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Chapter 9

Stronger T cell allo-reactivity and diminished suppressive capacity of peripheral regulatory T cells in infertile women undergoing *in vitro* fertilization

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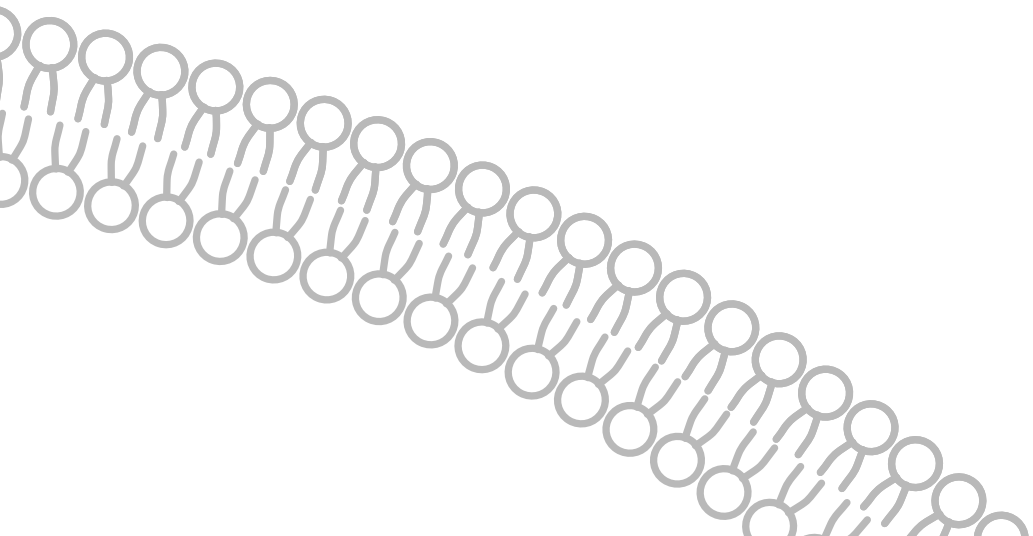

Abstract

Problem

Increasing evidence suggests modulation of the maternal immune response to be essential for successful pregnancy. We studied the immunophenotypic profile and function of peripheral blood T lymphocytes in infertile women undergoing IVF and fertile control population

Method

We collected peripheral blood mononuclear cells (PBMC) from infertile patients with recurrent implantation failure (RIF), infertile patients with successful IVF (IVFs) and normal fertile women. Cells were phenotypically analyzed and the proliferative response and cytokine production was studied in mixed lymphocyte cultures (MLC), using lymphocytes of the own partner, or a third party male as stimulators cells. To examine the suppressive capacity of regulatory Tregs we performed MLC studies with a CD45+ fraction depleted for CD4+CD25bright T cells.

Results

No significant differences in proportions of subsets of circulating T lymphocytes were observed. The proliferative allo-immune response of PBMC of IVF women (RIF and IVFs) was significantly higher, with higher production of Th1- and Th2 cytokines, compared to the fertile women. This difference in proliferation and cytokine production was associated with a diminished suppressive capacity of Tregs in these women

Conclusion

The higher allo-immune response of the IVF women compared to fertile women might be the result of a diminished suppressive capacity of Tregs and emphasizes the important role of Tregs during conception

Introduction

Since 1978 the technique of in-vitro fertilization (IVF) has been adjusted and improved. Still pregnancy rate remains under 30% [115]. The major rate limiting step in IVF success seems the failure of embryos to implant, which even exceeds the percentage in spontaneous cycles [391].

Successful implantation is however a complicated process that requires the orchestration of a series of events involving both the embryo and the endometrium [392]. Failure of any of the factors in these events might reduce the overall implantation rate. Increasing evidence suggests that an unbalanced maternal immune response towards the embryo may cause its rejection [89,393]. Indeed, the induction of fetus specific tolerance is important for normal implantation and recent evidence support epidemiologic data that already before implantation fetus specific tolerance is induced [394]. This activation of mother's adaptive immune system probably occurs during conception and prior exposure to semen [395]. Thus, even though the implantation actually has not yet taken place, an aberrant modulation of the maternal immune system to her future fetus could play a role in the mechanism of implantation failure.

