factors as compared to baseline. The discrepancy between our results and that of previous in vitro study [40] could be due to differences in in vivo effects in physiological conditions versus in vitro effects in selected culture conditions. Furthermore, LMWH/aspirin combination may act differently than LMWH and aspirin alone. We surmise that clinical improvement after LMWH/aspirin combination therapy is probably related to the modulation of Th1/Th2 responses.

To reiterate the strengths of our study, we used thus far the largest primary APS-RPL cohort to examine Th functional responses in vivo. We defined Th1/Th2 responses by circulating cytokine levels and flow cytometry as well by transcription factor gene expression. Furthermore, we evaluated immune effects in the context of a clinical outcome by a commonly used treatment.

Finally, we are cognizant of the limitations of our study. Firstly, our results do not provide the direct proof that combination therapy restores Th1/Th2 imbalance. It remains to be determined whether other effective therapies for APS will also restore Th1/Th2 imbalance in APS-RPL patients. Nevertheless, our robust findings form the basis for future studies to examine whether and how LMWH/aspirin combination therapy directly modulates Th cell differentiation. Secondly, most (69%) patients in our cohort were positive for one aPL, 30% patients were positive for two aPLs, and all three aPLs were positive in one patient. A similar low frequency of triple positive aPLs was reported in a

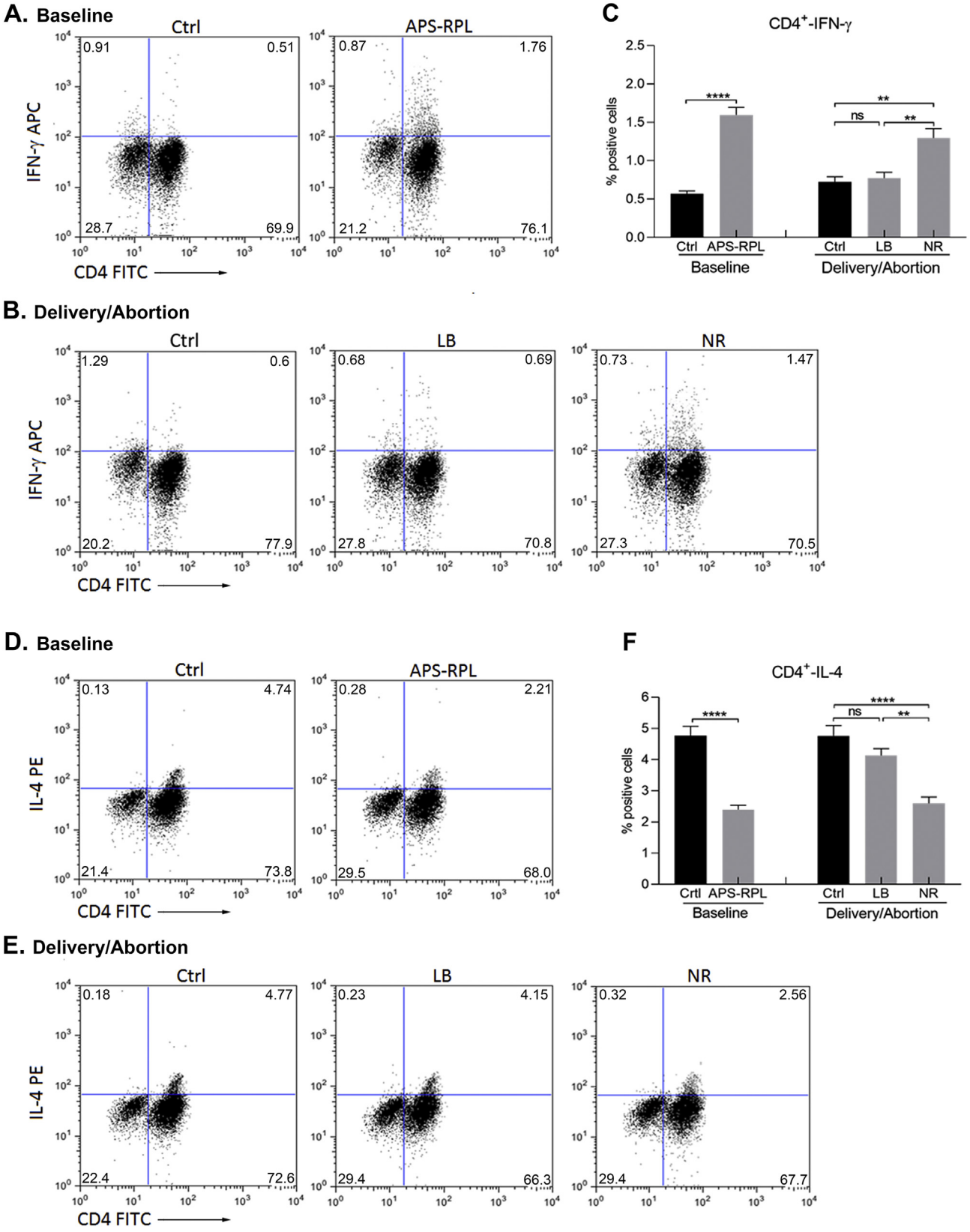

Fig. 2. Th1/Th2 cell subsets in pregnant women. PBMCs were obtained from pregnant women (healthy and APS-RPL) at the baseline (A, D) and at the time of delivery/abortion (B, E), and stimulated as described in Methods. Stimulated cells were stained for CD3, CD4, and INF-γ or IL-4, and analyzed by flow cytometry. Representative dot plots illustrate $CD4^+INF\text{-}\gamma^+$ cells (A, B) and $CD4^+IL\text{-}4^+$ cells (D, E) on gated live lymphocytes. Combined data with statistics are shown in C and F for IFN-γ and IL-4, respectively. Data are presented as the mean ± SD. *p < .05, **p < .01, ***p < .001, ****p < .0001, Bonferroni t-test (n = 31 healthy control women at baseline and at delivery, 89 APS-RPL patients before starting LMWH/aspirin combination treatment, 72 APS-RPL patients at deliverly who responded favorably to therapy (LB), and 17 APS-RPL patients at abortion (NR) who had no response. Ctrl, control; LB, live birth; NR, no response; ns = not significant.

