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ORIGINAL ARTICLE

Immunotherapy of Patients with Recurrent Spontaneous Miscarriage and Idiopathic Infertility: Does the Immunization-Dependent Th2 Cytokine Overbalance Really Matter?

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Abstract Recurrent spontaneous miscarriage (RSM) and idiopathic infertility (IIF) are partially caused by immunologic disturbances. Paternal lymphocyte immunization (PLI) is proposed for restoration of the proper Th1/Th2 balance in these patients, but still there are controversies on PLI mechanism, its efficacy and identification of patients who may benefit from this therapy. The study group consisted of $n = 34$ RSM and $n = 42$ IIF women with unexplained miscarriage or IIF. PLI was offered as a treatment in both groups. Peripheral blood lymphocyte (PBL) populations ($CD3^+$, $CD3^-/CD19^+$, $CD3^+/CD4^+$, $CD3^+/CD8^+$, $CD3^-/CD16^+CD56^+$) were studied before immunization, while PBL cytokine secretion (IFN-γ, TNF-α, IL-10, IL-5, IL-4, IL-2), before and after immunization, pre-conceptionally in both groups. The reference PBL ratio and cytokine levels were adopted from previously studied normal fertile women. PBL populations, concentration and ratio of Th1/Th2 cytokines did not differ between RSM and IIF patients. Compared to the results observed in normal fertile women the levels of IFN-γ, TNF-α and IL-2 were higher, while IL-10 lower in both RSM and IIF patients ($p < 0.01$). After immunization a decrease of IFN-γ (RSM and IIF groups) and IL-4 and IL-10 (RSM group) were observed, as well as an increase in TNF-α/IL-4 ratio (RSM group) ($p < 0.05$). No differences in Th1/Th2 concentration and ratio between patients with successful and unsuccessful pregnancy were observed. No significant correlations between success and particular cytokine concentration were observed. Concentrations of Th1/Th2 cytokines and PBL populations did not differ between RSM and IIF women. Th1 shift in both RSM and IIF patients was observed in comparison to fertile women. Treatment with PLI-induced pre-conceptionally cytokine changes which neither indicated Th2 shift nor correlated with subsequent pregnancy success.

Keywords Recurrent spontaneous miscarriage · Idiopathic infertility · Cytokines · Paternal lymphocyte immunization

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Abbreviations

RSM	Recurrent spontaneous miscarriage
IL	Interleukin
TNF-α	Tumor necrosis factor-alpha
IFN-γ	Interferon-gamma
IIF	Idiopathic infertility
ART	Artificial reproductive techniques
RIF	Recurrent implantation failures
PLI	Paternal lymphocyte immunization
IUI	Intrauterine insemination
IVF-ET	In vitro fertilization-embryo transfer
PBL	Peripheral blood lymphocyte
BV	Bacterial vaginosis
ACA	Anticardiolipin antibody

ANA	Antinuclear antibody
ASA	Antisperm antibody
ATA	Antithyroid antibody
TPOa	Peroxidase antibody
TGa	Thyroglobulin antibody
LA	Lupus anticoagulant
LCT	Lymphocytotoxic test
PE	Phycoerythrin

Introduction

Apart from parental chromosomal aberrations, uterine defects and thrombophilic disorders, both auto and alloimmunological disturbations are well-recognized reasons for recurrent spontaneous miscarriage (RSM) (Toth et al. 2010). Since approval of Wegmann's theory (Wegmann et al. 1993), the consequence of imbalanced Th1/Th2 cytokine activity has become one of the most discussed causes for the rejection of fetal allograft in case of unexplained or alloimmunological RSM. Classical experiments on abortion-prone mating of CBA/J × DBA/2 mice demonstrated activation of Th1 cytokines [interleukin (IL)-1, IL-12, tumor necrosis factor (TNF)-α, interferon (IFN)-γ] at the implantation site in CBA/J females (Chaouat et al. 1990). Similar Th1 overbalance was reported in the studies of peripheral blood lymphocytes (PBLs) in RSM women "in vivo" and "in vitro" (Hill et al. 1995; Marzi et al. 1996; Raghupathy et al. 2000). Some authors remarked that some other mechanisms like function of regulatory T $CD4^+CD25^+FoxP3^+$ cells (Tregs), expression of indoleamine 2,3-dioxygenase and heme-oxygenase-1 or regulatory properties of molecule CD200 could also be disturbed in RSM (Clark et al. 2005; Clark and Chaouat 2005; Mellor and Munn 2004; Yang et al. 2008; Zenclussen et al. 2005).

Idiopathic or unexplained infertility (IIF) is a condition defined as a lack of pregnancy despite the absence of reproductive pathology in the investigated couple. In many IIF women qualified for artificial reproductive techniques (ART), precise pre-conceptional monitoring demonstrates the existence of recurrent implantation failures (RIF). At least part of reproductive failures may be caused by immunological disturbances (Plaks et al. 2008), akin to Th1 overbalance observed in RSM women (Aboul Enien and El Metwally 2005; Hanzlikova et al. 2009; Kwak-Kim et al. 2003; Srivastava et al. 2008). However, the cytokine environment that is demanded for successful pregnancy in IIF patients is more complex. Immunological intrauterine milieu during implantation does not seem to favor Th2 shift, but instead engages both Th1 and Th2 activity (Ashkar and Croy 2001; Ashkar et al. 2000; Chaouat et al. 2004; Parr et al. 1995; Robertson et al. 1994).

