Altered Th17/Treg Ratio in Recurrent Miscarriage after Treatment with Paternal Lymphocytes and Vitamin D3: a Double-Blind Placebo-Controlled Study

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ABSTRACT

Background: Recurrent miscarriage (RM) affects 2-5% of pregnant women. Paternal lymphocyte immunotherapy is a common treatment for RM patients but the outcome has not been consistent. Therefore, combined therapy with other immunosuppressive drugs such as 1*a*, 25-dihydroxy-vitamin-D3 (vitamin D3) may improve the outcome. **Objectives:** To investigate the effect of vitamin D3 on the balance of two essential T cells subsets, T helper (Th) 17 and T regulatory (Treg) cells, which contribute to the immune tolerance during pregnancy. **Methods:** The expression levels of CD4 and forkhead box protein 3 (FOXP3) in Treg cells, and the expression levels of CD4 and IL-17 in Th17 cells, were evaluated pre- and 3 months post-immunotherapy in RM patients treated with a combination of paternal lymphocytes and vitamin D3 compared with RM patients receiving lymphocyte immunotherapy alone. **Results:** Vitamin D3 therapy decreased the frequency of Th17 cells in addition to reducing the Th17/Treg ratio in peripheral blood of RM patients compared with the control group ($p<0.05$). **Conclusion:** Considering that RM patients have a higher Th17/Treg ratio in peripheral blood, vitamin D3 may be a candidate therapeutic approach in this disease.

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Keywords: Paternal Lymphocyte Therapy, Recurrent Miscarriage, Regulatory T Cell, T Helper 17, Vitamin D3

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INTRODUCTION

Pregnancy is a unique immunological phenomenon. Healthy women usually carry a semi allogeneic fetus without immune rejection despite expression of different paternal antigens by the fetus (1). Recurrent miscarriage (RM) is defined as three or more consecutive miscarriages before the 20th week of gestation(2-4). The exact causes of RM are unknown but a significant proportion of miscarriages may be associated with immunological disturbance (5).

There are important evidence supporting the immune regulatory mechanisms in pregnancy(6). One of these regulatory mechanisms which may play an important role in tolerance to the fetus is the presence of regulatory T cells (Treg). It has been reported that peripheral blood Treg cells increase during early pregnancy, reach the highest level in the second trimester and then decline in the postpartum period (7,8). Possible explanations for the increase in Treg cells are hormonal changes and alterations in the expression of co-stimulatory molecules, (7). In addition, some studies have reported that Treg cells are reduced in the peripheral blood of RM patients (9,10). On the other hand, T helper (Th) 17 cells, which participate in inflammatory reactions, have also been shown to be critical in pregnancy outcome (11). Previous studies showed that Th17 cells were increased in the peripheral blood of RM patients compared to healthy pregnant women (10-12). The rise in Th17 cell number may play an important role in the inflammatory response and miscarriage. Imbalance of the Th17/Treg ratio has been demonstrated in several human diseases as a pathogenic mechanism. Recent studies showed that Th17/Treg ratio was increased in patients with RM compared to healthy pregnant women (10,13,14).

Lymphocyte Immune Therapy (LIT) has been introduced as a common treatment in RM patients although the efficacy of LIT remains controversial (2,3).It has been hypothesized that vitamin D3 may be a good candidate in the treatment of inflammatory-mediated disease by inhibiting inflammation and eliciting an anti-inflammatory response(15). Since a successful pregnancy is dependent on anti-inflammatory responses, vitamin D3 could potentially be an effective treatment in RM patients due to its immunomodulatory properties (15,16). Vitamin D3 has been shown to inhibit IL-17 expression *in vitro*(17), and to induce the reciprocal differentiation and/or expansion of fork head box protein 3 (FOXP3) regulatory T cells (18), both of which could help to generate an appropriate anti-inflammatory environment for pregnancy. Considering the imbalance of Th17/Treg in RM patients, and the effects of vitamin D3 on these two cell subsets, we designed this study to investigate the immunomodulatory effects of vitamin D3 on the frequency of peripheral blood Th17 and Treg cells when used in combination with conventional lymphocyte therapy in RM patients in comparison with RM patients receiving LIT alone.

