Seminal significance: the forgotten father in recurrent pregnancy loss

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CHAPTER 7

Impaired immunomodulatory effects of seminal plasma may play a role in unexplained recurrent pregnancy loss: results of an in vitro study

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ABSTRACT

Background

Seminal plasma contains signalling molecules capable of modulating the maternal immune environment to support implantation and pregnancy. Prior studies indicated that seminal plasma induces changes in gene transcription of maternal immune cells. Reduced immune suppressive capacity may lead to pregnancy loss. The aim of this study was to investigate the immunomodulating effects of seminal plasma on T cells and monocytes in the context of recurrent pregnancy loss (RPL).

Methods

Female T cells and monocytes were incubated with seminal plasma of 20 males in unexplained RPL couples (RPL males) and of 11 males whose partners had ongoing pregnancies (control males). The effect of seminal plasma on messenger RNA (mRNA) expression of immune cells was measured. Levels of mRNA expression were related to key signalling molecules present in the seminal plasma. Agglomerative hierarchical cluster analysis was performed on seminal plasma expression profiles and on mRNA expression profiles.

Results

Expression of CD25 and anti-inflammatory IL-10 by female T cells was significantly lower after stimulation with seminal plasma of RPL males compared to control males. Female monocytes treated with seminal plasma of RPL males showed an immune activation signature of relatively elevated HLA-DR expression. Expression of these T cell and monocyte components was particularly correlated with the amounts of TGF-β and VEGF in the seminal plasma.

Conclusion

Our findings indicate that seminal plasma has immunomodulating properties on female immune cells compatible with the induction of a more regulatory phenotype, which may be impaired in cases of unexplained RPL.

INTRODUCTION

Recurrent pregnancy loss (RPL) is a condition defined as the demise of two or more pregnancies before the fetus reaches viability.(1) Although multiple risk factors for RPL have been identified, including parental chromosomal translocations, uterine anomalies and several other maternal conditions, no explanation can be found in 60-70% of affected couples who undergo diagnostic investigations.(1, 2) Emerging evidence suggests that maternal immune response towards the embryo plays a pivotal role in at least part of the unexplained cases of RPL.(3-5) In fact, the phenomenon of a successful pregnancy involves the tolerance of a semi-foreign body by the maternal immune system. One potentially important player in attaining this state of immune tolerance towards the embryo is the seminal plasma.

The spermatozoa, carriers of the paternal genome, are surrounded by a nourishing and protecting fluid: the seminal plasma. The seminal plasma contains a wide variety of signalling molecules that are thought to exert their effects on female tissues directly after ejaculation.(6-8) For instance, TGF-β, prostaglandin E2 (PGE2) and soluble HLA-G (sHLA-G) are major tolerance-inducing agents present in high concentrations in the seminal plasma, while IFN-γ is a potent inhibitor of TGF-β and was found to be increased in seminal plasma of males in subfertile and RPL couples.(5, 9-11) Signalling molecules in the seminal plasma are thought to induce gene expression and recruitment of immune cells in the female reproductive tract.(12) Regulatory T cells (Tregs) are considered essential in the fetal-maternal interface because of their suppressive capacity.(13) Studies in female mice showed expansion of CD4+CD25+Foxp3+ Tregs in uterus-draining lymph nodes after exposure to seminal plasma.(14) Moreover, in a human in vitro setting, incubation of peripheral blood T cells with seminal plasma led to increased messenger RNA (mRNA) expression of CD25, IL-10 and Foxp3, suggesting the induction of a Treg cell pool.(15) Besides activation of the adaptive immune system, it has been shown that seminal plasma has immunomodulating effects on cells of the innate immune system as well. In a previous study, culturing monocytes in the presence of seminal plasma led to a change in gene expression, compatible with a diminished extent of maturation and immune activating capacity of these cells.(16)

Although prior studies have indicated that seminal plasma exerts stimulatory effects on female immune cells and promotes suppressive activity, little is known on the role of seminal plasma in the specific context of RPL. Key questions include whether perturbations in the stimulatory capacity of seminal plasma may contribute to RPL, and if these perturbations exist, whether these could be attributed to disbalances in seminal plasma content. The aim of the current study was to investigate the effect of seminal plasma on mRNA expression of important activation markers in human T cells and

monocytes in an in vitro model. The stimulatory capacity of seminal plasma of males in RPL couples was compared with that of males whose partners had ongoing pregnancies. Furthermore, correlations between mRNA expression of immune cells after seminal plasma stimulation and signalling molecules present in the seminal plasma were studied.