Although being controversial for many years (Ober et al. 1999; Porter et al. 2006), paternal lymphocyte immunization (PLI) is still used in the management of RSM women (Takeshita 2004). The rationale for use of PLI originated from animal studies, which indicated that immunization of CBA/J females with lymphocytes of BALB/c males, prior to mating with DBA/2 males, reversed the tendency to RSM (Clark and Chaouat 2005). Most studies concluded that PLI-dependent restoration of proper Th2 activity before subsequent pregnancy was a key factor for efficient treatment of the RSM patients (Gharesi-Fard et al. 2008; Hayakawa et al. 2000; Makhseed et al. 2001). Considering the fact that Th2 hypoactivity could be a potential pathologic factor in at least some groups of IIF patients, PLI was also proposed as a therapeutic option for these women (Beer 2003; Drakakis et al. 2003; Michelon et al. 2003).

Taking into consideration that immunopathologic mechanisms observed in the group of IIF patients are probably much more heterogenic than these observed in RSM women, an interesting question arises. Is there enough immunological similarity between both groups to justify similar treatment like PLI? Therefore, the first aim of the presented study was to compare the immunologic parameters between the group of RSM and IIF patients before the treatment with PLI in order to check if they constituted two distinct immunopathologic populations. Another controversy arises from the recent doubts that "Th2 phenomenon" is a neccessary component of normal pregnancy (Chaouat et al. 2004). Similarly, the PLI-dependent Th2 shift observed in some studies before conception (Gharesi-Fard et al. 2008; Hayakawa et al. 2000; Makhseed et al. 2001; Yokoo et al. 2006) might not be an indispensable condition for pregnancy success. Therefore, we aimed to investigate the influence of PLI on PBL cytokine secretion and to correlate it with the pregnancy success in both studied groups.

Materials and Methods

Patients

The study groups were recruited from $n = 91$ patients diagnosed for the cause of RSM or unknown infertility in two outpatient clinics: "Gameta" Fertility Center and "Vita-Med" Outpatient Gynecological Clinic in Lodz, Poland, between 2008 and 2009. Patients gave written consent for participation in the study, as well as the approval of the Ethics Committee was obtained.

According to the new recommendations, RSM was defined as at least two consecutive abortions before 22nd week of gestation (Practice Committee of the American Society for Reproductive Medicine 2008). The RSM

patients were subjected to precise examination and laboratory testing in order to recognize the cause for recurrent pregnancy loss (genetic, anatomical, infectious, hormonal, autoimmunological, thrombophilia or diabetes; Table 1). Only the group of patients without known cause for RSM and diagnosed as "unexplained RSM" were included in the study ($n = 34$). History of miscarriages in this group of women indicated that maximal duration of previous pregnancies was 12 weeks, minimal duration was 5 weeks and the most frequent (35% of pregnancies) time for pregnancy loss was between 7 and 9 weeks of gestation. Fifty percent of patients ($n = 17$) had history of two consecutive miscarriages, 44.1% ($n = 15$) of three, 2.9% ($n = 1$) of four and 2.9% ($n = 1$) of five, respectively. The total number of documented pregnancies was $n = 88$. Except for 3.4% ($n = 3$) patients who had successful first pregnancy with another partner, none of the women reported the history of viable birth. There were three forms of miscarriage: 19.3% ($n = 17$), intrauterine fetal death ("obsolete pregnancy"); 71.6% ($n = 63$), spontaneous abortion, and 5.7% ($n = 5$), anembryonic pregnancy ("blighted ovum").

Infertility was defined as a failure to conceive successfully after at least 12 months (or after at least 6 months for women over age 35) of regular unprotected intercourse (Practice Committee of the American Society for Reproductive Medicine 2008). Couples with male infertility described as: serious semen pathology (azoospermia, as well as terato-, astheno- and oligozoospermia according to the World Health Organization criteria), presence of antisperm antibodies in semen and/or cystic fibrosis gene mutation were excluded from the study (Table 1). Only the group of patients after meticulous evaluation (Table 1) that did not allow to recognize the infertility reasons were included in the study—IIF group ($n = 42$). The IIF group consisted of two kinds of patients. The first were infertile women who prior to enrolment into our study had got at

