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The role of T helper subsets in the pregnancy outcomes of a pregnant pristane-induced lupus mouse model

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Abstract

This research aimed to identify the role of T helper (Th) subsets in the pregnancy outcomes of a pregnant pristane-induced lupus mouse model. Thirty Balb/c mice were separated into healthy pregnant and lupus pregnant groups. For lupus induction, 0.5 cc of pristane was administered; the mice were mated 12 weeks later, and gestational day 0 (GD0) was determined by the presence of a vaginal plug. Blood pressure and urine albumin were measured at GD17. At GD18, the mice were euthanised, and serum samples, spleens and foetuses were collected. The foetal weights and lengths, number of viable foetuses and resorption were measured. The lupus group had higher systolic ($p = 0.017$), diastolic ($p = 0.011$), urine albumin ($p = 0.004$) and serum anti double stranded DNA levels ($p = 0.000$) and higher foetal resorption ($p = 0.000$). In contrast, it had a lower foetal weight ($p = 0.004$), length ($p = 0.046$) and number of viable foetuses ($p = 0.005$). Absolute numbers and percentages of Th1 ($p = 0.001$), Th2 ($p = 0.000$) and Th17 cells ($p = 0.000$) were also significantly higher in the lupus group. The Th1/Th2 ($p = 0.012$) and Th1/Th17 ($p = 0.031$) ratios in the lupus group were significantly higher compared with those of the healthy group. Correlation analyses revealed that Th1, Th2 and Th17 had significant correlations with foetal and maternal pregnancy outcomes. Th1/Th2/Th17 cells were elevated in the pregnant pristane-induced lupus mouse model and correlated with poor pregnancy outcomes.

Keywords: Systemic lupus erythematous; Pregnancy outcomes; T helper; Mouse Model

1. Introduction

An autoimmune disease that often occurs in women is systemic lupus erythematosus (SLE) [1]. The onset during reproductive years, coupled with improved survival, has led to increased numbers of pregnancies in SLE. The pregnancy outcomes have also recently improved, with the pregnancy loss rate decreasing from 43% to 17% [2]. However, SLE patients still carry a high risk of complications for both mother and child. The risks of maternal and foetal complications are still high in certain populations, including a higher risk of lupus flares, preeclampsia, hypertension, higher rates of preterm delivery and the presence of anti-phospholipid antibodies [3,4]. Yet, the pathogenesis of complications related to SLE during pregnancy is still poorly understood.SLE is considered a T helper 2 (Th2)-driven disease due to

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overexpression of Th2 cytokines and increased Th2 polarisation [5]. However, recent studies have shown some conflicting results for determining the significance of other Th subsets. In some reports, the differentiation and production of Th1-type cytokines were increased in SLE patients [6].These data indicate the important roles of Th subsets in the pathogenesis of SLE.

Pregnancy is a physiological condition in which several immunological response changes occur to maintain the immunosuppression and tolerance by the immune system to paternal and foetal antigens [7,8]. The most significant immunological change in normal pregnancy is Th2 polarisation [8]. The consequence of this process is that cellular immunities mediated by Th1 are inhibited, whereas humoral immunities mediated by Th2 are enhanced [9]. Recent studies have also shown that the frequency of circulating Th17 is extremely low in healthy subjects, especially during the third trimester of pregnancy [10].

Abnormality of Th polarisation during pregnancy may contribute to a poor pregnancy outcome [10]. Predominant Th1-type immunity is observed in abortion [11], but predominant Th2-type immunity is also reported in recurrent pregnancy loss [12]. Moreover, recent data have shown an increased prevalence of Th17 in the decidua and peripheral blood of spontaneous abortion patients that has not yet been explained [13]. Although Th1/Th2/Th17 imbalance has been documented in various clinical settings with poor pregnancy outcomes [9,11–13], the role of the Th subsets is still poorly investigated in terms of the pregnancy outcomes of SLE during gestation. Thus, the aim of this research was to evaluate Th1/Th2/Th17 during pregnancy in a mouse model of lupus induced by pristane to identify which factors could account for the poor pregnancy outcomes in this model.