MATERIALS AND METHODS

Ethics approval

This study was approved by the Medical Research Ethics Committee of the Leiden University Medical Center (reference numbers P11.196 and P19.014). All participants provided informed consent to take part in the study.

Study participants

RPL group

Seminal plasma samples of 20 male partners in couples with unexplained RPL were used for this study (RPL males). These couples visited the specialized RPL clinic of the Leiden University Medical Center between 2012 and 2019. They had a minimum of three pregnancy losses before 20 weeks of gestation. No underlying condition for RPL was identified, i.e. there was no evidence of maternal antiphospholipid syndrome, uterine anomalies, anti-thyroid peroxidase (TPO) antibodies or parental chromosomal translocations (following the recommended diagnostic investigations of the ESHRE guideline on RPL(1)). They had no anamnestic symptoms of genital tract infection and did not use any immune-modifying medications.

Control group

Seminal plasma samples of 11 healthy fertile males who had one or more live births with their partner and no history of pregnancy loss were used as a control group (control males). They had no anamnestic symptoms of genital tract infection and did not use any immune-modifying medications.

Semen collection

After 48-72 hours of sexual abstinence, semen samples were collected by masturbation. Complete ejaculates were collected directly in a plastic-free container and processed within 120 min after collection. To remove sperm and cellular debris, samples were centrifuged at 600 g for 10 min and the supernatant (seminal plasma) was aliquoted in 100 µL volumes and stored at-20°C until analysis.

Detection of signalling molecules in the seminal plasma

Key seminal plasma signalling molecules were selected based on previous studies.(7, 11, 12, 17) Levels of IL-1β, IL-6, IL-8, IL-10, IL-12, IL-16, IL-18, TNF-α, IFN-γ, VEGF, sHLA class I and TGF-β1, TGF-β2 and TGF-β3 were measured using the Bio-Plex Luminex™ system assay (Bio-Rad, Veenendaal, the Netherlands), following the manufacturer's instructions and as previously described by Meuleman et al.(15) To measure total levels of TGF-β isoforms including the latent precursor form, tests were performed with and without prior acid activation. Prostaglandin E2 (PGE2) and sHLA-G were detected using

an enzyme-linked immunosorbent assay (ELISA). To measure sHLA class I, an earlier described in-house developed assay was used(18). All assay specifications are shown in Supplementary Table 1. Concentrations are expressed in picograms per milliliter (pg/ml) unless indicated otherwise.

Stimulation of T cells and monocytes with seminal plasma

Human peripheral blood mononuclear cells (PBMCs) were isolated by means of density gradient centrifugation (Ficoll separation solution, pharmacy Leiden University Medical Center, the Netherlands) from a single buffy coat obtained from one anonymous healthy female donor (Sanquin Blood Supply, Amsterdam, the Netherlands) after informed consent. PBMCs were purified by the depletion of either non-T cells or non-monocytes using magnetic cell sorting (EasySep Human T Cell Enrichment Kit and EasySep Human Monocyte Enrichment Kit, STEMCELL Technologies, Köln, Germany) following the manufacturer's protocol. PBMC enriched T cells (CD3+ fraction) and monocytes (CD14+ fraction) were separately incubated in flat-bottom 48-well plates (Costar) at a density of 0.5 × 106 cells per well for 24 hours. Seminal plasma was added at the beginning of the culture at a concentration of 1:500 in 500 µl culture medium containing RPMI-1640 with 10% human serum and 1% L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, United States). The 1:500 seminal plasma concentration was based on previous (unpublished) pilot studies, as this concentration induced the highest cell responses, without being toxic to immune cells. As a negative control, cells were cultured with culture medium alone (without seminal plasma). After 24 hours, cells were harvested and stored in 300 µl of RNAlater (RNA stabilization buffer, Qiagen, Venlo, the Netherlands) at -20°C.