Table 1 Exclusion criteria for RSM and infertility patients

RSM testing	IIF testing
Hysteroscopy: septate uterus, adhesions	Hysteroscopy: congenital anomalies, adhesions
Genetic counseling	
Parental karyotypes: chromosomal aberration	Hysterosalpingography or hysterosalpingosonography: tubal impermeability
TORCH screening: active infection	TORCH screening: active infection
$OGTT_{75}$ test: glucose intolerance or DM	$OGTT_{75}$ test: glucose intolerance or DM
Female hormonal profile: disturbed	Female hormonal profile: disturbed
5–7 day of cycle: LH, FSH, PRL, TSH, fT_3, fT_4, testosterone	5–7 day of cycle: LH, FSH, PRL, TSH, fT_3, fT_4, testosterone
22–24 day of cycle: progesterone and endometrial sampling	22–24 day of cycle: progesterone and endometrial sampling
Chlamydia trachomatis screening: active infection	TVUS ovulation monitoring: anovulation
BV screening: active infection	Genetic counseling
	Parental karyotypes: chromosomal aberration
	CFTR mutation: positive
Thrombophilias: positive screening	BV screening: active infection
Hyperhomocysteinemia	*Chlamydia trachomatis* screening: active infection
V Leiden mutation	
PT 20210 mutation	
Concentration of protein C	
Concentration of protein S	
Autoantibodies: (+) in high level/titers	Autoantibodies: (+) in high level/titers
ACA	Serum ASA: female
ASA	Semen ASA: male
ANA	
ATA	
LA	
LCT-positive	Computed semen analysis: serious semen pathology

TORCH toxoplasmosis, rubella, cytomegaly; *$OGTT_{75}$* glucose curve after oral use of 75 g of glucose; *DM* diabetes; *LH* luteinizing hormone; *FSH* follicle-stimulating hormone; *PRL* prolactin; *TSH* thyroid-stimulating hormone; *fT_3* free triiodothyronine; *fT_4* free thyroxine; *TVUS* trans-vaginal ultrasound; *BV* bacterial vaginosis; *CFTR* cystic fibrosis gene; *PT* prothrombin; *ACA* anticardiolipin antibody; *ANA* antinuclear antibody; *ASA* antisperm antibody; *ATA* thyroid peroxidase antibody (TPOa) and thyroglobulin antibody (TGa); *LA* lupus anticoagulant; *LCT* lymphocytotoxic test

least 3-year history of treatment for IIF, decided to conceive without ART and had got more than one positive blood pregnancy test in that period. These women were diagnosed again and after passing through our inclusion criteria were qualified for immunological assay. The second kind of patients were infertile women who had got a history of infertility and after the confirmation of its unknown cause were subsequently qualified for the ART procedures which were all unsuccessful (at least two consecutive failures). These patients were immunologically tested after the unsuccessful completion of ART. The total number of 29 intrauterine inseminations (IUI) and 23 embryo transfers (IVF-ET) were done in the IIF group. Thirty-one percent of IUI procedures were done twice, 13.8%—three times and 55.2%—four times or more, whereas 65.2% of IVF-ET procedures were done twice and 34.8% done three times or more.

All studied RSM and IIF women who fulfilled inclusion criteria were qualified for immunological tests which consisted of PBL population profile and the PBL Th1/Th2 cytokine secretion upon mitogen stimulation. Women were asked not to be ill or in early convalescence during blood collection. All tests were performed in the midsecretory phase.

Immunologic Tests

Both immunologic assays and immunization were done in "APC" Medical Analyses laboratory that participates in a quality control procedures. The everyday work at the laboratory is under routine supervision of a stuff who was formerly working in the diagnostic immunologic division in the research institute.

Antibodies

The presence of serum anticardiolipin IgM/IgG autoantibodies (ACA), antisperm IgA/IgM/IgG antibodies (ASA), as well as antithyreoglobuline (TGa) and thyroid peroxidase antibodies (TPOa) was quantified with immunoenzymatic ELISA assay. Standardized commercial Varelisa Cardiolipin Antibodies, Varelisa Sperm Antibodies, Varelisa TPO Antibodies and Varelisa TG Antibodies microplate tests (Pharmacia Deutschland GmbH, Diagnostics Division, Germany) were used. Absorbance of the samples was measured by a DS2 counter (Dynex Technologies, Inc., USA) using the light of 450-nm length. The results were read from standard curves. Results were presented in U/ml and the adopted ranges of positive values were as follows: ACA > 15 GPL-U/ml, ASA > 20 U/ml, TGa/TPOa > 100 IU/ml. The concentration of serum lupus coagulant (LA) was estimated with LAC screen and LAC confirm tests (Instrumentation Laboratory, Lexington, USA) and ACL9000 analyzer. Results were given as normalized LAC coefficient [LA(−) <1.2, LA(+) 1.2–1.5, LA(++) 1.5–2.0, LA(+++) >2.0]. The level of serum antinuclear antibodies (ANA) was measured by indirect immunofluorescence assay with the use of NOVA Lite™ HEp-2 (INOVA Diagnostics Inc., San Diego, USA). The fluorescence of the sample was estimated in CX41 microscope (Olympus Corporation, Germany). The result was presented as a dilution titer and type of fluorescence (low/medium titers 1:80–1:160). For the measurement of serum anti-HLA class I IgG (ELISA lymphocytotoxic test) QUICK Screen Solid Phase HLA Antibody Screening Kit QS12G (GTI, Crossroads Circle Waukesha, USA) was used. Absorbance of the samples was measured by a DS2 counter (Dynex Technologies Inc., USA) using the light of 450-nm length. Lymphocytotoxic test (LCT) value of <0.30 U/ml was considered to be negative.