Earlier studies indeed showed that the immune reactivity in women with recurrent pregnancy losses is different as reflected by higher Th1/Th2 ratios in the peripheral blood of women with recurrent implantation failure (RIF), defined as repeated consecutive failure of high quality embryos to implant (following three attempts of IVF), and women with recurrent spontaneous miscarriages (RSM) [396-398]. Furthermore, peripheral T cells from patients with implantation failure express higher levels of the activation markers HLA-DR, CD154 and CD69 [399,400]. T cells with immune suppressive functions are pivotal for an appropriate immune regulation and only recently the essential role of these regulatory T cells in pregnancy has been appreciated [79,394]. In decidua and peripheral blood of RSM patients the proportion of $CD4^{+}CD25^{bright}$ regulatory T cells was significantly lower compared to elective abortions or non-pregnant multiparous women [99,174,401,402]. An important role of these regulatory T cells in the pathogenic mechanism of recurrent implantation failure is suggested by the fact that administration of anti-CD25 monoclonal antibody prior to the implantation period induced implantation failure in mice [403]. Furthermore, transfer of Tregs from normal pregnant mice was shown to prevent fetal resorption in mice [96,97]. In humans, elevated levels of $CD4^{+}CD25^{+}FoxP3^{+}$ Treg cells at the time of oocyte retrieval was associated with increased rates of pregnancy and live births in IVF treatment [404].

In this study we determined the immunophenotypic profiles and function of peripheral blood T lymphocytes in women with recurrent implantation failure after *in vitro* fertilization and compared those with control populations, with a special emphasis on the role of regulatory T cells.

Method

Subjects

We selected 54 women and their partners undergoing conventional IVF or intra cytoplasmatic sperm injection (ICSI) treatment in the Leiden University Medical Center (LUMC) from January 2005 to December 2009 (stimulation protocol in supplement). The patient group (n=22) consisted of couples with recurrent implantation failure (RIF), defined as ≥3 consecutive IVF failures with high quality and fresh embryos. The control group (n=32) consisted of couples with success after IVF treatment (IVFs), which was defined as a live child birth after the first IVF or ICSI treatment. Exclusion criteria were women with known autoimmune diseases (antiphospholipid syndrome, SLE, thyroid autoimmunity), endometriosis, use of immunosuppressive medication and couples who applied to egg or spermatozoa donation. A second control group (n=21) from the study of van den Boogaardt et al [23] consisted of normal fertile couples with at least 1 spontaneous pregnancy and live child birth in medical history and no record of secondary infertility. None of the female participants was pregnant at inclusion. Medical records were reviewed and clinical data were summarized. The study protocol was approved by the ethics committee of the LUMC (P08.228), and informed consent of every couple was obtained.

Lymphocyte isolation and HLA typing

Peripheral blood samples were obtained from the women and their partners. The blood samples were processed within 24hr on a Ficoll Hypaque (LUMC pharmacy; Leiden, The Netherlands) gradient for density gradient centrifugation at room temperature (20min/800g). After centrifugation peripheral blood mononuclear cells (PBMC) were isolated from the interface, and, after washing procedures, frozen in liquid nitrogen. The individuals were HLA typed for the loci HLA-A, -B, -C, -DQ and -DR using the Sequence Specific Oligonucleotides PCR technique and the number of paternal-maternal HLA DR mismatches was calculated at the national reference laboratory for histocompatibility testing (LUMC).

Flowcytometry

The PBMC of the women were used for phenotypic characterization by flowcytometry. The following directly conjugated mouse-anti-human monoclonal antibodies were used

for four-color immunofluorescence surface staining of the PBMCs: CD45-APC, CD14-FITC, CD19-PE, CD3-PerCP, CD4-APC, CD8-PE, CD16-PE, CD25-PE, CD28-APC, CD56-FITC, CD69-FITC and HLA-DR-FITC (Becton Dickinson, Franklin Lakes, NJ, USA), used in concentrations according to manufactures instructions. Flowcytometry was performed on a FACS Calibur using FlowJo-V10 Cytometry Analysis (Tree Star Inc, Ashland, USA). Gating strategies were performed as previously described [23]. In short, CD45+CD14- cells were selected within a density plot, back gated in a scatter plot and the lymphocyte gate was set around the viable lymphocytes. Percentages were calculated within gates set around the viable lymphocytes (in FCS/SSC dotplot) and based upon CD45, CD14 and CD3 expression. Percentages of CD4+CD25dim and CD4+CD25bright cells were calculated within the CD3+CD4+ cell population and percentages of CD8+CD28- cells within the CD3+CD8+ cell population.

