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# Increased T helper 1 cytokine responses by circulating T cells are present in women with recurrent pregnancy losses and in infertile women with multiple implantation failures after IVF

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**BACKGROUND: We aimed to study T-helper 1 (Th1) and Th2 intracellular cytokine expression in peripheral blood lymphocytes of women with recurrent spontaneous abortions (RSA) or infertility with multiple implantation failures after IVF cycles. METHODS: Twenty-six women with three or more RSA and 23 with two or more IVF failures (14 with no history of spontaneous abortion (SAB) and nine with more than one SAB) comprised the two study groups. Twenty-one non-pregnant healthy multiparous women served as controls. Proportions (%) of lymphocytes containing IFN-γ, TNF-α, IL-4 and IL-10 and the Th1/Th2 ratios of IFN-γ/IL-4, IFN-γ/IL-10, TNF-α/IL-4 and TNF-α/IL-10 in CD3+, CD3+/CD8– (T helper) and CD3+/CD8+ (T suppressor) cells were measured by 4-colour flow cytometry. RESULTS: RSA women demonstrated significantly higher Th1/Th2 ratios of IFN-γ/IL-4 ($P < 0.01$), TNF-α/IL-4 and TNF-α/IL-10 ($P < 0.05$ each) in CD3+/CD8– T helper cells than those of controls. The proportion of TNF-α producing CD3+/CD8– cells ($P < 0.05$), and the Th1/Th2 ratios of TNF-α/IL-4 ($P < 0.05$) and TNF-α/IL-10 ($P < 0.005$) in CD3+/CD8– cells were significantly higher in women with multiple IVF failures without SAB as compared with those of controls. CONCLUSIONS: The prevalence of dominant Th1 immune responses in peripheral blood lymphocytes may reflect the systemic contribution of Th1 cytokines to RSA or multiple implantation failures in IVF cycles.**

*Key words:* implantation failures/IVF failures/recurrent pregnancy losses/Th1/Th2 cytokines

## Introduction

Subpopulations of T helper lymphocytes (CD3+/CD4+) can be classified as either T helper 1 (Th1) or T helper 2 (Th2) cells depending on their cytokine profiles. Th2 cells selectively produce interleukins (IL)-4, IL-5, IL-6, IL-9, IL-10 and IL-13, and are involved in the development of humoral immunity against extracellular pathogens but inhibit several functions of phagocytic cells. In contrast to this, Th1 cells produce interferon-γ (IFN-γ), IL-2 and tumour necrosis factor-α (TNF-α) and evoke cell-mediated immunity and phagocyte-dependent inflammation (Mosmann and Coffman, 1989; Romagnani, 2000).

Recently, significantly higher serum levels of Th2 cytokines, IL-6 and IL-10, were detected in normal pregnancy compared with unexplained recurrent pregnancy losses and significantly higher serum levels of the Th1 cytokine, IFN-γ, were present in women with recurrent pregnancy losses compared with normal pregnancy (Raghupathy *et al*., 1999). These results suggested the notion that women with normal pregnancy have a Th2 bias, while women with a history of recurrent pregnancy losses have a bias toward Th1-type reactivity.

In pregnant mice, the injection of each Th1 cytokine, such as IFN-γ, TNF-α and IL-2, or co-administration of those significantly increased fetal resorption (Chaouat *et al*., 1990; Clark *et al*., 1998). It is suggested that Th1 cytokines trigger thrombotic/inflammatory processes at the maternal utero-placental blood vessels by activation of vascular endothelial cell procoagulant (Clark *et al*., 1998). By contrast, Th2 cytokines inhibit Th1-induced tissue factor production by monocytes (Del Prete *et al*., 1995).

Recurrent spontaneous abortion (RSA) is a common complication of pregnancy that may affect as many as 2% of women in reproductive age (Mills *et al*., 1988; Coulam, 1991). Although genetic, anatomic and hormonal causes have been implicated in the aetiology of RSA (Stray-Pedersen and Stray-Pederson, 1984; Carp *et al*., 1990), >60% of cases remain unexplained. Various immunological abnormalities have been reported in women with RSA of unknown aetiologies including