Lymphocyte Populations

The percentage of main T lymphocyte populations (total T CD3, T CD4, T CD8), B lymphocytes and NK cells was measured in whole heparinized peripheral blood using Multitest 6-Color TBNK Reagent Kit (Becton–Dickinson and Co., BD Biosciences, San Jose, USA) with standard monoclonal antibodies: CD3FITC, CD19APC, $CD16^+$ CD56PE, CD45 PerCP-Cy5.5, CD4Pe-Cy7 and CD8APC-Cy7. Tests were performed with the use of FacsCanto cytometer (Becton–Dickinson and Co., BD Biosciences, San Jose, USA) and BD FACSCanto Software v. 2.4. The reference values adopted as "normal" were: $CD3^+$ (60–82%), $CD3^-/CD19^+$ (7–23%), $CD3^+/CD4^+$ (30–51%), $CD3^+/CD8^+$ (19–39%), and $CD3^-/CD16^+/56^+$ (NK) (7–24%). The PBL populations were studied only before paternal lymphocyte immunization.

Th1/Th2 Cytokine Profile

Lymphocytes for the Th1/Th2 cytokine secretion assay were isolated from heparinized whole blood by Lymphoprep (Nycomed Pharma, Lyszkowice, Poland) gradient separation (density 1.077 g/cm^3). After being rinsed twice in phosphate buffer solution (PBS) buffer (Wytwórnia Surowic i Szczepionek, Lublin, Poland) lymphocyte suspensions of 1×10^6 cells were prepared. Lymphocytes were then cultured in medium containing RPMI 1640 enriched with 2 mM L-glutamine (Wytwórnia Surowic i Szczepionek, Lublin, Poland), 10% fetal calf serum (Sigma, St. Louis, USA) and antibiotics (100 U/ml Penicyllin, 10 μg/ml Streptomycin; Sigma, St. Louis, USA) in flat-bottom plates (BD Falcon™, Becton–Dickinson and Co., BD Biosciences, San Jose, USA) in triplicate using 1×10^5 lymphocytes suspended in 100-μl culture medium per well. Stimulation of cultures was performed with phytohemagglutinin in mitogenic dose of 5 mg/ml (Sigma,

St. Louis, USA). Cultures were incubated for 24 h in humid air enriched with 5% CO_2, at 37°C temperature. Then the plates were centrifuged for 10 min and the supernatant was collected for the cytokine assay. Cytokine production was estimated using BD™ Cytometric Bead Array Human Th1/Th2 Cytokine kit (Becton–Dickinson and Co., BD Biosciences, San Jose, USA) according to the instruction manual. The CBA kit enables simultaneous cytometric survey of six cytokines in 50-µl sample: IFN-γ, TNF-α, IL-10, IL-4, IL-5 and IL-2. Falcon (BD Falcon™, Becton–Dickinson and Co., BD Biosciences, San Jose, USA) tubes were filled with the mixture of 50 µl of cytokines, 50 µl of monoclonal antibodies conjugated with phycoerythrin (PE) and 50 µl of a tested sample (culture supernatant). After 3-h incubation in darkness at the room temperature, samples were rinsed in buffered PBS (Becton–Dickinson and Co., BD Biosciences, San Jose, USA), centrifuged and resuspended in 300 µl of PBS. Tests were performed on FacsCanto cytometer (Becton–Dickinson and Co., BD Biosciences, San Jose, USA) using FCAP Array Software v.1.01. Concentration of cytokines was given in pg/ml with a range of the test from 20 to 5,000 pg/ml. Tests were performed before PLI in all patients and in more than 50% of patients 4 weeks after PLI. All tests were performed before conception.

The cytokine reference values adopted as "normal" for the purposes of this study were previously determined in the group of fertile Polish women ($n = 90$) who delivered healthy mature neonates, and were as follows: IFN-γ 290–1,045 pg/ml, TNF-α 323–1,107 pg/ml, IL-10 1,534–3,824 pg/ml, IL-5 23–93 pg/ml, IL-4 35–120 pg/ml, IL-2 20–43 pg/ml (Banasik 2007).

Partner Lymphocyte Immunization

Both partners were tested for the presence of syphilis, HBV, HCV or HIV infection. After the presentation of negative results, PLI was proposed and pros and cons of this method were thoroughly discussed with couples. PLI was performed intra/subcutaneously two times before the planned pregnancy in 4-weeks interval, with 150–250 $\times$ 10^6 of isolated fresh, X-irradiated partner's lymphocytes. 3–4 weeks after the last pre-conceptional alloimmunization, the PBL Th1/Th2 cytokine production upon mitogen stimulation was tested again; however, in only $n = 25$ (74%) RSM and $n = 23$ (55%) IIF women it was able to complete both Th1/Th2 cytokine results before and after the PLI procedure. Apart from minor local irritation and swelling, no short- or long-time adverse effects after PLI were noticed. All immunized RSM women conceived spontaneously, while all IIF women were qualified for ART procedures. In the group of RSM and IIF patients who conceived successfully, additional single busting alloimmunization was performed in 6–8 weeks of gestation. No additional immunological testing was performed during pregnancy. During 2 years of the study 38% ($n = 13$) of RSM and 12% ($n = 5$) of IIF patients finished their pregnancies successfully. Median time of delivery was 39.0 ± 2.0 and 37.0 ± 2.0 weeks, median birth weight was 3,100 ± 450 g and 2,600 ± 400 g, whereas the cesarean section rate was 54 and 80% in the group of RSM and IIF patients, respectively. All neonates born were healthy and no congenital malformations were found.