MATERIALS AND METHODS

Patients. This study was a double blind placebo-controlled clinical trial that was performed in Isfahan University of Medical Sciences from October 2013 to September 2014. Forty four patients with primary recurrent abortion (without a live birth) with a mean age of 27.2 ±5 years were included in the study and randomly assigned to the treatment (n=22) and control (n=22) groups.

Patients were eligible for inclusion in the study if they had a history of at least three unexplained consecutive miscarriages with the same partner. The second inclusion criteria was a negative or normal result in a screening panel including hormone tests (Follicle-stimulating hormones; FSH, Luteinizing hormone; LH), male and female karyotypes, antinuclear antibodies, and spacious thrombophilia screen (anti phospholipid antibodies, anti-cardiolipin antibodies, and lupus anticoagulant antibodies). Furthermore, RM patients were analyzed for the presence of serum blocking antibodies and only those with negative results were included. In addition, RM patients and their partners were screened for infections with Human immunodeficiency virus (HIV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), and TreponemaPallidum particle agglutination assay (TPPA) and only those couples with negative results were included included in this study. Serum vitamin D3 levels of all RM patients was measured by ELISA (IDS 25-Hydroxy vitamin DEIA kit, IDS 25-Hydroxy vitamin DEIA kit IDS, Boldon, UK) and those who were deficient (less than 20 ng/ml) and had not received vitamin D3 in past 3 months were included in the clinical trial. Patients were excluded from the trial if they had one or more of the following conditions: fewer than three consecutive miscarriages, positive or abnormal RM screening tests, a positive infectious test, not deficient in vitamin D3, or had recently consumed vitamin D3.

Study Design. Identified study subjects were randomly and consecutively assigned to treatment and control groups. Randomization was performed by a statistician in binary blocks. The subjects and investigator were blind to randomized categories. Both groups of RM patients were immunized with mononuclear cells from their partners. All patients were given three courses of LIT at 4 week intervals. The treatment group was administered one 300,000 IU dose of intramuscular vitamin D3 (PakhsDaru, Karaj, Iran); while the control group was administered one dose of packaged inert placebo. All patients were advised to use a safe contraception method (3) to prevent pregnancy for 3 months post-injection because of the possible toxic effect of vitamin D3 on the fetus. Vitamin D3 levels were measured 3 months post injection of vitamin D3 or placebo in both groups. After explaining the purpose and risks of this protocol, written and verbal informed consent was obtained from all patients participating in this study. The protocol for the present study was approved by the Ethics Committee of the Isfahan University of Medical Sciences (Isfahan, Iran). This trial was registered under number IRCT201201258820N1 atwww.irct.ir (www.irct.ir/fa #IRCT201201258820N1).

Combined Immunotherapy with Paternal Mononuclear Cells and Vitamin D3. Fresh heparinized peripheral blood samples (250ml) were obtained from RM patients' partners and peripheral blood mononuclear cells (PBMCs) were isolated under sterile conditions using Ficoll-Hypaque (Lymphopreb, Oslo, Norway) density centrifugation. Cells were separated and washed and then suspended in 3ml of sterile saline. RM patients received 1ml of this suspension subcutaneously in two different areas and 1ml intravenously. All patients were given paternal mononuclear cells at 4 week intervals until Anti-Paternal Cytotoxic Antibodies (APCA) were detected in the cross match (up to three courses) (3). At the same time, immunized RM patients were randomized to either the treatment group that received one dose of vitamin D3 (300,000 IU) intramuscularly or the control group who were given a placebo injection.

Complement Dependent Cytotoxocity (CDC). Post-LIT, patients were screened for positive serum blocking antibodies such as APCA. Maternal serum was incubated with paternal PBMCs (2×10^6) at room temperature. CDC results were determined by

calculating the percentage of killed cells by inverted microscopy. Presence of more than 20% killed cells was considered a positive CDC result (19).