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autoimmune abnormalities such as positive antiphospholipid antibodies, anti-nuclear antibodies, anti-thyroglobulin antibodies and anti-microsomal antibodies, and increased cellular immunity such as elevated natural killer (NK) cell levels and NK cytotoxicity (Kwak *et al.*, 1995; Ruiz *et al.*, 1996). Interestingly, these immunological abnormalities also occur in infertile women who have implantation failures after multiple IVF cycles (Beer *et al.*, 1996; Coulam *et al.*, 1997). These immunological similarities between women with RSA and infertility due to implantation failures led us to speculate that the proclivity to Th1 cytokine responses by circulating T cells in women with RSA (Raghupathy *et al.*, 2000) may exist in women with infertility of implantation failures. Previous studies of cytokine synthesis in peripheral blood of women with normal pregnancies or recurrent spontaneous aborters (Hill *et al.*, 1995; Raghupathy *et al.*, 1999) were designed to measure the total secreted cytokines from mononuclear cells but failed to discriminate the lymphocyte subpopulations. Thus studies investigating Th1/Th2 immune regulation in women with RSA or infertility of implantation failures by specifically defining the intracellular cytokine expression of CD3+/CD4+ T helper cells have not been reported. In this study, we have directly tested the Th1/Th2 paradigm in women with a history of RSA and women with multiple (two or more) implantation failures after IVF cycles by measuring intracellular Th1 and Th2 cytokine expression in CD3+, CD3+/CD8– (considered equivalent to CD3+/CD4+) or CD3+/CD8+ cells, and their ratios in subsets of peripheral blood T lymphocytes using 4-colour flow cytometry.

## Materials and methods

### *Population*

The study design was a prospective controlled study. Study groups and controls were enrolled consecutively if they met the study inclusion criteria. The study was performed at the Division of Reproductive Medicine, Department of Microbiology and Immunology, Finch University of Health Sciences/The Chicago Medical School. All the study and control subjects had signed an informed consent prior to entering the study. Blood was drawn prior to any treatment including IVF cycles. No one was on any medication.

Inclusion criteria for this study were: (i) fertile women with three or more RSA of unknown aetiology or (ii) infertile women with two or more implantation failures after IVF cycles, who had two or more embryos transferred per each IVF cycle, excluding donor oocyte cycles; (iii) not pregnant; (iv) no more than one live born infant; (v) age ranges 25–45 years old; and (vi) no active disease including autoimmune disease. To investigate a possible medical condition, physical examination, past history review, review of system and blood tests were performed. Blood tests were comprehensive metabolic panel, complete blood count, thyroid function test [free thyroxine (T4) and thyroid-stimulating hormone], antinuclear antibody, antibodies to single-stranded DNA, double-stranded DNA and histone.

The recurrent abortion group included 26 women with three or more RSA of unknown aetiology with the same partner. Three had one child. All had pregnancy losses during the first trimester of gestation. None had active autoimmune disease or a history of autoimmune disease. No one with evidence of active autoimmune disease was included in this study. No apparent causes of recurrent abortion such as chromosomal, endocrine, anatomical, or infectious aetiologies were documented for previous pregnancy losses. None had infertility or received IVF cycles. Table I lists age, obstetric histories of RSA groups and normal fertile controls. Age distribution is comparable between women with RSA and normal fertile controls.

**Table I.** Age and obstetric histories of women with three or more recurrent spontaneous abortions (RSA) and normal fertile controls

| | Controls ($n$ = 21) | RSA ($n$ = 26) | $P$-value |
|---|---|---|---|
| Age (years) | 38.3 ± 8.0 | 34.4 ± 5.6 | NS |
| Gravidity | 2.6 ± 0.9 | 4.6 ± 2.0 | < 0.05 |
| SAB | 0.0 ± 0.0 | 4.3 ± 1.7 | < 0.001 |

Values are mean ± SD.
SAB = spontaneous abortions; NS = not significant.

A total of 23 women with infertility of implantation failures after two or more IVF cycles comprised the implantation failure groups. Based on their history of spontaneous abortion (SAB), women with implantation failures were divided to two subgroups. Multiple IVF failures without SAB group had 14 women. None had a history of spontaneous abortion and two had one live child (one had eight IVF failures and the other had five IVF failures). Multiple IVF failures with SAB group included nine women. Two had one live child (one had six IVF failures and the other had three IVF failures). In women with multiple IVF failures, no one had active autoimmune disease or a history of autoimmune disease. No apparent cause for IVF failures has been documented in these women. Women with male factor infertility and donor oocyte cycles were excluded. Table II lists age, obstetric histories and IVF histories including their primary infertility diagnosis.

Controls were 21 normal multiparous healthy non-pregnant women with documented uncomplicated pregnancies (⩾1, ranges 1–3). All controls were interviewed, during which time personal and family histories were ascertained. None had an active disease including autoimmune disorder. All had a history of one or more normal deliveries. None had a history of pregnancy loss, infertility or implantation failures (Table I).