Functional assays

For functional analysis of the allo-immune response a lymphocyte fraction containing all $CD45^+$ cells and a $CD45^+$fraction depleted for $CD4^+CD25^{bright}$ T cells were tested as previously described [79]. In short, cells were stained for CD4-FITC, CD25-PE and CD45-APC and FACS sorted with a FACS Aria II system with BD FACS Diva software (Becton Dickinson, Franklin Lakes, NJ, USA). The lymphocytes were gated using a FCS/SSC plot followed by doublet discrimination. Singlet lymphocytes were gated for $CD4^+CD25^{bright}$ expression to identify Tregs and these Tregs were than depleted in a yield mode. The depleted fractions were centrifuged and washed with culture medium containing RPMI 1640 with 10% human serum and L-glutamine. In order to confirm that this procedure indeed leads to the depletion of regulatory T cells both fractions of PBMC of RIF, IVF control and fertile control women were stained with two additional markers. Regulatory T cells are generally distinguished on basis of their high expression of the IL-2 receptor CD25; to further identify these cells we used the intracellular marker FoxP3 and the IL-7 receptor CD127. The expression of FoxP3 was determined using an APC-conjugated anti-human FoxP3 staining set (eBioscience, San Diego, CA, USA), cell surface staining of CD127 was performed with anti-CD127-FITC (Becton Dickinson, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. We performed this sorting procedure for 10 patients (RIF) and 10 IVF controls (IVFs) with unexplained infertility as indication for the IVF, and ten fertile control women. Unexplained infertility was defined as the inability to conceive within one year despite evidence of ovulation, tubal patency and normal semen parameters.

The $CD45^+$ fraction and $CD45^+$ fraction depleted for $CD4^+CD25^{bright}$ cells were both stimulated in a one-way mixed lymphocyte culture (MLC) with cells from their partner and with third party cells as follows: MLC were set up with $1x10^5$ female PBMC in culture medium added in triplicate wells in a round-bottom 96-well plate (Costar) to $1x10^5$ irradiated (3000 Rad) PBMC of their male partner or $1x10^5$ irradiated PBMC of a third

party male. This third party male was selected from the subjects on basis of the number of HLA-DR mismatches between the female and her own partner, because a positive correlation between the allo-reactivity and number of HLA-DR mismatches exists [175]. PHA stimulation, addition of 50µl of phytohemagglutinin in a dilution of 1:400 (PHA; 0.4 mg/ml, Wellcome, Dartford, UK) to the cultures, was used as positive control. Culture medium alone was used as a negative control.

Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 5 days. Cultures were pulsed with 20 µCi/well ^{3}H-thymidine diluted in RPMI 1640 medium for the last 18 hours of incubation. Just before pulsing, 50 µl of supernatant was removed from each well and stored at -20°C until cytokine analysis. ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy using a betaplate counter (Perkin Elmer, Waltham, MA, USA). The results were expressed as the median counts per minute (cpm) for each triplicate culture. The stimulation index (SI) was calculated by dividing the proliferation of stimulator and responder by the sum of proliferation of stimulator and responder separately. The suppression index (SUI) of $CD4^{+}CD25^{bright}$ T cells is depicted as the ratio of proliferation or cytokine production of the $CD45^{+}$ fraction depleted for $CD4^{+}CD25^{bright}$ cells and that of the total $CD45^{+}$ fraction.

Cytokine Analysis

Harvested supernatants were tested for the presence and concentrations of cytokines with a human cytokine standard 27-plex assay (Bio-Rad Laboratories, Veenendaal, The Netherlands) following manufacturer's instructions. Samples were analyzed using a Bio-Plex tm Array Reader with Bio-Plex software. The ratio of IFN-γ and IL-4 was calculated to evaluate the Th1/Th2 cytokine ratio.

Statistical Analysis

Descriptive statistical analysis was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) and SPSS Statistics 20 (IBM SPSS Software).

To analyze differences between the three groups the non-parametric ANOVA (Kruskal-Wallis) test was performed for continuous data. When significant, the Dunn's test was used. For categorical variables the Chi-squared test and thereafter the Fisher-exact test was used. Differences between the experimental- and control group were determined with the non-parametric Mann-Whitney test for continuous data and Chi-squared test for categorical data. Multiple -comparison correction was applied for the cytokine analysis by multiplying p values by the number of all tested cytokines (n=17). For all tests a value of $p<0.05$ was defined as significant.

Results

Subjects

The clinical characteristics of the participants are shown in Table 1. With respect to the fertile control group, only the female age at first pregnancy was known. The median age of this second control group was 28 years [24-38] and was significantly lower than the IVF groups. Between the RIF patient and IVF control group no differences in clinical characteristics were found. The groups were also comparable for ethnicity and social economic status (data not shown). The median of number of transfer cycle numbers in the RIF patient group was 5, with a maximum of 18 transfers. The proportion of HLA-DR mismatches between women and their partners was not significantly different between the RIF, IVF control and fertile control group (data not shown).