2. Material and methods

2.1. Mice and lupus induction

Thirty female Balb/c mice aged 9–10 weeks were obtained from a Veterinary Centre in Surabaya, Indonesia. All the mice were kept under pathogen-free conditions and given food plus water ad libitum at the Laboratory of Pharmacology, Brawijaya University. The mice were separated into the two following groups: a healthy pregnant group and pregnant SLE group. A single intraperitoneal injection of 0.5 cc of pristane was given to the mice to induce lupus [12]; weeks after the injection, the mice were mated. To standardise the pregnancy days, males were kept in the breeding cages for only 1 night. Gestation day 0 (GD0) was defined as the presence of a vaginal plugAnimal Trials

2.2. Monitoring of clinical manifestation and tissue collection

All the mice from each group were monitored for clinical features, including blood pressure and urine albumin levels. The measurement of systolic and diastolic blood pressure of the mice was performed on the tail by the CODA mouse rat tail-cuff system (Kent Scientific Corporation, USA) at GD17. All the mice were kept in the collecting cages for 24 h to collect the urine samples for urine albumin level measurement. At GD18, all the mice were euthanised by chloroform inhalation. All the specimens, including serum, spleen and foetuses were collected. The foetal number, weight and length were measured. The presence of foetal resorption was also documented.

2.3. Flow cytometry assay

Flow cytometry assay was performed to measure the percentages and absolute numbers of Th subsets, including Th1, Th2 and Th17, from the spleen. Single cell suspensions were prepared from the spleen using 100-µm cell strainers (protocol from BD Biosciences, USA). Detection of Th subsets from the suspension was conducted using extracellular and intracellular staining according to the protocol from Biolegend, USA. First, the suspension was stained with extracellular staining of fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 antibody (protocol from Biolegend). Prior to intracellular staining, cells were stimulated and fixed-permeabilised for 5 h with phorbol myristate acetate (Sigma-Aldrich, USA), ionomycin (Sigma-Aldrich) and brefeldin-A (BD Biosciences). Next, the cells were stained with phycoerythrin-conjugated anti-mouse interferon γ (IFNγ) antibody to detect Th1, Peridin-Chlorophyll-Protein-conjugated anti-mouse interleukin 4 (IL-4) antibody (Biolegend) to detect Th2 and phycoerythrin-conjugated IL-17A antibody (Biolegend) to detect Th17. Quantification was done in 10^5 cells with a FACSCalibur (BD Biosciences).

2.4. Enzyme-linked immunosorbent assay (ELISA)

The measurement of serum anti-dsDNA and urine albumin levels was analysed by enzyme-linked immunosorbent assay (ELISA). A mouse anti-dsDNA ELISA kit (MyBioSource, USA) was used to measure the serum anti-dsDNA level, while a mouse albumin ELISA kit (Elabscience, USA) was used to measure the urine albumin level. All the ELISA procedures were done according to the manufacturers' protocols.

2.5. Statistical analyses

Before analysing the data using parametric statistical tests, the data were analysed first with the parametric prerequisite test. Normality testing was conducted using the Shapiro-Wilk test. Since the data were normally distributed (p> 0.05), the parametric test was used in this study. The comparison between the healthy and lupus pregnancy groups was carried out via the independent *t*-test, while comparison for the number of foetal resorptions was done by chi-square analysis. Correlation analysis between clinical manifestations with the Th subsets was done using Pearson correlation analysis. All statistical analyses were performed using*Statistical Package for the Social Sciences*version 20.

3. Results

3.1. Maternal and foetal outcomes in the healthy and SLE pregnancy groups

Maternal outcomes from each group are shown in Table 1. The blood pressure measurement revealed that systolic and diastolic blood pressures from the SLE group (116.2 ± 30.5 mmHg and 79.7 ± 18.3 mmHg, respectively) were significantly higher compared with the healthy group (87.7 ± 9.2 and 60.8 ± 7.7 with p = 0.017 and p = 0.011 respectively). The serum anti-dsDNA level in the healthy group was significantly lower compared with that of the SLE group (0.470 ± 0.02 vs 0.532 ± 0.05, p = 0.004). Urine albumin levels were highly increased in the SLE group and statistically significantly different compared with the healthy group (1402.3 ± 401.5 ng/ml vs 132.3 ± 197.9, p = 0.000). Correlation analyses revealed that some of the maternal outcomes were significantly correlated with each other. The serum anti-dsDNA level was significantly correlated with the urine albumin level (p =0.002, r = 0.574). In addition, there was a significant correlation between the urine albumin level and systolic blood pressure (p =0.046, r = 0.395).