### *Laboratory*

#### *Cell separation and staining*

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Biotech, Sweden) density centrifugation, and prepared and stained as previously reported (Ng *et al.*, 2002). The cytokine expression levels were undetectable without stimulation after 5 h of culture with monensin only (Pharmingen, USA). Therefore, cells were stimulated using 25 ng/ml phorbol myristate acetate (PMA) and 1 μmol/l ionomycin in the presence of 2 μmol/l monensin, which inhibits cytokine secretion. Cells were stained according to the manufacturer's instruction with the Cytofix/Cytoperm kit (Pharmingen). To detect intracellular cytokines, 0.2 μg of monoclonal antibody/$\times 10^6$ cells for IL-4 and 0.5 μg of monoclonal antibody/$\times 10^6$ cells for IFN-γ, TNF-α and IL-10 were used. Anti-cytokine antibodies were: [phycoerythrin (PE)-anti-human IFN-γ, clone 4S.B3; PE-anti-human TNF-α, clone Mab11; PE-anti-human IL-4, clone 8D4-8; PE-anti-human IL-10, clone JES3-19F1; PE-mouse IgG1 isotype, clone MOPC-21; PE-rat IgG2 isotype, clone R35-95 (Pharmingen). Corresponding isotype controls were utilized for each antibody and for each patient.

It has been shown that following stimulation of lymphocytes with PMA and ionomycin, a rapid down-regulation of CD4 molecules on the surface of lymphocytes occurs. In our hands, a decrease of CD4 occurred as rapidly as 4 h after stimulation. Therefore, a negative



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**Table II.** Age, obstetric and IVF cycle histories of infertile women with multiple implantation failures after two or more IVF cycles with and without a history of spontaneous abortion (SAB)

| | Infertility ($n$ = 23) (mean ± SD) | | $P$-value[a] |
|---|---|---|---|
| | No history of SAB ($n$ = 14) | History of SAB ($n$ = 9) | |
| Age (years) | 36.9 ± 4.6 | 35.2 ± 4.6 | NS |
| Gravidity | 0.5 ± 0.6 | 2.8 ± 1.1 | < 0.000 |
| SAB | 0.0 ± 0.0 | 2.5 ± 1.1 | < 0.000 |
| No. of failed IVF cycles | 4.3 ± 1.9 | 4.5 ± 1.6 | NS |
| No. of mature oocytes/cycle | 9.1 ± 5.0 | 15.3 ± 5.9 | 0.002 |
| No. of oocytes fertilized/cycle | 5.5 ± 4.1 | 9.4 ± 6.7 | NS |
| No. of embryos transferred/cycle | 3.1 ± 1.6 | 3.6 ± 1.5 | NS |
| Primary infertility diagnosis | | | NS[b] |
| Endometriosis (no.) | 2 | 1 | |
| Tubal factor (no.) | 5 | 2 | |
| Unexplained (no.) | 7 | 6 | |

[a]Two-sample two-tailed $t$-test was applied except for the primary infertility diagnosis.
[b]$\chi^2$-Test.
NS = not significant.

gating strategy was used to measure intracellular cytokine expression in CD3+CD4+ cells. Cells are reacted with phycoerythrin–Texas Red (ECD)-anti-CD3 (Beckman–Coulter, USA) and fluoroscein isothiocyanate (FITC)-anti-CD8 (clone T8; Beckman–Coulter) and cells that were CD3+ but not CD8+ were considered to be CD3+CD4+ cells (Rostaing *et al*., 1999). In our study, the percentages of Th2 cytokine (IL-4 and IL-10)-producing cells were relatively low. Although the same pattern has been reported previously (Tsuda and Yamasaki, 2000), we verified IL-10 and IL-4 data by analysing isotype controls. Non-specific intracellular staining with isotype control antibodies was 0.19 ± 0.15% for the IL-10 expression study and 0.17 ± 0.13% for the IL-4 expression study.

### *Acquisition and analysis of flow cytometric data*

The stained and fixed samples were analysed on a Coulter XL flow cytometer using XL software (Coulter Corp., USA). Fluorescence from the FL1 (FITC), FL2 (PE), FL3 (ECD) and FL4 [phycoerythrin–cyanin (PC5)] channels were used to measure cell surface and intracellular fluorescence as previously reported (Ng *et al*., 2002). The number of events acquired for each sample was 40 000. A region based on light scatter [forward angle light scatter (FALC) versus side scatter (SS)] was drawn around the major lymphocyte population. This population was used to obtain the gated fluorescence plot of CD3 versus CD8. Rectangular regions were set to include all the CD3+CD8+ bright cells and all the CD3+CD8– (CD3+/CD4+) bright cells obtained by negative gating strategy (Figure 1). All data are expressed as the percentage of cytokine-positive CD3+/CD8– or CD3+/CD8+ bright cells. A cell surface activation antigen (CD69, recognized by Pc5-anti-human CD69, clone TP.55.3; Beckman–Coulter) was also used on these permeabilized and fixed cells to show the activation status of the CD3+/CD8– (CD3+/CD4+) and CD3+/CD8+ cells within the tight lymphocyte light scatter gate used for the analysis of intracellular cytokines.