Cell Pareparation for Flowcytometry. Fresh blood samples were obtained from RM patients and collected into tubes containing 0.2ml sodium Heparin before and after immunotherapy. PBMCs were separated using standard Ficoll-Hypaque density centrifugation (as above). The cells were washed twice with sterile saline and aliquoted into separate tubes for Treg and Th17 analysis. For the analysis of Th17, PBMCs of each RM patient, were resuspended at a density of 2×10^6 cells/ml in complete culture medium (RPMI 1640 supplemented with 2mM glutamine, 10% heat-inactivated fetal calf serum Gibco BRL, 100 U/ml penicillin, 100 µg/ml streptomycin) and then transferred to each well of 24-well plates. For induction of Th17cells to produce IL-17, PBMCs were stimulated with ionomycin (1µM) plus PhorbolMyristate Acetate (PMA 50ng/ml) for 4 hours in the presence of monencin (500 ng/mL) as golgistop. Cells were incubated at 37^{o}C in 5% CO_2-in-air. After 4 hours, cells were transferred to 5ml sterile tubes and washed once in Phosphate buffered saline (PBS) at 252×g for 5min(20).

Flowcytometric Analysis. All antibodies, isotype controls and buffers were purchased from eBioscience (San Diego, CA, USA). Cells were incubated with anti-human CD4 antibody (clone RPA-T4) conjugated to Peridinin-cholorophil-protein with cyanin-5.5 (PerCpCy5.5) at 4^{o}C for 30 min in separate tubes for Th17 and Treg staining. Fixation and permeabilization buffers were used according to manufacturer's instructions. Intracellular staining was performed with Fluorescein isothiocyanate (FITC)-conjugated anti-human IL-17A antibody (clone eBio64DEC17) for Th17 detection and phycoerythrin (PE)-conjugated anti-human FOXP3 antibody (clone PCH101) for detecting Treg(20). Isotype controls were used to enable correct compensation and to confirm antibody specificity. Flow cytometric analysis was performed on FACSCalibur Flow cytometer (BD Bioscience) by accumulating up to 100,000 events per tube. Flow cytometric analysis was performed for all patients pre- and 3 months post-LIT.

Statical Analysis. Calculation of Th17/Treg ratio made through dividing the percentage of Th17 to Tregin each patient. The overall Th17/Treg ratio expressed as median of all patients in each group.The comparison of Th17, Treg cells and Th17/Treg ratio in the treatment and control group, pre- and 3 months post-LIT, and between these groups pre- and post-combinational therapy with LIT and vitamin D3 or placebo, were performed using the Wilcoxon signed-rank test and the Mann-Whitney U test, respectively. Results were expressed as median values. All data analyses were processed by SPSS statistical software (version 20). A p value less than 0.05 was considered to be statistically significant.

RESULTS

Forty four patients with primary recurrent abortion (without a live birth) with a mean age of 27.2 ±5 years were included in the study and randomly assigned to the treatment (n=22) and control (n=22) groups. The average time of miscarriage was 15 and 12 weeks for treatment and control groups, respectively. No significant differences were observed in the serum levels of vitamin D3 between treatment and control groups pre-LIT. All participating subjects (n=44) received LIT. The mean levels of vitamin D3 was measured in treatment (28.29±7.73ng/ml) and control groups (14.88±8.2ng/ml) post treatment (Table1).The mean percentage of the Th17 and Treg frequency, as well as the

Th17/Treg ratio in peripheral blood was calculated for subjects pre- and 3 months post-treatment. All samples were taken from RM patients in follicular phase.

Table 1. Clinical characteristics of RM women enrolled in this study.

Clinical characteristics	Treatment N=22	Control N=22
Age (Year)	26.59±4.68	27.81±5.6
Mean of abortion	3.45±0.8	3.36± 0.65
Dose of vitamin D3 (IU/ml)	300,000	-
Serum Vitamin D3 level baseline (ng/ml)	14.23± 5.7	14.57±8.4
	28.29±7.73	14.88±8.2
Serum Vitamin D3 level after treatment (ng/ml)	15	12
Average of miscarriage weeks		

The Frequency of Th17 Cells in Peripheral Blood of RM Patients before and after Immunotherapy. The flowcytometric results indicated that the mean percentage of Th17 cells significantly decreased post-LIT in the treatment group (0.93% vs. 0.43%, p = 0.001) and control group (0.92% vs. 0.65%, p=0.001) when compared to the Th17 cell percentages before therapy. However, the decline in Th17 cell frequency was significantly more in the treatment group than in the placebo group when compared to baseline values (p=0.01; Table 2) (Figure 1).