	RIF (n=22)	IVFs (n=32)	p-Value
Female age at first ART (yr)*	35 [26-41]	34 [24-39]	ns
Female BMI (kg/m^2)*	23.3 [19.7-35.1]	22.2 [18.4-30.3]	ns
Smoking (%)	2 (9.1)	1 (3.1)	ns
Nulli para Primi para	18 (81.8) 4 (18.2)	25 (78.1) 7 (21.9)	ns
Indication of ART (%) Andrological Tubal pathology Unexplained infertility	 11 (50.0) 1 (4.5) 10 (45.5)	 19 (59.4) 3 (9.4) 10 (31.2)	 ns
Type of ART (%) IVF ICSI	 14 (63.6) 8 (36.4)	 12 (37.5) 20 (62.5)	 ns

Table 1 Clinical data of included patients *Values are medians with minimum and maximum. ns = not significant, ART= assisted reproductive technique

Phenotypic analysis

A phenotypic analysis using flowcytometry was performed to compare the frequency of T cell subpopulations in the peripheral blood between the different groups. With respect to the percentage of CD3+ T cells no significant differences were observed (Supplement Figure S1a). Analysis of the different subsets of (CD3+) T cells revealed no difference in %CD4+ or %CD8+ T cells (Supplement Figure S1b), nor a difference in CD4+/CD8+ ratio (data not shown). The activation state of the CD3+ T cells was studied by measuring CD69 expression (early marker of activation), IL-2R expression (CD25) and HLA-DR expression (late marker of activation). No differences were observed between the groups

with respect to these markers (Supplement Figure S1c). The CD4+ T cell population with low expression of CD25 (CD25low; Figure 1a), high expression of CD25 (CD25bright; Figure 1b) and the ratio of CD4+CD25dim: CD4+CD25bright (Supplement Figure 1c) was comparable between the groups. Finally, the percentage of CD8+CD28- T cells, another T cell population with a possible suppressive capacity, was also not different between the groups (Supplement Figure S1d).

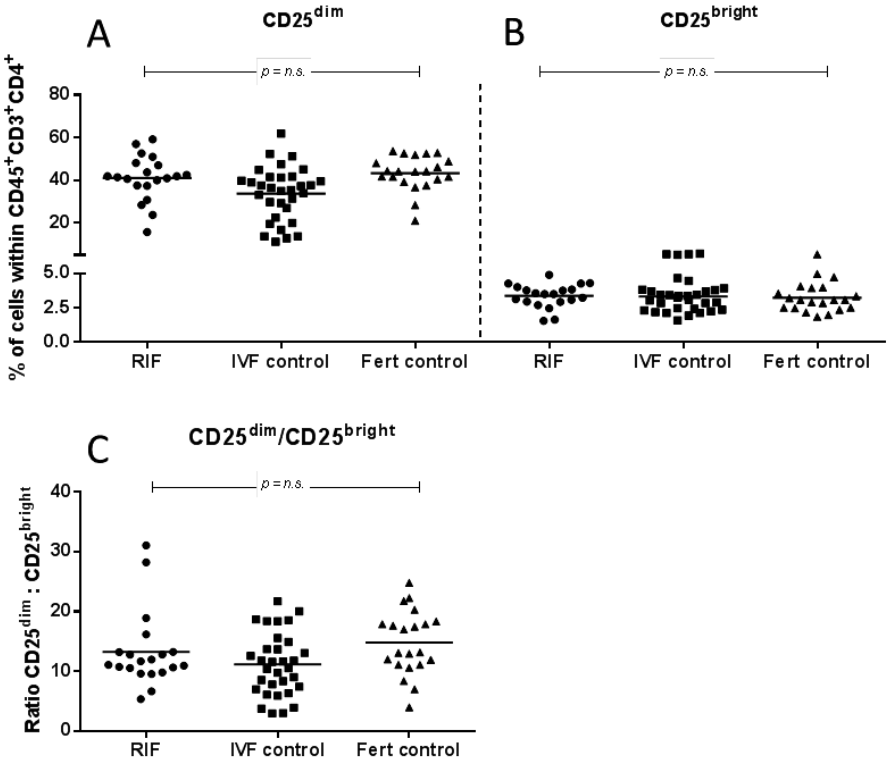

Figure 1. CD4+CD25+ cells in peripheral blood of women with RIF (n=22), IVF controls (n=32) and fertile controls (n=21). Lines are median of values. n.s. = not significant. Percentage of CD25dim or CD25bright within CD45+CD3+CD4+ cells in RIF (41.7% resp 3.5%), IVF control (35.9% resp 3.3%) and fertile control (44.1% resp 3.1%)

Functional analysis of $CD4^+$ and $CD4^+CD25^{bright}$ cells

The proliferative response to PHA (positive control), autologous cells and culture medium alone (negative control) of female peripheral blood mononuclear cells was similar in the three groups (Supplement Figure S2).