### *Statistical analysis*

The statistical analysis was performed using SPSS/PC+™ program. Since women with RSA do not have any infertility or implantation failure histories, two separate statistical analyses were performed: (i) the study results of women with RSA were compared with those of normal fertile controls, and (ii) the study results of infertile women with multiple implantation failures with SAB, without SAB and normal fertile controls were analysed.

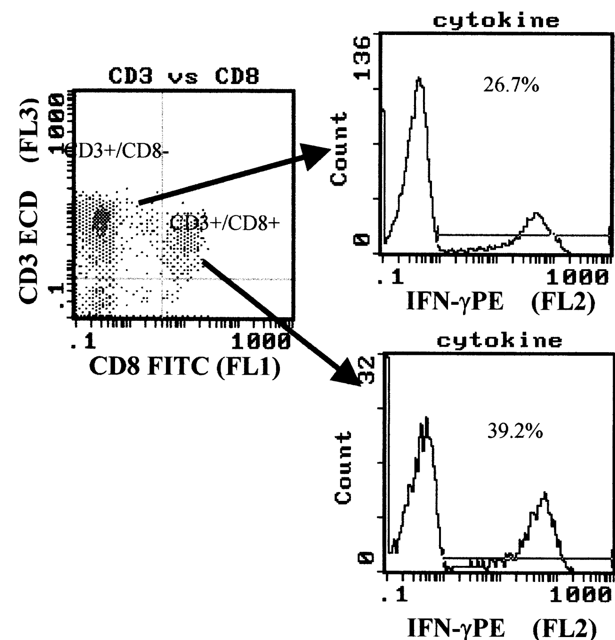


**Figure 1.** Gating strategy for flow cytometric analysis of CD3+/CD8– (for CD3+/CD4+ analysis) and CD3+/CD8+ cells for intracellular cytokine expression. ECD = phycoerythrin–Texas Red; FITC = fluorescein isothiocyanate; PE = phycoerythrin; IFN-γ = interferon-γ.

Unpaired two-tailed $t$-test was applied for comparisons of intracellular cytokine expression and proportion of T cell subsets between women with RSA and normal fertile controls. Th1/Th2 ratios in CD3+, CD3+/CD8– and CD3+/CD8+ cells of women with RSA were compared with those of normal fertile controls using unpaired two tailed $t$-test. If the population variances in the two groups were equal, the pooled-variance $t$-test was applied. If the population variances in the two groups are different, the separate variance $t$-test was applied. $P \leqslant 0.05$ was considered significant.

For the comparison of intracellular cytokine expression, T cell subsets and the Th1/Th2 ratios among women with multiple implantation failures without SAB, with SAB and normal fertile

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**Table III.** Intracellular cytokine expression in CD3+, CD3+/CD8– (T helper) and CD3+/CD8+ (T suppressor) lymphocytes in women with recurrent spontaneous abortion (RSA), multiple implantation failures after two or more IVF cycles with and without a history of spontaneous abortion (SAB) and normal fertile controls