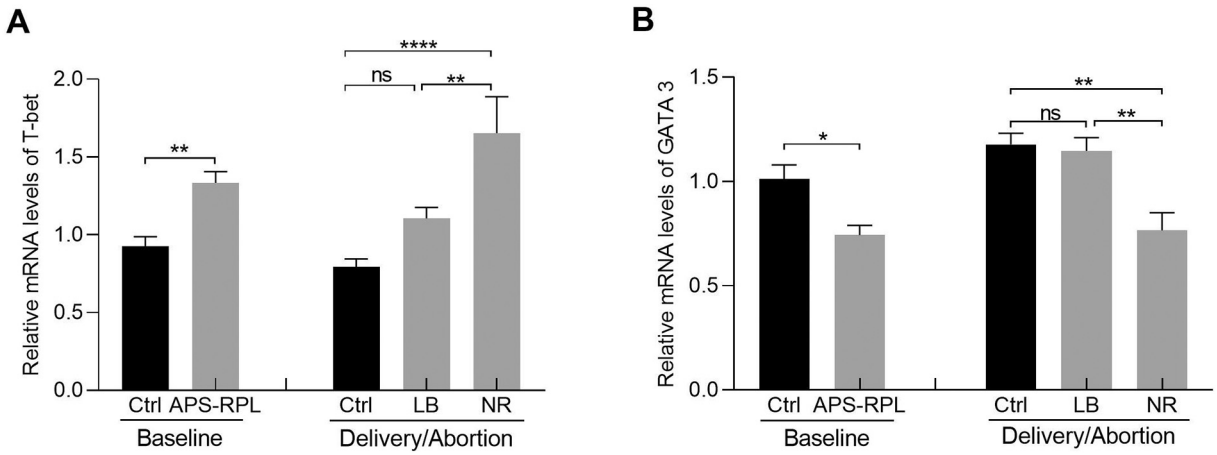

Fig. 3. Gene expression of Th1/Th2 cell-specific transcription factors in pregnant women. RNA extracts from 240 PBMC samples were analyzed for the relative mRNA expression levels of T-bet (A) and GATA3 (B). PBMCs were obtained at 5 weeks of gestation and at the time of delivery from healthy pregnant women who did not receive any medications (n = 31), APS-RPL patients before starting treatment (n = 89), LB group who responded favorably to LMWH/aspirin combination therapy (n = 72), and NR group who had no response (n = 17). Data are presented as the mean ± SD. *p < .05, **p < .01, ***p < .001, ****p < .0001, Bonferroni t-test. Ctrl, control; LB, live birth, NR, no response, ns = not significant.

large multi-center cohort, PREGNANTS (Pregnancy in Women with Antiphospholipid Syndrome), where only 2.7% of 750 singleton pregnancies were triple positive [41]. Notably, the rate of triple aPL positivity has varied in different study populations from 57% in APS patients with thrombosis to 2.7% in APS patients with obstetric events alone [41–46]. We selected patients based on the history of recurrent miscarriages. Although, we did not deliberately exclude patients with arterial or venous thrombosis, none of our APS-RPL patients had thrombotic events. Prospective studies in large, unselected cohort of APS patients may help distinguish whether triple aPL positivity is a feature of APS-thrombosis, whereas APS-RPL patients have single or double positive aPLs. Thirdly, we did not test the effect of LMWH/aspirin therapy on other T cell subsets, including regulatory T cells (Treg) and Th17 cells. A previous study has implicated an altered balance between Th17 cells and Treg cells in the pathogenesis of reproductive failure in APS patients [47]. However, another study found increased Th17/Treg ratio in SLE patients but not in primary APS patients [48]. Comprehensive analyses of these and additional T cell subsets in circulation and at maternal-fetal interface in APS-RPL patients may further highlight the roles of T cells in the pathogenesis of APS-RPL.

In conclusion, our data demonstrate increased Th1 responses in patients with APS-RPL, suggesting a possible role of Th1 inflammatory milieu in the pathogenesis of APS-induced pregnancy loss. This assumption is further strengthened by our finding that such Th1 polarization is reversed in patients who attain a successful pregnancy outcome after LMWH/aspirin combination therapy. Taken together, these data provide a new understanding of potential immunomodulatory mechanism of LMWH/aspirin combination therapy in APS-RPL.

Author contributions

MYW designed the research, created figures, and wrote the manuscript. PZ analyzed data. SYY collected clinical data. GMZ and JYL performed laboratory experiments. DN assisted with figure preparation and edited English language. CSG assisted with research design. QWW was responsible for the supervision of subject recruitment and patient care. RRS assisted with research design and data interpretation, and revised the completed manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Study approval

This study was approved by the Ethical Review Committee of Peking University Shenzhen Hospital, and informed consent was obtained from all pregnant patients.

Declaration of Competing Interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.108259.

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