Table 2. Peripheral blood Th17, Treg, and Th17/Treg values in RM women before and after vitamin D3 or placebo therapy.RM women in treatment and control groups received vitamin D3 and placebo, respectively.

	Control				Treatment				
Parameters	**Before**		**After**		**Before**		**After**		p
	Med	**Mean ± SD**	**Med**	**Mean ± SD**	**Med**	**Mean ± SD**	**Med**	**Mean ± SD**	
Th17	0.92%	1.17±0.84	0.65%[a]	0.8±0.56	0.93%	1.27±0.94	0.43%[a]	0.57±0.55	0.01*
Treg	2.46%	2.76±1.19	3.34%[a]	3.86±1.35	2.21%	2.61±1.32	4.02%[a]	4.04±1.66	0.14
Th17/Treg ratio	0.32**	0.46±0.37	0.17[a]**	0.21±0.15	0.48**	0.51±0.29	0.12[a,]**	0.15±0.13	0.001*

a :Significant in comparison with baseline value (P value < 0.05)

* Significant reduction of Th17 and Th17/Treg ratio in treatment group compared to control group

** Th17/Treg ratio was achieved through dividing the percentage of Th17 to Treg in each patient and the reported values, presented as median of Th17/Treg ratios in each group before or after treatment.

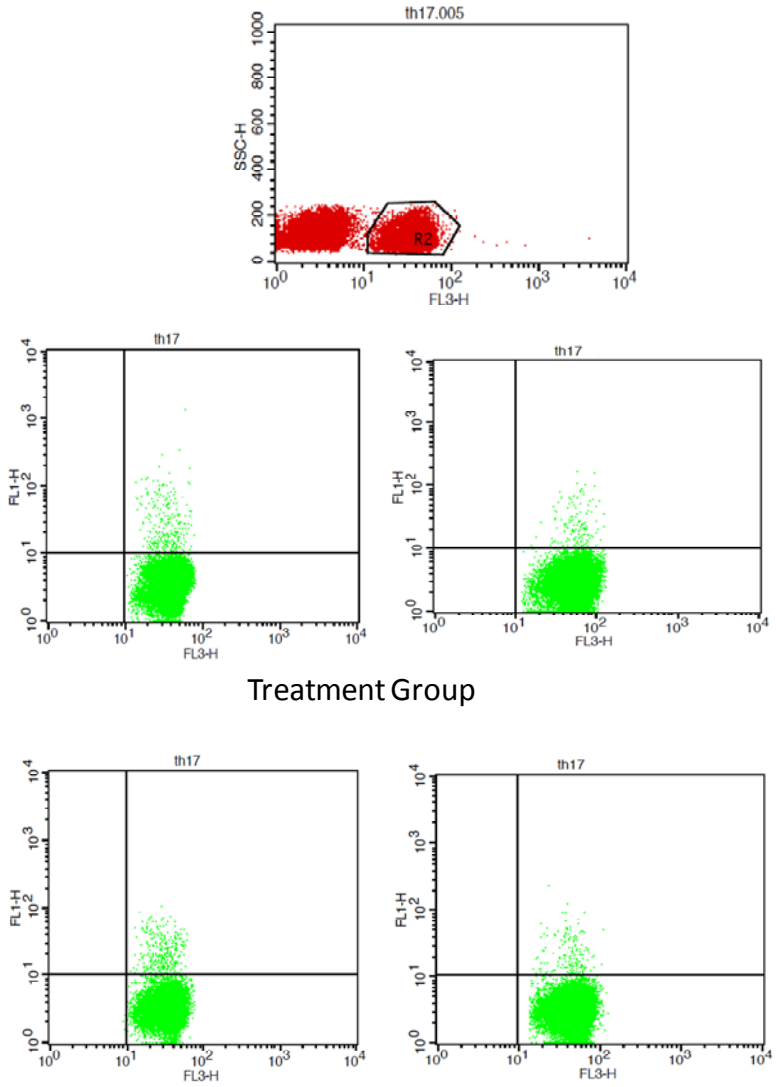

Figure 1. Flowcytometric analysis of Th17 cells in representative patient before and after immunotherapy (treatment and control groups). (A and B) evaluation of Th17 cell percentage in CD4+ lymphocyte population before and after treatment in control group. (C and D) evaluation of Th17 cell percentage in CD4+ lymphocyte population before and after treatment in treatment group. Plots shown were gated on CD4+ lymphocytes population (R2). X and Y axes are anti-human CD4 antibody conjugated to Peridinin-cholorophil-protein with cyanin-5.5 (PerCpCy5.5) and Fluorescein isothiocyanate (FITC)-conjugated anti-human IL-17A antibody respectively.

The Frequency of Treg Cells in Peripheral Blood of RM Patients after Immunotherapy. The frequency of Treg cells was significantly increased post-LIT in both the treatment (2.21% vs. 4.02%, p=0.001) and the control group (2.46% vs. 3.34%, p=0.001). There was no significant difference found for post-treatment peripheral blood Treg percentage between the treatment group and the control group (p=0.14; Table 2).

Th17/Treg Ratioin Peripheral Blood of RM Patients before and after Immunotherapy. The ratio of Th17/Treg in peripheral blood of RM patients was significantly decreasedpost-LIT in both the treatment (0.48 vs. 0.12, p=0.001) and the control group(0.32 vs. 0.17 p=0.001**)**. The Th17/Treg ratio decline, in the treatment

group was significantly higher than in the control group after vitamin D3 therapy (p=0.001; Table 2).