To investigate the partner-specific allo-immune response of these PBMC, female cells were stimulated with irradiated PBMC of their partner and a third party (3p) male in mixed lymphocyte cultures. In none of the groups a significant difference in proliferation was observed between stimulation with the own partner, or a third part as stimulator (Figure

2). However, the response of female PBMC after stimulation with cells from her own partner or cells from an 3p male was significantly higher for the RIF and IVF control group, compared to the spontaneous fertile women (Figure 3). There was no difference between women with recurrent implantation failure and women with a successful implantation after IVF (IVFs).

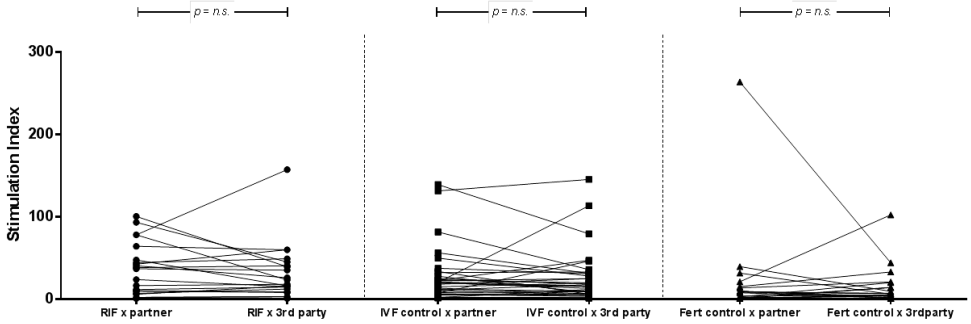

Figure 2. Proliferation of female PBMC to partner and third party male in a mixed lymphocyte culture

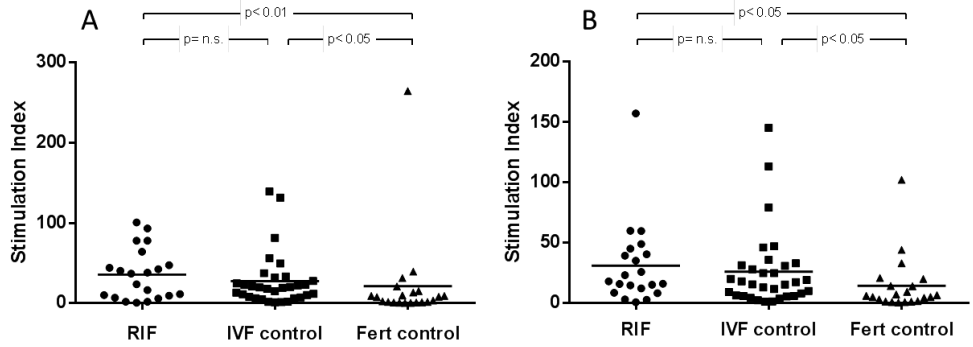

Figure 3. Proliferation of female PBMC to partner (a) and third party male (b) of women with RIF (n=22), IVF controls (n=32) and fertile controls (n=21). Overall comparison (ANOVA) for graph A; $p<0.005$ and for graph B; $p<0.04$

Analysis of the cytokines produced confirmed the data on the proliferative response. After multiple testing correction, a significant higher production of IL-6, IL-10, GM-CSF, IFNγ and TNFα was observed for the RIF and IVF control women compared to the fertile control women after proliferation with either the own partner or a 3p partner (Table 2).
The Th1/Th2 ratio was investigated by dividing the production of IFNγ, a cytokine produced by Th1, with IL-4, secreted by Th2 cells. Significant higher ratios were found for the RIF and IVF control group after stimulation with PHA or mixed lymphocyte culture with the own partner, or a 3p male, compared to spontaneous fertile women (Supplement Figure S3).