| Cytokine | Controls | RSA | *P*-value[a] | IVF failures (*n* = 23) | | *P*-value[b] |
|---|---|---|---|---|---|---|
| | (*n* = 21) | (*n* = 26) | | No history of SAB (*n* = 14) | History of SAB (*n* = 9) | |
| CD3+ T cells | | | | | | |
| IFN-γ | 19.40 ± 8.31 | 18.79 ± 8.78 | NS | 20.26 ± 9.63 | 18.62 ± 4.26 | NS[b] |
| TNF-α | 24.61 ± 10.23 | 25.50 ± 11.61 | NS | 33.42 ± 12.94 | 26.37 ± 8.67 | NS |
| IL-4 | 2.46 ± 0.78 | 2.14 ± 0.95 | NS | 2.69 ± 1.62 | 2.42 ± 1.07 | NS |
| IL-10 | 0.95 ± 0.41 | 0.75 ± 0.33 | NS | 0.77 ± 0.33 | 0.69 ± 0.22 | NS |
| CD3+CD8– T helper cells | | | | | | |
| IFN-γ | 14.23 ± 6.52 | 14.47 ± 6.22 | NS | 17.59 ± 9.05 | 14.92 ± 5.42 | NS |
| TNF-α | 24.80 ± 10.23 | 27.0 ± 12.85 | NS | 38.29 ± 16.42* | 28.36 ± 8.99 | 0.011 |
| IL-4 | 2.75 ± 0.93 | 2.36 ± 1.04 | NS | 2.67 ± 0.97 | 2.53 ± 0.77 | NS |
| IL-10 | 0.90 ±0.41 | 0.75 ± 0.36 | NS | 0.69 ± 0.21 | 0.68 ± 0.23 | NS |
| CD3+/CD8+ T suppressor | | | | | | |
| IFN-γ | 34.82 ± 13.34 | 31.97 ± 17.20 | NS | 29.23 ± 13.97 | 30.64 ± 13.46 | NS |
| TNF-α | 24.73 ± 13.19 | 21.83 ± 12.08 | NS | 22.92 ± 14.11 | 21.84 ± 13.33 | NS |
| IL-4 | 2.04 ± 1.18 | 1.63 ± 1.07 | NS | 1.47 ± 1.06 | 1.45 ± 0.87 | NS |
| IL-10 | 1.20 ± 0.59 | 0.83 ± 0.39 | 0.013 | 1.00 ± 0.78 | 0.69 ± 0.26 | NS |

[a]Comparisons were made between women with RSA and normal fertile controls using two-tailed *t*-test.
[b]Comparisons were made using one way analysis of variance with the Scheffé multiple comparison test in women with IVF failures without SAB, with SAB and normal fertile controls.
*$P < 0.05$ as compared with controls by the Scheffé multiple comparison test.
IFN = interferon; TNF = tumour necrosis factor; IL = interleukin; NS = not significant.

controls, one-way analysis of variance with the Scheffé multiple comparison test was applied. The Scheffé multiple comparison test was applied to protect against identifying too many differences as significant. This test allows more stringent criteria for declaring differences significant than the usual *t*-test (Norusis, 1986).

## Results

### *T cell subsets and activation status*

Peripheral blood T lymphocyte subpopulations were determined by flow cytometric analysis. There was no statistically significant difference in the proportion (%) of CD3+, CD3+/CD8– and CD3+/CD8+ cells between women with RSA and normal fertile controls, and women with infertility of implantation failures without SAB, with SAB and normal fertile controls.

To measure intracellular cytokines, resting lymphocytes must first be activated for 5 h with PMA and ionomycin. Less than 2% of CD3+ cells demonstrated CD69 expression without stimulation. For the confirmation of lymphocyte activation, CD69 expression on T cell subpopulations was measured in study and control subjects. A total of 88.2 ± 4.3% (mean ± SD) cells of women with RSA, 88.8 ± 4.1% cells of women with infertility of implantation failures without SAB, 88.6 ± 4.0% cells of women with infertility of implantation failures with SAB, and 87.4 ± 6.0% of normal fertile controls were activated as judged by the expression of the early activation antigen CD69 after 5 h incubation with PMA and ionomycin. The proportions of activated cells (CD69+) in study and control groups were not significantly different. These results suggested that lymphocyte stimulation with PMA and ionomycin was similar in study and control groups.

### *Intracellular cytokine expression*

The proportion (%) of TNF-α-, IFN-γ-, IL-4- and IL-10-producing CD3+, CD3+/CD8–, CD3+/CD8+ cells in women with RSA, infertile women with multiple implantation failures without a history of SAB, with a history of SAB and normal fertile controls are listed in Table III. The proportion of IL-10-producing CD3+/CD8+ cells was significantly lower in women with RSA as compared with that of normal fertile controls ($P = 0.013$). The proportion of TNF-α-producing CD3+/CD8– cells was significantly different among women with implantation failures without SAB, with SAB and normal fertile controls ($P = 0.011$).

### *Th1/Th2 cytokine ratios*

To compare the proportion of T cell subsets synthesizing Th1 cytokines versus Th2 cytokines in each woman, the ratios of Th1/Th2 were calculated by dividing the proportion of Th1 cytokine producing cells by the proportion of Th2 cytokine producing cells with the following combination: IFN-γ/IL-4, IFN-γ/IL-10, TNF-α/IL-4 and TNF-α/IL-10. After calculating each study subject's Th1/Th2 ratios, the mean and SEM of each study group was calculated. Figure 2 (CD3+ cells), Figure 3 (CD3+/CD8– cells) and Figure 4 (CD3+/CD8+ cells) plot the Th1/Th2 cytokine ratio of women with RSA and normal fertile controls.