Statistical Analysis

Results were reported as the group mean ± standard deviation. For analysis of variance, Fisher's multiple comparison test was used; for normality distribution analysis the Shapiro–Wilk test and for the statistical significance of differences between groups the Student's t test was performed as appropriate. Correlations between parameters were performed using Spearman test. Tests with $p < 0.05$ were accepted as significantly different. We used licensed Statistica 7.0 for Windows.

Results

Serum Antibodies Levels/Titers in RSM and IIF Patients Before Alloimmunization

Concentrations of studied ACA, ASA, ATA antibodies, values of LA coefficient and LCT test, as well as percentage of ANA positive (low titers 1:80–1:160) patients are shown in Table 2. The differences between RSM and IIF patients were statistically insignificant, although the serum levels of TGa antithyroid antibodies were lower in IIF group ($p = 0.06$). Patients with positive low level/titers of autoantibodies constituted for 58.8% of RSM and 42.8% of IIF patients, respectively.

Table 2 ACA, ANA, ASA antibodies and values of LA coefficient and LCT test in RSM and IIF patients

Parameter	RSM ($n = 34$) mean ± SD	IIF ($n = 42$) mean ± SD	p
ACA IgM (U/ml)	2.5 ± 5.5	1.7 ± 1.0	NS
ACA IgG (U/ml)	4.3 ± 9.3	2.6 ± 3.6	NS
ASA (U/ml)	0.5 ± 2.1	1.5 ± 3.6	NS
TGa (U/ml)	81.9 ± 150.4	22.2 ± 25.4	0.06
TPOa (U/ml)	104.6 ± 336.5	63.9 ± 195.2	NS
ANA(+) (%)	30.0 ± 46.6	27.8 ± 45.4	NS
LA	1.1 ± 0.2	1.0 ± 0.1	NS
LCT (U/ml)	0.09 ± 0.12	0.05 ± 0.01	NS

SD standard deviation, *NS* difference not significant

PBL Populations in RSM and IIF Patients Before Alloimmunization

Before alloimmunization PBL populations percentage of T, B, T $CD4^+$, T $CD8^+$ and NK cells did not differ significantly between RSM and IIF patients and stayed within normal range (Table 3). Compared to the reference values the percentage of NK cells was increased, however, insignificant in both RSM and IIF group (results not shown). The ratio of patients possessing more than 18% of peripheral blood NK cells was 20.6% in RSM and 14.3% in IIF group, respectively.

Th1/Th2 Cytokine Profile in RSM and IIF Patients Before Alloimmunization

Before qualification to PLI method, mean concentrations of IFN-γ, TNF-α, IL-10, IL-5, IL-4 and IL-2 cytokines produced by mitogen-stimulated PBL, as well as Th1/Th2 ratio (IFN-γ/IL-10, IFN-γ/IL-4, TNF-α/IL-10 and TNF-α/IL-4) did not differ significantly between RSM and IIF patients (Table 4). High levels of IFN-γ (>5,000 pg/ml) were observed in 35.3% of RSM and 19% of IIF women, respectively. The percentage of women with high levels of TNF-α (>5,000 pg/ml) was 2.9% in RSM and 2.4% in IIF group. Compared to reference concentrations of cytokines, levels of IFN-γ, TNF-α and IL-2 were significantly higher, while the level of IL-10 was significantly lower in both RSM and IIF patients before the PLI procedure (results not shown).

Table 3 Peripheral blood lymphocyte populations in RSM and IIF patients before partner lymphocyte immunization

Parameter	RSM ($n = 34$) mean ± SD	IIF ($n = 42$) mean ± SD	p
T $CD3^+$ (%)	71.7 ± 8.5	72.8 ± 7.9	NS
B $CD3^-/CD19^+$ (%)	12.2 ± 6.2	12.0 ± 3.4	NS
T $CD3^+/CD4^+$ (%)	45.4 ± 9.3	46.9 ± 8.9	NS
T $CD3^+/CD8^+$ (%)	25.8 ± 7.6	24.3 ± 5.5	NS
NK $CD3^-/CD16^+56^+$ (%)	14.6 ± 6.0	14.3 ± 8.0	NS

NS difference not significant

Table 4 Mitogen-stimulated PBL cytokine concentrations and Th1/Th2 ratio in RSM and IIF patients before partner lymphocyte immunization