	MLC; female vs partner*	Post test	MLC; female vs 3p party*	Post test
IL-2	ns	-	ns	-
IL-4	ns	-	ns	-
IL-5	ns	-	ns	-
IL-6	0.002	RIF vs IVFs ns RIF vs fertile** IVFs vs fertile***	0.002	RIF vs IVFs ns RIF vs fertile** IVFs vs fertile***
IL-10	0.002	RIF vs IVFs ns RIF vs fertile*** IVFs vs fertile***	0.002	RIF vs IVFs ns RIF vs fertile** IVFs vs fertile***
IL-12	ns	-	ns	-
IL-13	ns	-	ns	-
IL-17	ns	-	ns	-
GM-CSF	0.002	RIF vs IVFs ns RIF vs fertile*** IVFs vs fertile***	0.002	RIF vs IVFs ns RIF vs fertile** IVFs vs fertile***
IFNγ	0.002	RIF vs IVFs ns RIF vs fertile** IVFs vs fertile***	0.002	RIF vs IVFs ns RIF vs fertile** IVFs vs fertile***
TNFα	0.002	RIF vs IVFs ns RIF vs fertile*** IVFs vs fertile***	0.002	RIF vs IVFs ns RIF vs fertile *** IVFs vs fertile***

Table 2. Cytokine production after proliferation of PBMC of RIF, IVF control (IVFs) and fertile control

*ANOVA test result after correction for multiple testing. ns= not significant. For 6 cytokines > 50% of the values were out of range and therefore not applicable

To examine the suppressive capacity of Tregs, proliferation tests were also performed with a CD45+ fraction depleted for CD4+CD25bright T cells. We validated the sorting procedure by staining of the fractions with FoxP3 and CD127 (Supplement Figure S4). The control analysis showed indeed depletion of cells with immunophenotypic characteristics of regulatory T cells after our sorting procedure.

The proliferative capacity and the production of IFNγ and IL10 of PBMC after PHA stimulation was not affected by the depletion of CD4+CD25bright T cells (Supplement Figure S5a-c). The suppressive capacity of the CD4+CD25bright regulatory T cells was expressed as the suppression index (SUI). There were no significant differences with regard to proliferation, and e.g. IFNγ- or IL10 production (Supplement Figure S5d-f). Besides IFNγ and IL-10 all other cytokines (IL-2, IL-4, IL-5, IL-6, IL12, IL-13, IL-17, GM-CSF and TNFα) were analyzed but also no significant differences in these cytokine concentrations were observed in the CD45+ fraction compared to the CD4+CD25bright depleted fraction.

In contrast, the depletion of CD4+CD25bright T cells leads to a significant increase in proliferation to both lymphocytes of the partner and a third party male in the fertile control group, which was not the case in the women undergoing IVF (both RIF and IVFs)

(Figure 4a-b). The capacity to suppress the partner specific proliferation was significantly ($p<0.05$) higher in the fertile control women compared to both the women in the RIF and IVFs group. Also the suppressive capacity of the Tregs against 3p male lymphocytes was significantly ($p<0.05$) higher for regulatory T cells in women in the fertile control group compared to both RIF and IVFs women (Figure 4c-d).