Foetal outcomes from the healthy and SLE groups, including foetus numbers, number of resorptions and foetal body length and weight, are shown in Table 1. The numbers of viable foetuses from the SLE group were significantly lower compared with those of the healthy group (8.2 ± 2.5 vs 11.5 ± 2.0, p =0.005). In addition, no resorption was found in the healthy group, which means that all foetuses from this group were viable; in contrast, 16 foetuses (20.7%) were resorbed in the SLE group (p = 0.000). Foetuses from the SLE pregnancy group exhibited reduced length (2.0 ± 0.4 vs 2.3 ± 0.2, p =0.046) and weight (0.8 ± 0.2 vs 1.1 ± 0.1, p =0.004) compared with those from the healthy group. Correlation analyses revealed some significant correlations between the foetal and maternal outcomes. Foetal body weight was significantly correlated with serum anti-dsDNA (p =0.026, r = −0.497) and urine albumin levels (p =0.016, r = −0.530). Foetal body length was significantly correlated with the urine albumin level (p =0.021, r= −0.513). Finally, the number of viable foetuses was significantly correlated with the urine albumin level (p =0.015, r = −0.537) and blood pressure (systolic: p =0.015, r = −0.535; diastolic: p =0.018, r = −0.522).

Table 1 Clinical Characteristic of Mice in Each Group

Clinical Characteristic	**Healthy Pregnant (n=10)**	**Pregnant SLE (n=10)**	**p**
Systolic blood pressure (mmHg)	87.7 ± 9.2	116.2 ± 30.5	0.017
Diastolic blood pressure (mmHg)	60.8 ± 7.7	79.7 ± 18.3	0.011
Serum anti-dsDNA level (optical density)	0.470 ± 0.02	0.532 ± 0.05	0.004
Urine albumin level (ng/ml)	132.3 ± 197.9	1402.3 ± 401.5	0.000
Foetus profiles			
Foetus numbers	11.5 ± 2.0	8.2 ± 2.5	0.005
Number of resorption [n (%)]	0 (0)	16 (20.7)	0.000
Foetal body length (cm)	2.3 ± 0.2	2.0 ± 0.4	0.046
Foetal body weight (gr)	1.1 ± 0.1	0.8 ± 0.2	0.004