Messenger RNA transcript analysis

RNA extraction was performed with NucleoSpin® columns (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Quantity of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Complement DNA (cDNA) synthesis and real-time quantitative PCR were performed as described in more detail elsewhere.(15) Briefly, to synthesize cDNA, RNA was combined with oligo dT (Invitrogen; 0.25 mg) and random nucleotide hexamers (Invitrogen; 0.25 mg). Quantitative PCR was performed on a ViiA7 (Life Technologies, Carlsbad, California, USA) using specific primers and SYBR Green (BioRad) for general fluorescence detection. For each sample, levels of target mRNA transcripts were standardized to beta-actin (ACTB) and glyceraldehyde-3-phoseminal plasmahate dehydrogenase (GADPH) reference genes using the ΔCq method and the formula $\Delta Cq = 2^{-(Cq\ [transcript] - AVG\ Cq\ [references])}$. Cq values for GADPH and ACTB were stable across all samples and highly correlated with each other (coefficient of variation = 0.05 for both factors; r = 0.96). Primer sequences for the selected mRNA transcripts are shown in Supplementary Table 2.

Data analysis and statistics

Analyses were performed in R studio version 1.3.9.50 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 8.4.2 (GraphPad Software, San Diego, California USA). For calculations, measurement levels of seminal plasma factors below the detection limit were set to one half the detection limit and measurement values that were marked as out of range were replaced by the highest extrapolated value for that particular factor (similar to methods used in previous studies(7, 17)). To visualize seminal plasma expression profiles and mRNA expression profiles of female T cells and monocytes, heatmaps were created using R studio packages gplots and ComplexHeatmap. First, all values were log transformed and subsequently scaled using Z-scores. To identify expression patterns, both rows and columns were clustered using unsupervised agglomerative hierarchical cluster analysis with Ward's minimum variance method and 1-Spearman's rank correlation coefficient (ρ) as distance metric.(19).

Standardized signals of mRNA expression were statistically compared between the RPL group, the control group and the negative control group with a Kruskal Wallis test for unpaired non-parametric data. To explore correlations between individual seminal plasma factors and standardized signals of mRNA expression, Spearman's rank correlation coefficients were calculated and shown in a correlation matrix. These correlations were only assessed in the selection of seminal plasma factors and mRNA transcripts that significantly differed between the RPL group and the control group. Correction for multiple comparisons was performed using the Benjamini-Hochberg method. Statistical significance was inferred when (adjusted) $P<0.05$.

RESULTS

Characteristics of study participants

Clinical characteristics of RPL males (*n* = 20) and control males (*n* = 11) are shown in Table 1. No significant differences were found between the two groups for age, body mass index, smoking and history of fertility treatment.

Table 1. Characteristics of study participants

	RPL males (*n* = 20)	Control males (*n* = 11)	*P*-value
Age (years) mean (SD), range	36.07 (4.09), 29-45	32.9 (3.88), 29-40	0.06
BMI (kg/m²) Mean (SD), range	24.56 (2.73), 18-29	22.38 (3.29), 19-30	0.08
Smoking *n* (%)	3 (15)	0 (0)	0.18
History of fertility treatment* *n* (%)	3 (15)	0 (0)	0.18
Number of pregnancy losses median (interquartile range)	3 (3-4)	-	-

BMI = body mass index, RPL = recurrent pregnancy loss, SD = standard deviation.
Control males: males whose partners had ongoing pregnancies.
*Fertility treatment: intrauterine insemination or in vitro fertilisation

Seminal plasma expression profiles of immunological factors differ between RPL males and control males

In Table 2, descriptive statistics for all factors measured in the seminal plasma are shown for RPL males and control males. Concentrations of TGF-β1, TGF-β2, VEGF and sHLA-G were significantly lower in RPL males compared to control males. In Figure 1, the seminal plasma expression profiles of RPL males and control males are shown in a heatmap. The clustering algorithm separated the samples in three subgroups. Two subgroups mainly contained semen samples of RPL males (in orange and yellow), while one subgroup mainly contained samples of control males (in green). One subgroup of RPL samples (in orange) showed relatively high concentrations of pro-inflammatory cytokines, including IL-12, IL-18, IL-16, IL-8, IL-16, IL-1β, IFN-γ and TNF-α. In contrast, in the majority of the control samples, levels of pro-inflammatory cytokines were low, while levels of TGF-β, VEGF, sHLA-G and sHLA class I were relatively high compared to the RPL samples.