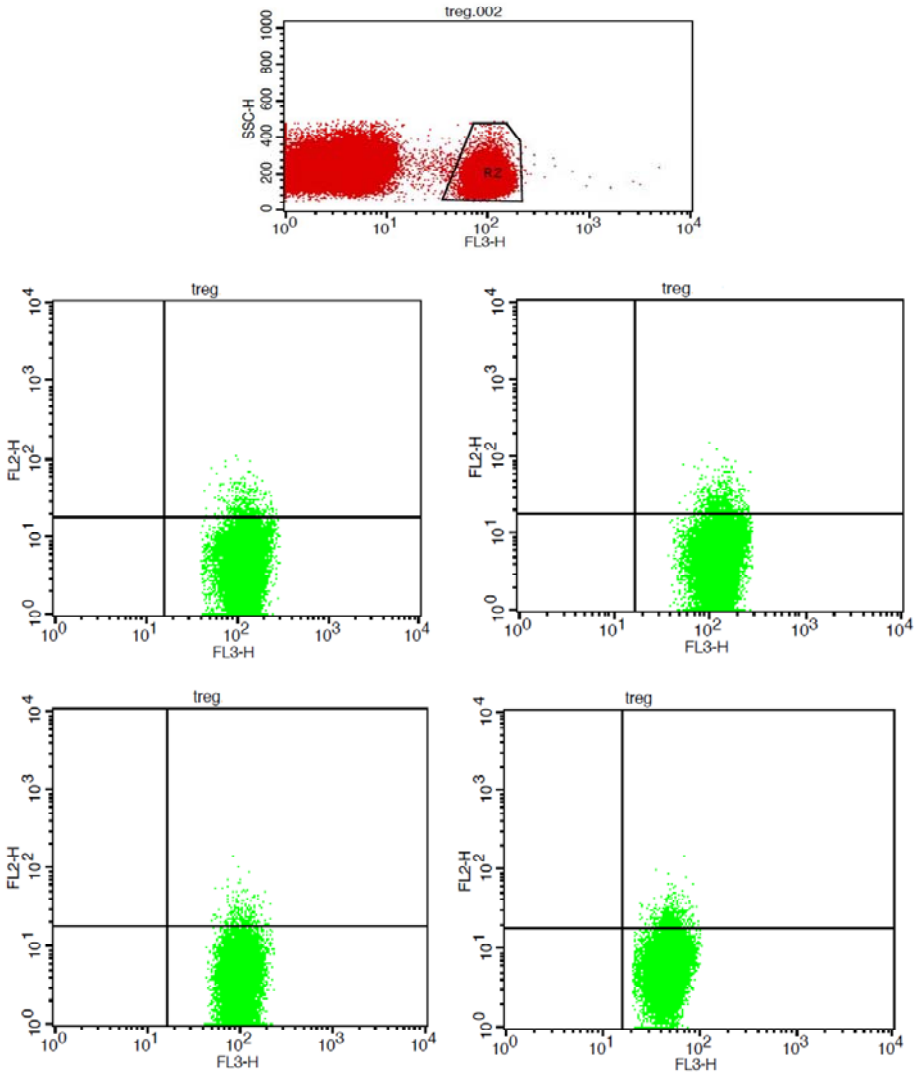

Figure 2.Flowcytometric analysis of Treg cells in representative patient before and after immunotherapy (treatment and control groups). (A and B) evaluation of Treg cell percentage in CD4+ lymphocyte population before and after treatment in control group. (C and D) evaluation of Treg cell percentage in CD4+ lymphocyte population before and after treatment in treatment group. Plots shown were gated on CD4+ lymphocytes population (R2). X and Y axes are anti-human CD4 antibody conjugated to Peridinin-cholorophil-protein with cyanin-5.5 (PerCpCy5.5) and phycoerythrin (PE)-conjugated anti-human FOXP3 antibody respectively.

Pregnancy Outcome. At the end of at the end of 7 months of follow up, months follow up, 8 of the 22 patients in the control group have become pregnant. The pregnancy continued in 2 of the 8 patients, while the remaining 6 cases experienced miscarriage at 6-12 weeks of gestation. Neither of the two patients in whom the pregnancy continued

has yet delivered. 10 of 22 patients in the treatment group have sofar experienced pregnancy without miscarriage. 5 of 10 pregnancies have delivered babies and the remaining are in gestational week 12-35.

DISCUSSION