3.2. Comparison of Th subset percentages and absolute number counts between groups

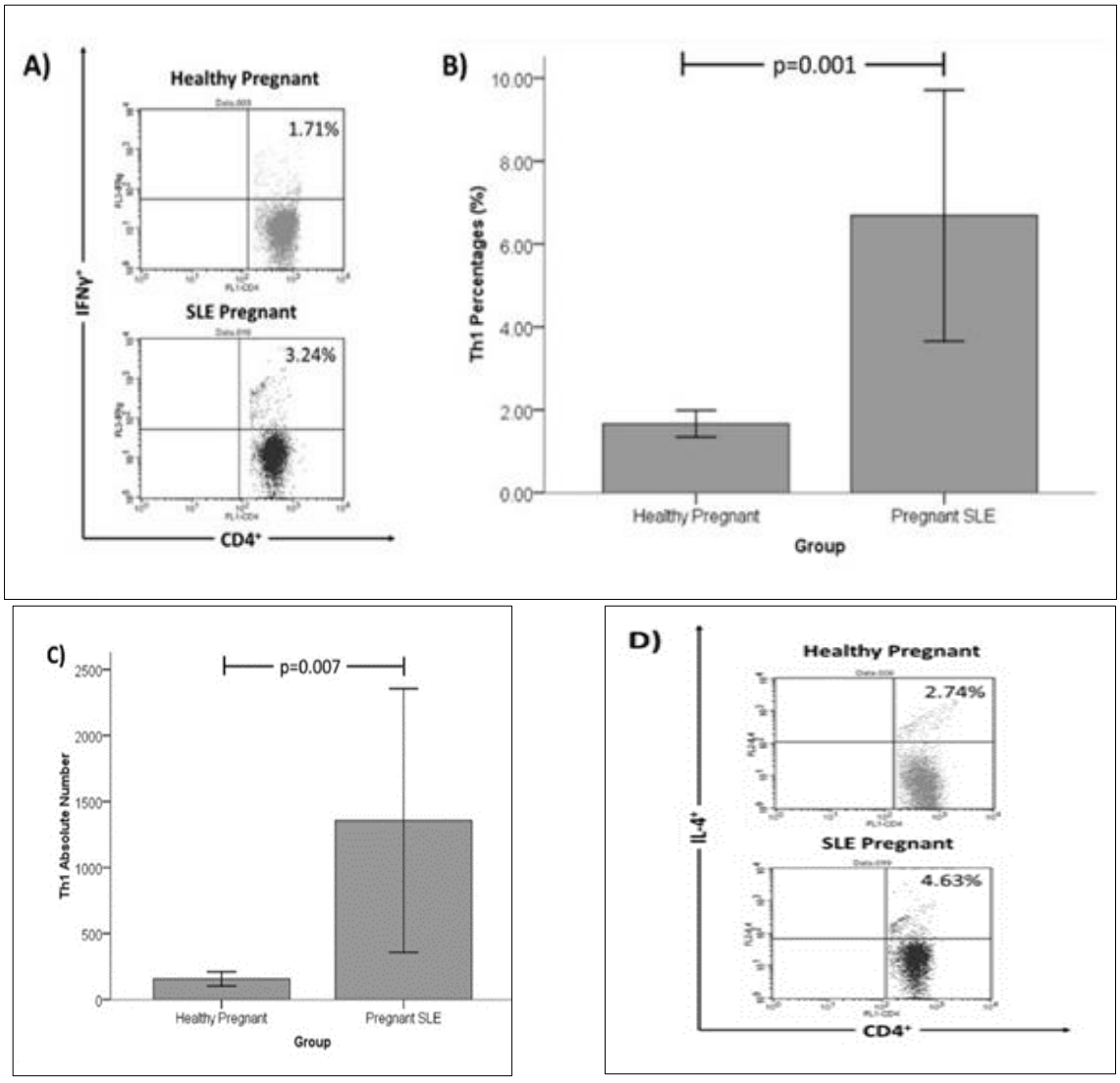
The Th subset percentage and absolute number measurement were determined by flow cytometry assay. Th1 was measured from cells that expressed CD4+ IFNγ+ (Figure 1A). The Th1 percentages from the SLE group were significantly

higher compared with those in the healthy group (6.6 ± 3.0% vs 1.7 ± 0.3%, *p* =0.001; Figure 1B). In the SLE group, the absolute Th1 numbers were also significantly higher than they were in the healthy group (1355.9 ± 998.8 vs 156.0 ± 53.2, *p* =0.007; Figure 1C).

As shown in Figure 1D, the Th2 measurement was assessed from cells expressing CD4+ IL-4+. Like in the case of Th1, in the SLE group, the percentages of Th2 were significantly higher (7.7 ± 3.6% vs 2.3 ± 1.3%, *p* =0.000; Figure 1E); the absolute numbers were also significantly higher in the pregnant SLE group (1304.0 ± 938.3 vs 196.1 ± 85.1, *p* =0.008; Figure 1F).

Finally, Th17 was measured from flow cytometry by measuring the cells expressing CD4+ IL-17+ (Figure 1G). In the SLE group, both the Th17 percentages and absolute numbers were statistically significantly higher, as shown in Figure 1H and 1I (4.6 ± 1.2 vs 1.5 ± 0.5, *p* =0.000 for Th17 percentages and 709.8 ± 377.3 vs 126.7 ± 57.2, *p* =0.002 for absolute Th17 numbers).

Representative of dot plot analysis of Th1, Th1 expressed CD4+ IFNγ+; B) Mean of Th1 percentages from each group; C) Mean of Th1 absolute numbers from each group; D) Representative of dot plot analysis of Th2, Th2 expressed CD4+ IL-4+; E) Mean of Th2 percentages from each group; F) Mean of absolute numbers from each group; G) Representative of dot plot analysis of Th17, Th17 expressed CD4+ IL-17A+; H) Mean of Th17 percentages from each group; I) Mean of Th17 absolute numbers from each group.