Table 2. Key seminal plasma immunological factors compared between RPL males and control males

Seminal plasma factor	RPL males (*n* = 20) Median (IQR)	Control males (*n* = 11) Median (IQR)	*P*-value
IL-1β	1.40 (0.64-2.52)	0.58 (0.37-0.91)	0.023
IL-6	10.3 (2.92-36.8)	5.56 (0.17-11.9)	0.086
IL-8	545.5 (284.7-888.4)	275.4 (57.0-551.7)	0.054
IL-10	4.00 (0.35-18.8)	0.35 (0.35-11.3)	0.359
IL-12 (p70)	3.60 (3.41-5.20)	4.55 (1.82-5.29)	0.640
IL-16	14.0 (5.36-44.4)	10.7 (4.43-43.6)	0.583
IL-18	2.70 (1.81-3.63)	2.23 (0.57-2.66)	0.169
IFN-γ	32.0 (19.9-104.0)	13.9 (2.15-40.3)	0.025
TNF-α	66.7 (36.1-155.7)	34.3 (0.57-80.9)	0.023
TGF-β1 (total)	122174 (80163-200642)	76297 (530961-773132)	<0.001*
TGF-β2 (total)	1116 (9475-13111)	28236 (22439-33229)	<0.001*
TGF-β3 (total)	127494 (51864-194999)	72433 (28869-528085)	0.984
PGE2‡	6008 (3758-10834)	7269 (3073-14541)	0.823
VEGF	18568 (13770-104158)	446075 (375250-528775)	<0.001*
sHLA-G‡	51.7 (26.7-80.2)	165.9 (70.2-1593)	0.007*
sHLA class I‡	434.4 (247.0-604.3)	1108 (358.7-2083)	0.044

IQR = interquartile range, RPL = recurrent pregnancy loss.
Control males: males whose partners had ongoing pregnancies.
These data are visualized in Figure 1.
* Significant after correction for multiple comparisons with Benjamini-Hochberg
Concentrations are shown in pg/ml, unless indicated with ‡ (ng/ml)

Stimulation with seminal plasma of RPL males induces different alterations in mRNA expression of female immune cells compared to stimulation with seminal plasma of control males

Figure 2 shows mRNA expression by female T cells and monocytes after stimulation with seminal plasma of RPL males, seminal plasma of control males and in the negative control group (cells incubated without seminal plasma). Stimulation with seminal plasma of control males induced several significant increases in mRNA expression by T cells: fold changes were 3.4 for CD25, 3.5 for IL-10 and 7.1 for Foxp3 (all fold changes reported in this paragraph are relative to mRNA expression in the negative control group). Stimulation with seminal plasma of RPL males also led to significantly increased mRNA expression of Foxp3 by T cells (fold change 7.1), but did not induce significant differences in IL-10 and CD25 mRNA expression. In monocytes, stimulation with seminal plasma of both control males and RPL males led to significantly decreased mRNA expression of HLA-DR, but HLA-DR expression was significantly less downregulated by seminal plasma of RPL males (fold changes-6.0 for control males and-3.2 for RPL males).