Table IV demonstrates the Th1/Th2 cytokine ratios of women with multiple implantation failures after IVF cycles without SAB, with SAB and normal fertile controls.

## Discussion

In this study, we report that intracellular Th1 cytokine expressions are increased over Th2 cytokine expressions in



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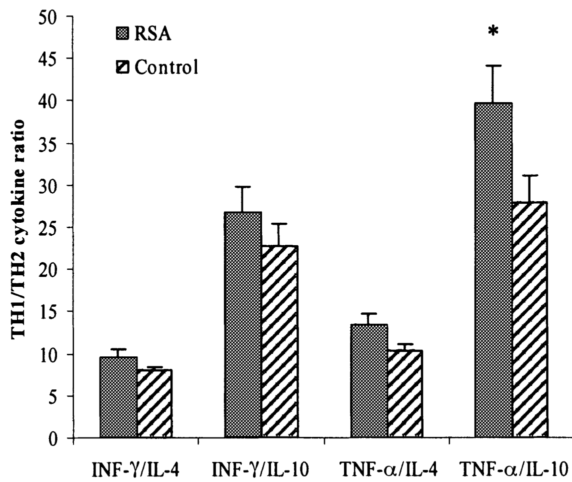


**Figure 2.** Comparison of T helper 1 (Th1)/Th2 cytokine-producing CD3+ cell ratios in women with three or more recurrent spontaneous abortions (RSA; $n = 26$) and normal fertile controls ($n = 21$). Values are mean ± SEM. $^*P < 0.05$.

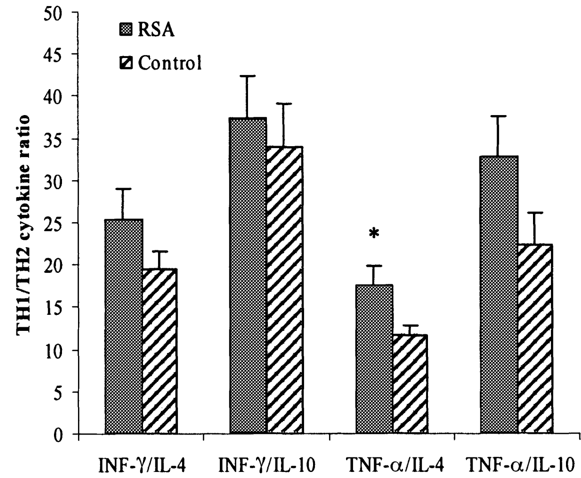


**Figure 4.** Comparison of Th1/Th2 cytokine-producing CD3+/CD8+ cell ratios in women with three or more recurrent spontaneous abortions ($n = 26$) and normal fertile controls ($n = 21$). Values are the mean ± SEM. $^*P < 0.05$. For abbreviations, see Table III.

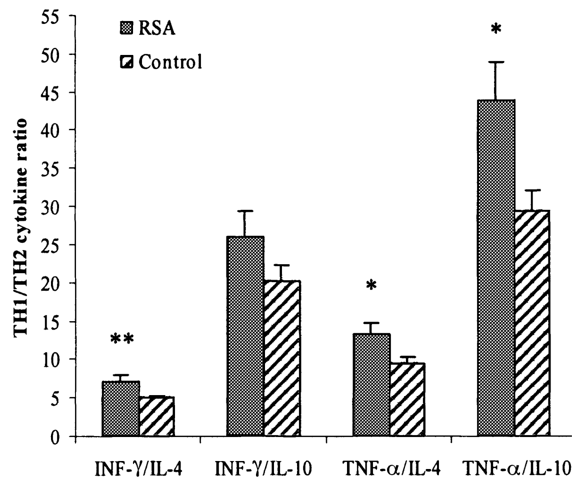


**Figure 3.** Comparison of Th1/Th2 cytokine producing CD3+/CD8– cell ratios in women with three or more recurrent spontaneous abortions ($n = 26$) and normal fertile controls ($n = 21$). Values present the mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$. IL = interleukin; for other abbreviations, see Table III.

women with RSA and infertility of multiple implantation failures. Our study is consistent with the previous reports of increased Th1 immune responses in women with recurrent pregnancy losses (Raghupathy, 1997a; Lim *et al.*, 2000). This proclivity to Th1 cytokine responses by T cells is mainly expressed in CD3+/CD8– cells, but also in CD3+/CD8+ cells in women with recurrent pregnancy losses and infertile women with multiple implantation failure after IVF cycles. These results were obtained by analysis of peripheral blood lymphocytes that reflect systemic expression and regulation. Indeed we found that the ratio of Th1/Th2 immune responses is more important than the expression of a single cytokine.