Parameter	RSM ($n = 34$) mean ± SD	IIF ($n = 42$) mean ± SD	p
IFN-γ (pg/ml)	3,308.4 ± 2,035.5	2,773.0 ± 2,035.3	NS
TNF-α (pg/ml)	1,375.3 ± 1,238.3	1,677.1 ± 1,574.7	NS
IL-10 (pg/ml)	675.6 ± 628.9	524.3 ± 544.9	NS
IL-5 (pg/ml)	81.8 ± 67.9	164.3 ± 236.9	NS
IL-4 (pg/ml)	114.4 ± 70.3	117.8 ± 135.2	NS
IL-2 (pg/ml)	162.8 ± 130.8	128.0 ± 143.5	NS
IFN-γ/IL-10	12.1 ± 14.2	8.9 ± 9.4	NS
IFN-γ/IL-4	34.7 ± 35.0	46.6 ± 63.2	NS
TNF-α/IL-10	3.8 ± 3.5	3.8 ± 3.0	NS
TNF-α/IL-4	12.1 ± 8.4	21.4 ± 24.3	NS

SD standard deviation, *NS* difference not significant

Th1/Th2 Cytokine Profile in RSM and IIF Patients After Alloimmunization

After the PLI procedure in $n = 25$ RSM women having complete (paired: pre- and post-immunization) results, significant decrease of IFN-γ, IL-10 and IL-4 and increase in TNF-α/IL-4 ratio were observed (Table 5). In the group of $n = 23$ IIF patients with complete results a significant decrease of IFN-γ after PLI was noted (Table 6).

Table 5 Change of concentration of cytokines and Th1/Th2 ratio in the group of $n = 25$ RSM women having complete (paired pre- and post-immunization) results, after the PLI procedure

Parameter	Before PLI mean ± SD	After PLI mean ± SD	p
IFN-γ (pg/ml)	3,529.8 ± 1,967.8	1,388.6 ± 1,492.9	<0.01
TNF-α (pg/ml)	1,464.0 ± 1,341.8	1,174.3 ± 1,201.4	NS
IL-10 (pg/ml)	765.4 ± 708.1	371.1 ± 421.2	<0.01
IL-5 (pg/ml)	80.1 ± 75.6	47.4 ± 42.0	NS
IL-4 (pg/ml)	122.9 ± 75.5	47.2 ± 25.7	<0.01
IL-2 (pg/ml)	155.7 ± 132.5	82.1 ± 88.9	NS
IFN-γ/IL-10	14.0 ± 15.9	8.9 ± 11.0	NS
IFN-γ/IL-4	35.6 ± 36.6	36.9 ± 58.9	NS
TNF-α/IL-10	4.1 ± 3.9	5.8 ± 6.3	NS
TNF-α/IL-4	11.8 ± 7.2	23.6 ± 16.2	<0.01

SD standard deviation, *NS* difference not significant

Table 6 Change of concentration of cytokines and Th1/Th2 ratio in the group of $n = 23$ IIF women having complete (paired pre- and post-immunization) results, after the PLI procedure

Parameter	Before PLI mean ± SD	After PLI mean ± SD	p
IFN-γ (pg/ml)	3,693.7 ± 1,721.6	2,063.7 ± 2,041.7	<0.05
TNF-α (pg/ml)	2,414.9 ± 1,767.9	1,345.5 ± 1,510.6	NS
IL-10 (pg/ml)	688.1 ± 580.4	360.3 ± 338.2	NS
IL-5 (pg/ml)	238.8 ± 236.5	107.4 ± 141.1	NS
IL-4 (pg/ml)	169.7 ± 127.6	99.4 ± 109.3	NS
IL-2 (pg/ml)	130.3 ± 120.2	167.2 ± 123.4	NS
IFN-γ/IL-10	11.1 ± 12.0	7.2 ± 6.8	NS
IFN-γ/IL-4	41.5 ± 48.3	45.3 ± 73.4	NS
TNF-α/IL-10	4.8 ± 4.3	4.3 ± 3.8	NS
TNF-α/IL-4	19.7 ± 24.8	19.5 ± 16.6	NS

SD standard deviation, *NS* difference not significant

Table 7 Initial concentration of cytokines and Th1/Th2 ratio in the combined group of RSM and IIF women ($n = 76$), with either successful or unsuccessful pregnancy after the PLI procedure

Parameter	Successful ($n = 18$) mean ± SD	Unsuccessful ($n = 58$) mean ± SD	p
IFN-γ (pg/ml)	2,982.2 ± 2,054.1	2,973.7 ± 2,089.5	NS
TNF-α (pg/ml)	1,125.2 ± 854.3	1,519.9 ± 1,460.0	NS
IL-10 (pg/ml)	726.2 ± 800.8	532.2 ± 507.6	NS
IL-5 (pg/ml)	67.1 ± 46.6	123.3 ± 183.4	NS
IL-4 (pg/ml)	75.7 ± 56.2	118.9 ± 109.7	NS
IL-2 (pg/ml)	120.1 ± 132.8	149.8 ± 134.8	NS
IFN-γ/IL-10	15.9 ± 15.7	9.8 ± 13.9	NS
IFN-γ/IL-4	50.8 ± 45.9	40.6 ± 57.9	NS
TNF-α/IL-10	4.8 ± 4.8	3.7 ± 3.3	NS
TNF-α/IL-4	17.7 ± 23.6	17.9 ± 23.4	NS

SD standard deviation, *NS* difference not significant

Th1/Th2 Cytokine Profile Before Alloimmunization in Patients with Subsequent Successful Versus Unsuccessful Pregnancy

In studied group of women (RSM and IIF together $n = 76$) there were no significant differences in initial concentration of cytokines and Th1/Th2 ratio between patients having successful or unsuccessful pregnancy (Table 7).