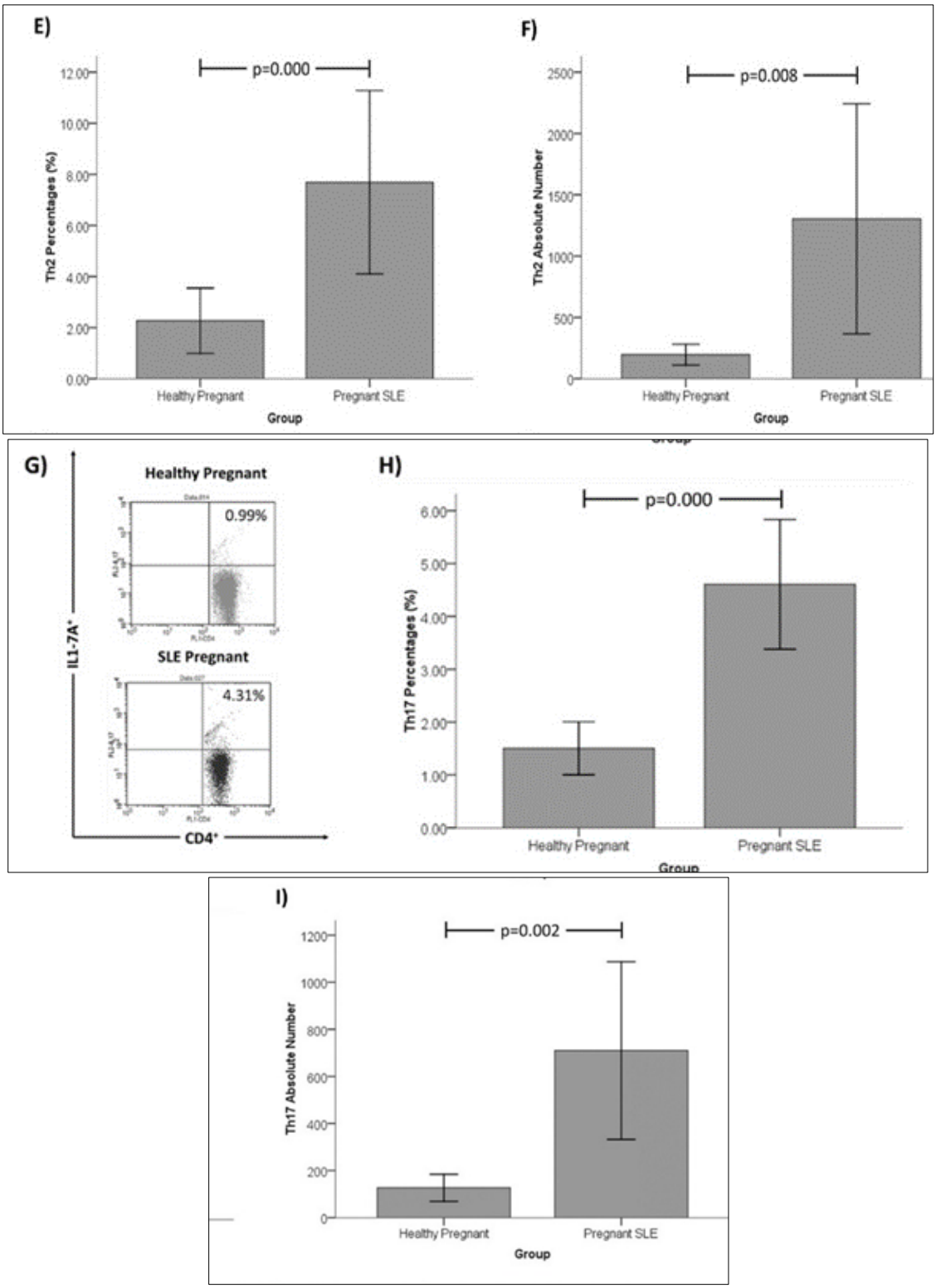


Figure 1 T helper subsets percentages and absolute number counts between groups

3.3. Comparison of the ratio of Th subsets between the healthy and SLE groups

To determine whether there was a shift in the balance in Th1/Th2/Th17, we also measured the ratio of Th1/Th2 and Th1/Th17 from each group. We found that, in the SLE group, the Th1/Th2 ratio was significantly higher than it was in the healthy group (1.1 ± 0.3 vs 0.7 ± 0.3, p =0.012); this was also the case with the Th1/Th17 ratio (1.9 ± 1.0 vs 1.0 ± 0.4, p =0.031).