Recent advances in immunoassays such as commercially available standardized cell permeabilization reagents rather than saponin, antibodies to cytokines which are directly conjugated to give low background fluorescence, and the use of different combinations of monoclonal antibodies for 4-colour flow cytometry, have led to greater flexibility and more consistency in determining intracellular cytokine expression (North *et al.*, 1996). In our study we investigated CD3+/CD4+ Th1 and Th2 cells by measuring CD3+/CD8– cells based on intracellular cytokine expression and also evaluated cytokine expression in CD3+/CD8+ cells in women with RSA or infertility of implantation failures. NK cells, which are CD3–, were not included in the flow cytometric analysis. A concern may be raised that CD3+/CD8– cells are measured for CD3+/CD4+ cells. These two cell populations are probably identical, although by definition of expression they are not.

In our study, lymphocytes were stimulated with PMA and ionomycin in the presence of a protein transport inhibitor monensin to allow cytokines to accumulate in levels that were high enough to be detected by intracellular flow cytometric analysis. We have demonstrated the prevalence of Th1 immune responses over Th2 immune responses in specific T cell subpopulations based on expression of each cytokine. Previous in-vitro studies utilized trophoblast antigens to activate lymphocytes of women with a history of RSA and reported the presence of cytokines in the supernatant that was injurious to the developing conceptus or trophoblast cell lines (Ecker *et al.*, 1993; Hill *et al.*, 1995). Our study suggests that altered Th1 immune responses can also be detected systemically as a result of an in-vivo challenge during pregnancy (RSA) or IVF cycles.

TNF-α is supposed to suppress the growth of trophoblasts (Todt *et al.*, 1996), possibly by inducing apoptotic changes in these cells (Yui *et al.*, 1994). TNF-α is present on the proliferating tips of anchoring villi, invasive interstitial cytotrophoblasts, and endovascular trophoblasts which invade spiral arteries (Lea *et al.*, 1997). These findings suggest a role for TNF-α in early invasion of trophoblasts. However, a decrease in the release of TNF-α from PBMC upon the



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**Table IV.** The ratios of Th1/Th2-related intracellular cytokine expression in CD3+, CD3+/CD8– (T helper), and CD3+/CD8+ (T suppressor) lymphocytes in women with two or more IVF failures and normal fertile controls

| T cell subsets | Cytokine ratio | Controls | IVF failures (*n* = 23) | | *P*-value[a] |
|---|---|---|---|---|---|
| | | | No history of SAB (*n* = 14) | History of SAB (*n* = 9) | |
| | | (*n* = 21) | | | |
| CD3+ | IFN-γ/IL-4 | 7.94 ± 0.46 | 8.61 ± 1.10 | 9.14 ± 1.49 | NS[b] |
| | IFN-γ/IL-10 | 22.67 ± 2.73 | 29.55 ± 3.67 | 29.68 ± 4.34 | NS |
| | TNF-α/IL-4 | 10.31 ± 0.80 | 15.88 ± 2.17 | 13.14 ± 2.80 | 0.050 |
| | TNF-α/IL-10 | 27.88 ± 3.27 | 46.37 ± 6.84* | 42.57 ± 7.73 | 0.030 |
| CD3+/CD8– (T helper) | IFN-γ/IL-4 | 5.02 ± 0.29 | 6.74 ± 0.75 | 6.33 ± 0.90 | NS |
| | IFN-γ/IL-10 | 20.05 ± 2.29 | 26.55 ± 3.29 | 26.03 ± 6.06 | NS |
| | TNF-α/IL-4 | 9.49 ± 0.79 | 15.96 ± 2.30* | 12.81 ± 2.52 | 0.028 |
| | TNF-α/IL-10 | 29.45 ± 2.60 | 60.05 ± 8.63** | 48.67 ± 10.08 | 0.0043 |
| CD3+/CD8+ (T suppressor) | IFN-γ/IL-4 | 19.55 ± 1.94 | 25.22 ± 4.23 | 23.29 ± 4.22 | NS |
| | IFN-γ/IL-10 | 34.04 ± 5.01 | 39.87 ± 7.52 | 51.17 ± 9.34 | NS |
| | TNF-α/IL-4 | 11.61 ± 1.27 | 20.32 ± 4.16 | 17.48 ± 4.10 | NS |
| | TNF-α/IL-10 | 22.42 ± 3.65 | 44.06 ± 10.12 | 40.56 ± 8.75 | 0.049 |