Th1/Th2 Cytokine Profile After Alloimmunization in Patients with Subsequent Successful Versus Unsuccessful Pregnancy

Similarly, in studied group of women with cytokine results after PLI procedure (RSM and IIF together $n = 48$) there were no significant differences in concentration of cytokines and Th1/Th2 ratio between patients with successful and unsuccessful pregnancy (Table 8).

Correlation between Success and Cytokine Concentration Before and After Alloimmunization

There were no significant correlations observed between the presence of successful pregnancy and cytokines' concentration either before or after the PLI procedure (Table 9).

Table 8 Concentration of cytokines and Th1/Th2 ratio after the PLI procedure in the combined group of RSM and IIF women having cytokine results ($n = 48$), with either successful or unsuccessful pregnancy

Parameter	Successful ($n = 16$) mean ± SD	Unsuccessful ($n = 32$) mean ± SD	p
IFN-γ (pg/ml)	1,636.4 ± 1834.8	1,380.9 ± 1,618.8	NS
TNF-α (pg/ml)	1,489.6 ± 1581.7	992.3 ± 989.3	NS
IL-10 (pg/ml)	429.2 ± 494.9	281.7 ± 260.6	NS
IL-5 (pg/ml)	56.2 ± 50.8	65.2 ± 87.0	NS
IL-4 (pg/ml)	54.1 ± 46.0	55.9 ± 65.4	NS
IL-2 (pg/ml)	98.8 ± 92.7	109.1 ± 108.6	NS
IFN-γ/IL-10	8.7 ± 10.5	6.4 ± 7.9	NS
IFN-γ/IL-4	43.3 ± 70.9	32.9 ± 48.4	NS
TNF-α/IL-10	5.8 ± 6.4	4.5 ± 4.3	NS
TNF-α/IL-4	25.2 ± 16.1	19.9 ± 14.4	NS

SD standard deviation, *NS* difference not significant

Table 9 Correlations between success and particular cytokine concentration before and after the PLI procedure in combined group of RSM and IIF women

Correlation of success with concentration of cytokine	r	p
Before PLI ($n = 76$) (pg/ml)		
IFN-γ	0.01	NS
TNF-α	−0.05	
IL-10	0.04	
IL-5	−0.08	
IL-4	0.06	
L-2	0.12	
After PLI ($n = 48$) (pg/ml)		
IFN-γ	0.06	NS
TNF-α	0.11	
IL-10	0.03	
IL-5	0.07	
IL-4	0.00	
IL-2	0.07	

r Spearman correlation coefficient, *NS* difference not significant

Discussion

Previous studies devoted to RSM patients demonstrated increased activity of CD4 Th lymphocytes, as well as cytotoxic T CD8 and NK cells, while decreased number of Tregs in peripheral blood (Coulam et al. 1995; Ramhorst et al. 2003; Saito et al. 2005). Similarly, laboratory tests in the group of infertile women qualified for IVF procedures demonstrated increased number of peripheral blood cytotoxic NK cells and lymphocytes bearing CD69 and HLA-DR activating markers (Chernyshov et al. 2010; Michou et al. 2003) when compared to fertile controls. The comparison of results of PBL populations between our study groups and reference values showed insignificant increase of NK cells in patients with both reproductive pathologies. However, all studied patients had PBL staying within normal reference range. The aim of our study was to compare the immunological status of RSM and infertility patients and in this context no significant differences according to PBL populations between both studied groups

were found. The possible explanation for this phenomenon might be that considerable parts of both RSM and IIF patients share similar immunological pathology.

In RSM women the Th1/Th2 imbalance occurs both spontaneously (elevated IL-2 and IFN-γ levels) and upon trophoblast stimulation (increased IL-2, TNF-α, IFN-γ and reduced IL-4 and IL-10 levels) in PBLs (Hill et al. 1995; Marzi et al. 1996; Raghupathy et al. 2000). It was also reported in endometrium (elevated levels of IL-13 and IL-15 levels) (Chegini et al. 2002). Peripheral blood Th2 hypoactivity was also observed in infertile women (Hanzlikova et al. 2009; Kwak-Kim et al. 2003), whereas serum Th1 overactivity after embryo transfer (Fasouliotis et al. 2004) or observed in uterine washings (Ledee-Bataille et al. 2004) predicted poor pregnancy outcome in these patients. Increased levels of Th1 pro-inflammatory cytokines were found in cervical mucus or uterine tissues of infertile women and correlated with bacterial vaginosis (BV) and *Chlamydia* infection (Aboul Enien and El Metwally 2005; Srivastava et al. 2008), suggesting that in some circumstances cytokine imbalance could be provoked by subclinical genital tract infection. Our study confirmed reports about Th1 overbalance existing in RSM and IIF women. Patients with both BV and *Chlamydia* infection were excluded from the study. Therefore, it is probably not an infection which is the trigger for the observed cytokine profile changes.