Pregnancy is considered to be a state of immunologic tolerance(6) Moreover, there is accumulating evidence indicating an increase in Th17/Treg ratio in RM patients (10,13,14). Although this imbalance is not the major cause of miscarriage(11), it may play a crucial role in the development of an inappropriate environment for the fetus and could be one of the reasons which increases the risk of recurrent miscarriage in each pregnancy (21,22).Thus, factors which inhibit Th17 cells or induce an anti-inflammatory response could be beneficial for term pregnancy. One of these factors is vitamin D3. The immunomodulatory effects of vitamin D3 led to a hypothesis presented by Bubanovic who introduced vitamin D3 as a new potential immunotherapy agent for RM patients (15). In the present study the Th17 and Treg frequency and Th17/Treg ratio in peripheral blood of the treatment group that received vitamin D3 was compared with the control group who only received treatment with paternal mononuclear cells.

Considering the similarities of autoimmune disease and RM, in which there is a predominance of inflammatory responses and imbalance in the Th17/Treg ratio, previous studies in autoimmune disorders could be compared with the present data. Results of the present study revealed that Th17 cell frequency reduced post-LIT in treatment and control groups. Moreover, vitamin D3 seems to significantly reduce the percentage of IL-17 producing cells in RM patients 3 months post-injection when compared with the control group. In another study conducted by Tian Y *etal*. in Behçet's disease, vitamin D3 seems to inhibit Th17 differentiation *in vitro*(23).