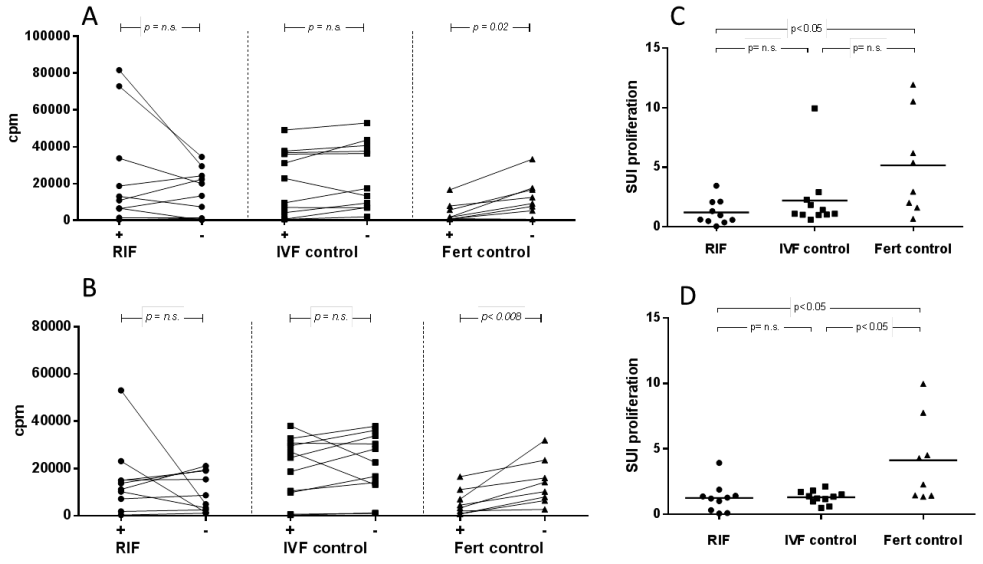

Figure 4. Mixed lymphocyte culture of CD45⁺ cells (+) and CD45⁺ cells depleted for CD4⁺CD25^{bright} cells (-) against own partner (A) or third party (B). The corresponding suppression indices (SUI) of proliferation against own partner (C) or third party (D). Overall comparison (ANOVA): p= 0.02 for C and p=0.009 for D

Finally, we examined the cytokine production after MLC of the depleted fraction against lymphocytes of the own partner and the third party male. The production of IL-10, a cytokine produced by regulatory T cells, was significantly decreased after depletion of CD4+CD25bright cells for all groups and for both MLC against the own partner, as a third party male. No significant differences were found between the groups (Supplement Figure S6). We also tested the production of TGFβ, another cytokine which may be produced by Tregs, but the level of this cytokine was too low for further analysis (data not shown).

Discussion

In vitro fertilization is a process in which oocytes are fertilized *ex vivo*, which gives an increasing number of couples with various causes of infertility the ability to conceive. However, as pregnancy rates remain <30% [147], this procedure is accompanied with considerable emotional aspects and morbidity, as well as financial consequences. To improve IVF success, a better understanding of the mechanisms responsible for implantation failure is necessary [116]. In this study we focused on the allo-immune response, as the semi-allogeneic embryo has to escape maternal immune rejection in order to implant successfully. We determined different subsets of leukocytes in the peripheral blood of women with RIF in comparison to women pregnant after IVF or spontaneous conception. We then performed functional analysis on the maternal peripheral blood mononuclear cells to evaluate their proliferative effect and cytokine profile when stimulated by allogeneic, paternal PBMC.

No significant differences in proportions of subsets of circulating T lymphocytes were observed between women with RIF and the control groups. This is in line with previous studies, in which no differences in percentages of $CD3^+$, $CD3^+CD4^+$ or $CD3^+CD8^+$ populations or difference in CD69 expression (after PMA and ionomycin stimulation) was observed [396,398,405]. Yang et al reported a similar expression of the activation marker $CD154^+$ in the T cell population for RIF patients, but a significantly higher expression of $CD69^+$, although their study group consisted of both RIF patients and patients with RSM [400]. However, a study comparing different cell subsets in a prospective cohort design showed a higher expression of HLA-DR on T cells in women with RIF that became pregnant in the following attempt [399].

To examine the allo-immune response functionally, we studied the proliferation and cytokine production of $CD4^+$ T cells upon stimulation with irradiated lymphocytes of the partner and of a third party male. The response of lymphocytes of patients with RIF, IVF control and fertile control women to cells of their own partner and to those of a third party was not different. This observation brings into question the validity of treating infertile patients with paternal leucocytes. In addition to this, randomized controlled trials did not show enhanced implantation rate or pregnancy rate after paternal leukocyte immunization [406,407] and the treatment has now been banned from the US Food and Drug Administration.

In general however, the allo-immune response was higher in the IVF women (RIF and IVFs) compared to the fertile control women. To further characterize this response we analysed the cytokine production after incubation with the different stimuli. Higher levels of both Th1- as Th2 cytokines were observed (in RIF and IVF control compared to fertile control) after mixed lymphocyte reaction with lymphocytes of either the own partner, as a third party. The Th1/Th2 ratio, expressed as the IFNγ/IL-4 ratio, was also significantly higher for

the IVF women compared to the fertile control women. There is however little consensus in literature on the role of various cytokines in IVF failure. The suggestion that pregnancy is a balance of the maternal immune system with a predominance of T helper 2 immunity is nowadays refuted [91-93] and other mechanisms have been proposed to be involved in the immunoregulation during pregnancy [11]. Moreover, recent studies suggest that Th1 cytokines actually play an essential role in an inflammatory-like reaction in preparation of implantation [93,408]. This was also shown in the analysis of endometrial expression of Th1 and Th2 cytokines [114,409].