[a]Comparisons were made using one-way analysis of variance with the Scheffé multiple comparison test.
$^{*}P < 0.05$, $^{**}P < 0.01$ as compared with controls by the Scheffé multiple comparison test.
IFN = interferon; TNF = tumour necrosis factor; NS = not significant.

recognition of HLA-G was a consistent finding among normal women, recurrent aborters, and men (Maejima *et al*., 1997). Perhaps the regulation of TNF-α synthesis may determine reproductive outcome. In our study, TNF-α expression in CD3+/CD8– cells from infertile women with implantation failures is significantly up-regulated as compared with that of normal controls. More importantly, the ratio of TNF-α to IL-4- or IL-10-expressing cells is persistently elevated in both women with RSA and infertility due to implantation failures with no history of SAB as compared with those of normal fertile controls, and infertile women with multiple implantation failures without SAB demonstrated the highest Th1/Th2 ratios, especially TNF-α related ratios. These findings are examples of the relationship between TNF-α, implantation and pregnancy outcome.

IL-10 is known to selectively suppress Th1-mediated cellular immunity by inhibiting the production of inflammatory cytokines such as IFN-γ, TNF-α and IL-1 (Mosmann and Moore, 1991). Decreased production of IL-4 and IL-10 by decidual T cells of women with unexplained RSA when compared with decidual cells of women with normal pregnancy has been reported (Piccini *et al*., 1998). Our study demonstrated a significant difference in IL-10 expression in activated peripheral blood CD3+/CD8+ cells in recurrent aborters.

In the mouse model, placental antigens from resorption-prone CBA/J×DBA/2 mating activate CD8+ T cells, which results in abortion (Raghupathy, 1997b). However, in-vivo injection of anti-CD8+ T cells into abortion-prone CBA/J×DBA/2 pregnancies either has no effect or boosts the abortion rate, dependent upon days of gestation (Chaouat and Menu, 1997). CD8+ T cells have apparently opposing effects, which may in part be explained by the Th1/Th2 paradigm and the fact that CD8+ T cells can potentially belong to either phenotype. In our study, TNF-α/IL-10 ratios in CD3+/CD8+ T cells differed significantly between in women with infertility of implantation failure as compared with those of normal controls. Our collateral study using the same population of patients demonstrated significantly elevated activated NK cells (CD56+/CD69+) in peripheral blood (Ntrivalas *et al*., 2001). The presence of activated NK cells in these women may be related to the activation status of CD3+/CD8+ cells, which have decreased Th2 cytokine production.

The underlying aetiology of Th1/Th2 polarization needs further investigation. A Th1 shift may be mediated by T cells or antigen-presenting cells that direct the differentiation of effector cells. From our study, it is interesting to notice that lymphocytes from women with implantation failures, who had never become pregnant and have had no chance to be exposed to trophoblasts antigens, or never had any history of pregnancy losses, demonstrated an increased Th1 shift in their cytokine expression. Therefore, it is plausible that increased synthesis of Th1 cytokines may be induced not only by trophoblast antigens, but also by antigen-non-specific cytokine/chemokine production in response to stress products of hormonally manipulated endometrium, hyperstimulated ovarian products or non-physiologically high levels of female sex hormones. In this study, incidence of endometriosis and tubal factor infertility in women without a history of SAB was higher than that of women with a history of SAB although the difference was not statistically significant. It is speculated that endometriosis and tubal inflammatory diseases may contribute to a Th1 shift in women with multiple implantation failures.

A question was raised as to whether women with implantation failure without a history of spontaneous pregnancy losses have a Th1 shift. In this study, women with implantation failures without a history of SAB were compared with women with implantation failures with a history of SAB and normal fertile controls. This study setting allowed us to investigate the impact of pregnancy losses and Th1 shift with regard to implantation failures. Interestingly, infertile women with multiple implantation failures without a history of SAB demonstrated significantly lower number of mature oocytes than infertile women with multiple implantation failures with



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SAB history in their previous IVF cycles. The possibilities that (i) pre-existing Th1 shift may cause low response to ovarian stimulation or (ii) higher dose FSH stimulation due to poor ovarian response may induce Th1 shift, should be explored in the future.

Since the introduction of IVF and embryo transfer technique, the implantation rate after IVF cycles has not markedly increased. Prenatal genetic diagnosis may explain this low implantation rate in part (Kahraman *et al*., 2000). However, our study also raises an important question for the role of Th1/Th2 immune responses in failure of embryonic implantation. Further study is needed to explore the relationship between T helper cell cytokine regulations, MHC complex, and reproductive outcome in women with altered Th1 immune responses.

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