3.4. Correlation analysis between the pregnancy outcomes and Th subsets

Correlation analysis between the clinical profiles, including maternal and foetal profiles, and the Th cell subsets was done to determine the role of the latter in influencing the clinical manifestation of a pregnant pristane-induced lupus mouse model. The correlation between the variables is summarised in Table 2. Th1 was significantly positively correlated with systolic blood pressure ($p = 0.039$, $r = 0.399$), serum anti-dsDNA ($p = 0.038$, $r = 0.401$) and urine albumin ($p = 0.000$, $r = 0.749$), while it was significantly negatively correlated with foetal body weight ($p = 0.002$, $r = -0.637$), body length ($p = 0.008$, $r = -0.577$) and the number of viable foetuses ($p = 0.016$, $r = -0.532$). In contrast, Th2 was only significantly positively correlated with urine albumin levels ($p = 0.001$, $r = 0.620$) and negatively correlated with foetal body weight ($p = 0.006$, $r = -0.589$) and the number of viable foetuses ($p = 0.037$, $r = -0.439$). Th17 was positively correlated with serum anti-dsDNA ($p = 0.010$, $r = 0.574$) and urine albumin ($p = 0.000$, $r = 0.795$), while it was negatively correlated with foetal body weight ($p = 0.006$, $r = -0.589$) and the number of viable foetuses ($p = 0.003$, $r = -0.624$).

Table 2 Correlation between Pregnancy Outcomes and T Helper Subsets

	Serum anti-dsDNA	Urine albumin	Systolic blood pressure	Diastolic blood pressure	Foetal body weight	Foetal body length	Number of viable foetuses
Th1	p=0.038*	p=0.000*	p=0.039*	p=0.053	p=0.002*	p=0.008*	p=0.016*
	r=0.401	r=0.749	r=0.399	r=0.377	r=-0.637	r=-0.577	r=-0.532
Th2	p=0.138	p=0.001*	p=0.110	p=0.074	p=0.006*	p=0.061	p=0.037*
	r=0.293	r=0.620	r=0.315	r=0.350	r=-0.589	r=-0.427	r=-0.469
Th17	p=0.010*	p=0.000*	p=0.110	p=0.155	p=0.006*	p=0.064	p=0.003*
	r=0.484	r=0.795	r=0.315	r=0.281	r=-0.589	r=-0.422	r=-0.624

*showed a significant correlation between two variables, with $p<0.05$

4. Discussion

In this research, we analysed pregnancy outcomes and alterations of Th subsets from a pregnant pristane-induced lupus mouse model. This model was established for mimicking the clinical characteristics of humanswith SLE [14]. However, few reports were found that analysed the clinical manifestations of the pregnant pristane-induced lupus mouse model. Our current study revealed elevated blood pressure, serum anti-dsDNA and urine albumin levels in pregnant mice with pristane-induced lupus. Hypertension and albuminuria in pregnant women with SLE have been documented as major complications of SLE during pregnancy [3]. Neuroendocrine and immunological changes during pregnancy, which cause many comorbidities like preeclampsia and worsening of renal disease, are the predisposing factors for these conditions [15]. Other predicting factors for maternal outcomes are active disease during conception and high titres of anti-dsDNA antibody [16]. We found a significant correlation between serum anti-dsDNA and urine albumin levels; however, no correlation was found between serum anti-dsDNA levels and blood pressure.

Like in our previous report [4], the foetal outcomes found in this study were low birth weight and length, increased foetal resorption and a low number of viable foetuses. Foetal outcomes from mothers with SLE are highly correlated with maternal conditions [16]. The presence of anti-Ro/SSA (Anti-Sjögren's-syndrome-related antigen A) antibodies, flare, renal disease and low complement levels are the predicting factors for foetal loss and poor foetal outcomes in SLE during pregnancy [16,17]. The main mechanism of how the maternal conditions can affect the foetal outcomes is the placental ischaemia and subsequent endothelial damage, resulting in either spontaneous abortion or intrauterine growth retardation[18]. In the present study, we found that birth weight was significantly correlated with serum anti-dsDNA and urine albumin levels, while the number of viable foetuses was significantly correlated with urine albumin levels and blood pressure. These data confirmed the influence of maternal conditions on foetal outcomes.