In this study we focused on the role of $CD4^+CD25^{bright}$ regulatory T cells (Treg), which are important players in regulating overstimulation of inflammatory immune responses [11]. Increasing evidence shows that regulatory T cells are necessary for the maternal immune system to tolerate paternal allo-antigens expressed by the fetus. These cells may affect the capacity to establish pregnancy even before conception, as in the lymph nodes of mice accumulation of regulatory T cells was detectable as early as 2 days after mating [95]. This early expansion of Tregs in the lymph nodes is accompanied by accumulation of $FoxP3^+$ cells and elevated FoxP3 mRNA expression in the uterus [410]. It is most likely that similar events occur in humans where seminal fluid is suggested to be necessary for the induction of Tregs [410,411].

In the current study, we did not find any differences in proportions of $CD4^+CD25^{bright}$ T cells in the peripheral blood of patients with recurrent implantation failures after IVF, women with pregnancy after one IVF treatment or normal fertile women. To study the contribution of Tregs in regulating the allo-immune response, we examined the suppressive capacity of these regulatory T cells. We compared the outcome of mixed lymphocyte cultures with a $CD45^+$ fraction and a $CD45^+$ fraction depleted for $CD4^+CD25^{bright}$ cells [79]. A significantly diminished suppressive capacity of the $CD4^+CD25^{bright}$ cells was found for the women with unexplained infertility RIF and IVFs), compared to fertile control women. To our knowledge, this study is the first to measure the suppressive capacity of Tregs in patients with successful and unsuccessful IVF treatment compared to a normal fertile control group. Our data are in line with observations in women with RSM, where a diminished suppressive capacity of decidual Tregs was shown by Bao et al [412]. In the present study, no difference in suppressive capacity was found between women with recurrent implantation failure after IVF and women with a live child birth after the first IVF treatment. The difference between women with unexplained infertility (RIF and IVFs) and normal fertile women suggests that suppression by Tregs might be more important at time of conception than during the 'window of implantation'. Indeed, in the endometrium regulatory T cells accumulate before ovulation and decrease in number during the luteal phase [46]. Furthermore, peripheral blood $CD4^+CD25^+FoxP3^+$ regulatory T cells expand just before ovulation [46], pointing to a key role of regulatory T cells during this time point. Infertility has also been associated with reduced expression of FoxP3 mRNA in endometrial tissue [413] and a reduced percentage of peripheral $CD4^+CD25^+FoxP3^+$Treg

cells in the follicular phase of the menstrual cycle was found in women with reproductive failure after repeated artificial insemination compared with parous healthy controls [414]. The observed difference could however also be explained by the difference in age between the IVF (RIF and IVFs) patients and the fertile control women. Moreover, the procedure of IVF, with hormonal treatment for oocyte stimulation and induction of a proper uterine environment, could contributed to the differences [415,416]. On the other hand, the proliferation and cytokine production after non-specific stimulation was similar for all groups. This indicates an equal capacity of the cells to proliferate and regulate upon stimulation. In addition, age at first pregnancy or IVF attempt and method of conception (IVF, ICSI or spontaneous) did not show an independent effect in an univariate logistic regression analysis on the suppressive capacity of regulatory T cells (dichotomous outcome; SUI>1 or <1) against partner or 3p male (data not shown).

To further address the suppressive mechanism of the regulatory T cells we studied the production of IL-10 and TGFβ. The level of the latter cytokine was very low and there is no clear indication for TGFβ production in this regulation of the alloimmune response. Regarding IL-10, we observed a decreased IL-10 production after depletion of $CD4^{+}CD25^{bright}$ cells after stimulation with either the own partner, or a third party male for all three study groups and observed no differences in suppressive capacity based on the IL-10 production. Actually, the exact mechanism how Treg mediate their suppressive action remain unclear; both secretion of the immunosuppressive cytokines IL-10 and TGFβ, as well as several direct cell-cell contact mediated functions are proposed [417]. The contact dependent mechanism of suppression results from ligation of a range of Treg surface molecules, including CTLA-4, membrane bound TGFβ and LAG-3 [418] and competitive deprivation of autocrine IL-2 to adjacent T cells [419]. Moreover, in the presence of anti-IL-10 and anti-TGFβ antibodies the proliferation of effector T cells was not completely inhibited by Treg cells [412], suggesting an important contribution of the cell contact dependent manner in the mechanism of suppression.

In conclusion, we found no differences in various T cell subsets in the peripheral blood of women with RIF, women with success after one IVF treatment or fertile controls. Nevertheless, the allo-immune proliferative response of the women with unexplained infertility, including an increased production of Th1- and Th2 cytokines, is significantly higher compared to fertile control women. This difference in proliferation and cytokine production might be the result of the diminished suppressive capacity of regulatory T cells, as suggested by the present results.