Studies performed so far have indicated that IIF could have much more complicated multifactorial pathophysiology than RSM (Kang et al. 2009; Salker et al. 2010). Therefore, similar cytokine secretion pattern in RSM and IIF women might be just an epiphenomenon resulting from underlying unidentified cause. The significant difference in pregnancy rates between RSA and IIF women observed in our study seems to confirm that notion. Alternatively, disturbed Th1/Th2 ratios may be partially explained by cytokine encoding genes polymorphism which favors Th1 secretion profile. This was confirmed in some RSM women (Amani et al. 2003; Daher et al. 2004; Liu et al. 2010) and could probably be also true for a group of IIF patients.

Partner lymphocyte alloimmunization is believed to provoke such immunological changes that improve the subsequent pregnancy outcome. The studies performed in RSM patients after PLI showed favorable immunocyte changes (Kwak et al. 1998; Szpakowski et al. 2001; Yang et al. 2008) and shifting of cytokine balance towards Th2 activity (Gharesi-Fard et al. 2008; Hayakawa et al. 2000; Makhseed et al. 2001; Yokoo et al. 2006). Paternal alloimmunization was also proposed as one of the therapeutic options for women who suffer from IIF and/or RIF (Beer 2003; Drakakis et al. 2003; Michelon et al. 2003). Our results did not confirm that alloimmunization had neither biased cytokine balance clearly towards Th2 activity nor that observed cytokine changes were connected with subsequent pregnancy success after immunization. In our opinion, cytokine profile modifications might be interpreted as a transient reaction for immunization itself. Moreover, some experiments and human studies seem to neglect the role of Th2 cytokines as the important players even during pregnancy. Based on animal studies, it was found that uterine regulatory NK cells infiltrating decidua produce IFN-γ and IL-18, which together with IL-15, IL-12 and TNF-α are the key regulators of angiogenesis in the implantation site (Ashkar and Croy 2001; Ashkar et al. 2000; Chaouat et al. 2004; Parr et al. 1995; Robertson et al. 1994). IL-12-, IL-15- or IL-18-ablated mice showed predisposition to early reproductive failure (Croy et al. 2003). It was shown that IL-4/IL-10 knockout mice can deliver healthy normal pups, which proved that both maternal and placental IL-4 and IL-10 production was not a crucial component of allogeneic pregnancy (Svensson et al. 2001). Similar mechanisms were described in humans (Murphy et al. 2009; Zenclussen et al. 2002). Patients with RIF after IVF-ET presented lowered levels of both IL-10 and IFN-γ (Inagaki et al. 2003). Moreover, monocytes during normal human pregnancy were primed to secrete IL-12 (Sacks et al. 2003), which indicates that different cell populations may behave in different manner without any harm to ongoing pregnancy. Clinical trials have also brought some criticism about the indispensable role for Th2 shift in successful reproduction. In the group of 54 women with IIF, significant differences in implantation and live birth rate between women with higher and lower Th1/Th2 ratio were not shown (Winger et al. 2009). The results of another study demonstrated that serum levels of IFN-γ and TNF-α did not correlate neither with implantation rate nor with miscarriage rate in women subjected to IVF-ET (Thum et al. 2007). These observations seem to support our results and let us conclude that cytokine changes should not be considered separately, but rather as a part of well orchestrated different mechanisms.

Although exposed to critical opinions and found as ineffective and controversial method by some authors and 2006 Cochrane Database (Ober et al. 1999; Porter et al. 2006), PLI still exists as an therapeutic option (Takeshita 2004). The results of both recent randomized trials and meta-analyses seem to confirm a benefit from PLI treatment (Check 2010; Christiansen et al. 2004; Pandey and Agrawal 2004; Pandey et al. 2004) under the condition that the immunization protocol is correct (Clark and Chaouat 2005). Non-randomized clinical trials demonstrated a benefit in 58–87% of alloimmunized compared to 36–46% of non-alloimmunized RSM women (Malinowski et al. 1997; Ramhorst et al. 2000; Sugi et al. 1991). Its efficacy was estimated to be 43–73% in IIF women (Beer 2003; Drakakis et al. 2003; Michelon et al. 2003). Our results

showed 38% of effectiveness in RSM and 12% of IIF women, respectively, with success defined as delivery of a viable neonate. As we performed PLI according to the international recommendations, low efficacy in RSM group is probably caused by the fact that for the purposes of the study patients were monitored only until the end of 2009. Some of them had successful pregnancy after this period. We conclude that due to low efficacy PLI should not be offered routinely to IIF patients as there are no accepted immunologic criteria for isolation of IIF patients who would get the greatest benefit from this treatment. There are some new therapeutic options tested in infertility patients nowadays (Clark 2010; Guerin et al. 2009).

The strength of our study is precisely defined exclusion criteria and unified and correct immunization protocol. The weakness of the study is a number of studied patients, especially those having paired complete pre- and post-immunization results. However, most other studies devoted to PLI were done on groups less than 80 women, and when only randomized trials are considered on 20–35 patients (Porter et al. 2006).

In summary, results of our study indicate that Th1/Th2 imbalance exists in RSM and IIF women. Treatment with paternal lymphocyte immunization induces some cytokine changes which are not identical with Th2 shift and do not correlate with subsequent pregnancy success.

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