Vitamin D3 reduces the production of PGE2, an effective mediator in the differentiation of Th17/Treg intermediate cells into Th17 cells(24). Th17/Treg intermediate cells could respond to vitamin D3 through their high content of Vitamin D Receptor (VDR) altering the expression pattern of different genes including the gene for *IL-17*(25). Regarding the role of vitamin D3 in PGE2 metabolism, it could be concluded that Th17 decrease after vitamin D3 injection may be due to PGE2 reduction. In a recent study, low levels of IL-17 referred to post-transcriptional modifications in response to vitamin D3. It seems vitamin D3 may induce over expression of C/EBP homologous protein (CHOP) which suppresses IL-17 mRNA translation to protein in these cells(25). Therefore, vitamin D3 is potentially reducing the Th17 cell-mediated inflammatory immune response which is associated with recurrent miscarriage.

Our study showed that the Treg cell frequency increased post-LIT in both the treatment and the control group. Previous studies introduced paternal lymphocyte immunotherapy as a method for induction of feto-maternal immunologic tolerance through elevation of Treg cells which are responsible for maintaining a tolerogenic environment needed for fetus retention(26). Furthermore, a molecular antagonism exists between Th17 and Treg cells' transcription factors which is responsible, at least in part, for $CD4^+$ cell fate (27), thus $FOXP3^+$Treg cells increase, leading to a tolerogenic circumstance which suppresses Retinoic acid-related orphan receptor γt (RORγt) expression in Th17/Treg intermediate cells. Moreover, a previous report demonstrated a large population of alloreactive effector T cells found in RM patients which respond to fetus antigens. LIT

could reduce the alloreactive effector T cell population, leading to augmentation of Treg cells in the whole lymphocyte population (26).

Although there is accumulating evidence assigning a positive effect to vitamin D3 for increasing number of $FOXP3^+$Tregs (17),there was no significant increase in Treg cell frequency observed between treatment and control groups. This is in good agreement with previous studies in relapsing-remitting multiple sclerosis (RRMS) patients who illustrated a negative correlation between vitamin D3 treatment and $FOXP3^+$ cell population size (28-30). In contrast, in experimental autoimmune encephalomyelitis (EAE) and Behçet's disease higher vitamin D3 serum levels were associated with an elevated number of $FOXP3^+$ cells. The discrepancy of the results may be due to a lack of differentiation in Treg subpopulations, since FOXP3 is expressed both in natural Treg (nTreg) and inducible Treg (iTreg) cells, so discrimination of nTreg and iTreg may possibly illustrate the vitamin D3 impact on these subsets and interpret the inconsistency of available data.

A significant reduction in the Th17/Treg ratio was observed in both the treatment and control group post-LIT. The Th17/Treg ratio has a great impact on the establishment of a pregnancy-specific immune tolerance(31). As previously reported, the Th17/Treg ratio in RM patients was significantly increased when compared to fertile controls(13), creating an improper milieu for the allogeneic fetus. Increased Th17 and reduced Treg cell frequency results in an inflammatory response leading to spontaneous abortion. Thus, alteration of the Th17/Treg ratio could be beneficial to pregnancy. As previously described, LIT is an effective way for induction of immunological tolerance through Treg cell increase, which in turn inhibits Th17 cell differentiation antagonistically (26). Furthermore, the reduction in the Th17/Treg ratio was significantly more in the treatment group which received LIT and vitamin D3 compared to the control group. Thus, it could be concluded that combinatorial therapy with vitamin D3 and LIT may play a more important role than LIT alone in Th17/Treg ratio reduction. According to the data presented herein, Th17 decrease subsequent to vitamin D3 consumption is of more importance in Th17/Treg ratio alteration, while Treg cell frequency showed no significant change following vitamin D3 injection.

To the best of our knowledge this is the first report evaluating the Th17/Treg ratio in RM patients treated with vitamin D3 and LIT. Considering the roles of Th17 and Treg cells in pregnancy outcome, alteration in this ratio may result in term pregnancy. In this combinatorial therapy, LIT seems important for providing basic requirements needed for an anti-inflammatory state as well as increasing levels of APCA, and the role of vitamin D3 seems to be to provide an anti-inflammatory condition which is in favor of pregnancy maintenance. Pregnancy outcome of the treatment and control groups is still under follow-up. Further investigation in a larger RM patient population is required to identify the optimal dose and time of vitamin D3 injection in RM patients for the best outcome.

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