At first, SLE was considered a Th2-driven disease. Most clinical and laboratory studies revealed predominantly Th2 polarisation and overproduction of Th2-type cytokines in SLE [5]. However, the present study showed a significant increase of both percentages and absolute numbers of Th1, Th2 and Th17 from the pregnant pristane-induced lupus mouse model. Our results indicated that not only Th2, but also Th1 and Th17, could become abnormal in SLE during pregnancy. Like this study, Chan et al. [19]. determined that there was an elevated level of Th1 transcription factors that correlated with SLE disease activity. Upregulation of Th1-type cytokines, such as IL-18 and IFNγ, was also reported

these markers were increased in lupus nephritis patients [6]. The role of Th17 in the progression of SLE was described in our previous study [20]. In SLE patients, there was significantly higher serum IL-17 levels and a positive correlation with the SLE disease activity index [21].

The significance of the Th subset balance is not limited to the natural history of some diseases but also has an important role in pregnancy homeostasis [13]. Our result showed that the ratio between Th1 and Th2 in normal healthy pregnant mice was slightly skewed towards Th2. This was a normal response to maintain the immunosuppression and tolerance by the immune system to paternal and foetal antigens during pregnancy.7 While Th2 is an anti-inflammatory agent, Th1 and Th17 are considered potent proinflammatory inducers [9]. In many cases, there was an increase of Th1 and Th17 polarisations that correlated with poor pregnancy outcomes [10–12]. Although we did find both Th1 and Th2 enhanced in pregnant SLE mice, we showed that the elevation of Th1 was more dominant than Th2, illustrating a significant increase of Th1/Th2 ratio in pregnant pristane-induced lupus mice. In healthy pregnant mice, we also found a balance between Th1 and Th17 numbers. However, Th1 polarisation was predominantly found in pregnant SLE mice as indicated by a significant increase of the Th1/Th17 ratio.

In the present study, we also revealed that Th1 had the most correlations with the pregnancy outcomes compared with Th2 and Th17. A previous study determined that predominant Th1-type immunity could be observed in abortion [11,22]. Another study found that Th1 activation was a predisposing factor for preeclampsia [23]. Secreted IL-12 and IL-18, which are mainly produced by Th1, are increased in preeclampsia and may suppress the tolerance immune system, resulting in preeclampsia [23,24] Although not as much as Th1, we revealed that Th17 had a correlation with pregnancy outcomes. Increased Th17 cell numbers in peripheral blood have been reported in preeclampsia [25]. Th17 also plays a significant role in complications of pregnancy, such as pregnancy loss and preterm labour. Recent data have shown that the recurrent spontaneous abortion and elevated prevalence of Th17 in decidua and peripheral blood are unexplained [13].

Th2 is considered a protective factor during pregnancy; however, predominant Th2-type immunity has also been reported in recurrent pregnancy loss [12]. We confirmed this result via a significant correlation between increased numbers of Th2 with an elevated level of urine albumin and decreased number of viable foetuses. Therefore, an adequate balance for Th1/Th2/Th17 immunity may be suitable for the maintenance of pregnancy. Overstimulation of one of the Th subsets may be harmful for successful pregnancy [9].

5. Conclusion

In conclusion, the numbers of Th1, Th2, and Th17 are elevated in a pregnant pristane-induced lupus mouse model. The most relevant elevation seems to be an increase of Th1, which is more dominant compared with Th2 and Th17. Elevation of Th subsets also correlates with poor pregnancy outcomes in the pregnant pristane-induced lupus mouse model. Thus, the results of this study suggest the important role of the Th subsets in modulating the pregnancy outcomes in SLE. However, further studies are still required to monitor not only the number but also the functions of Th subsets that may be abnormal in SLE during pregnancy.

Compliance with ethical standards

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Disclosure of conflict of interest

We warrant that the article is the Authors' original work and ensure no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is not under review at any other publication.

Statement of ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards at the University of Brawijaya, Malang, numbered 328/ EC/KEPK/05/2015.

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