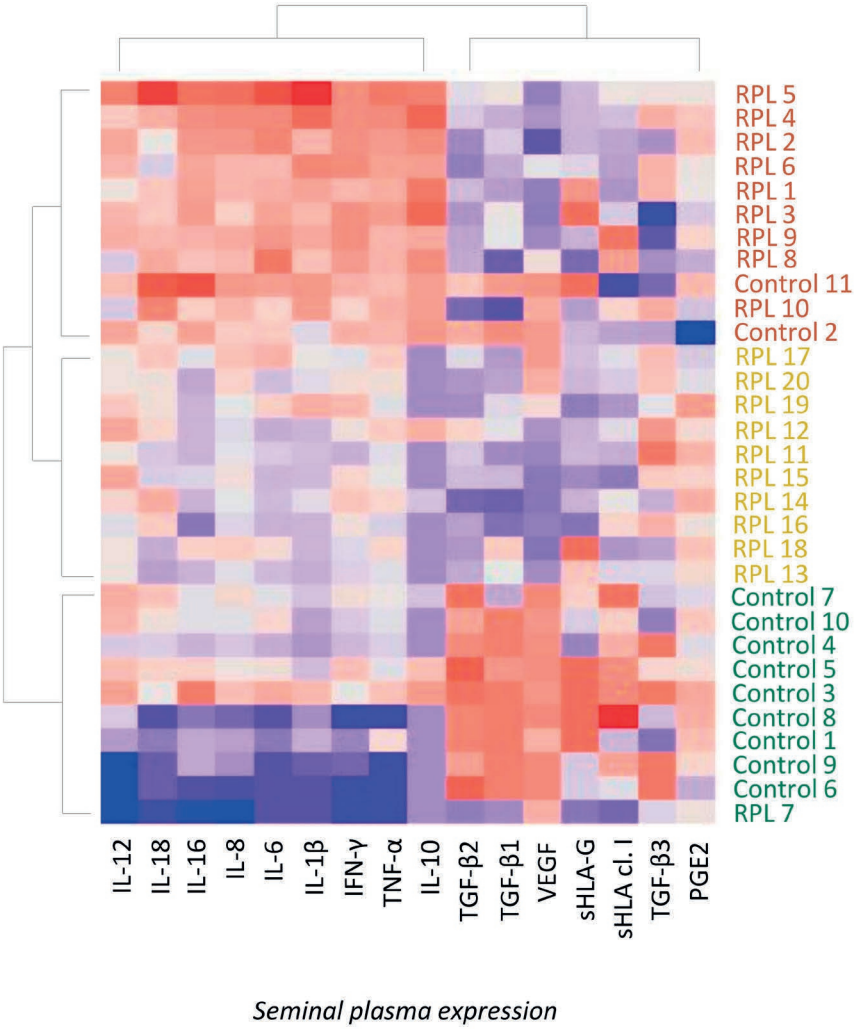


Figure 1. Cluster analyses of seminal plasma expression profiles and mRNA expression profiles of immune cells after stimulation with seminal plasma

Rows represent seminal plasma samples of males in couples with RPL (RPL males) and males whose partners had ongoing pregnancies (control males), and columns represent immunological factors present in the seminal plasma. Standardized concentrations are indicated in colours ranging from blue (low) to red (high). Both seminal plasma samples and seminal plasma factors were clustered with 1-Spearman's rank correlation distance and Ward's aggregation method. Dendrograms generated with this clustering method are shown in grey. Rows belonging to each cluster are labelled with colours orange, yellow and green, respectively.

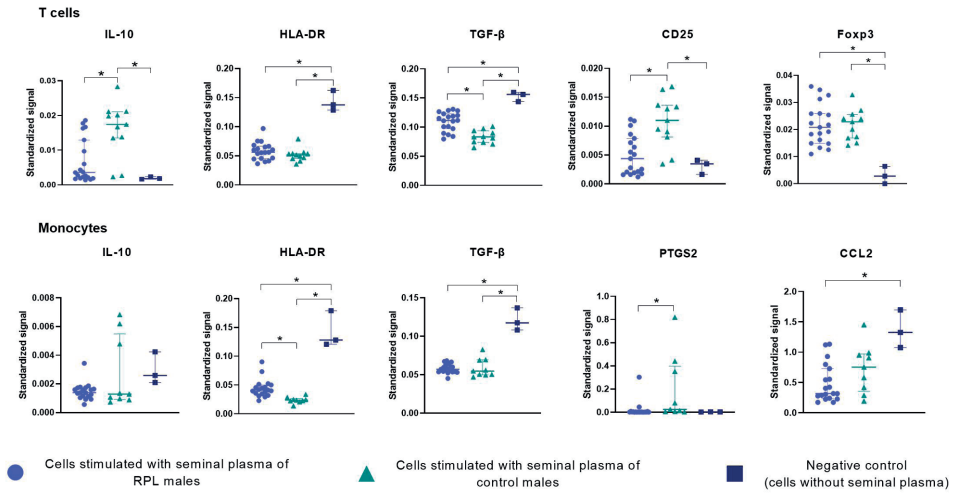
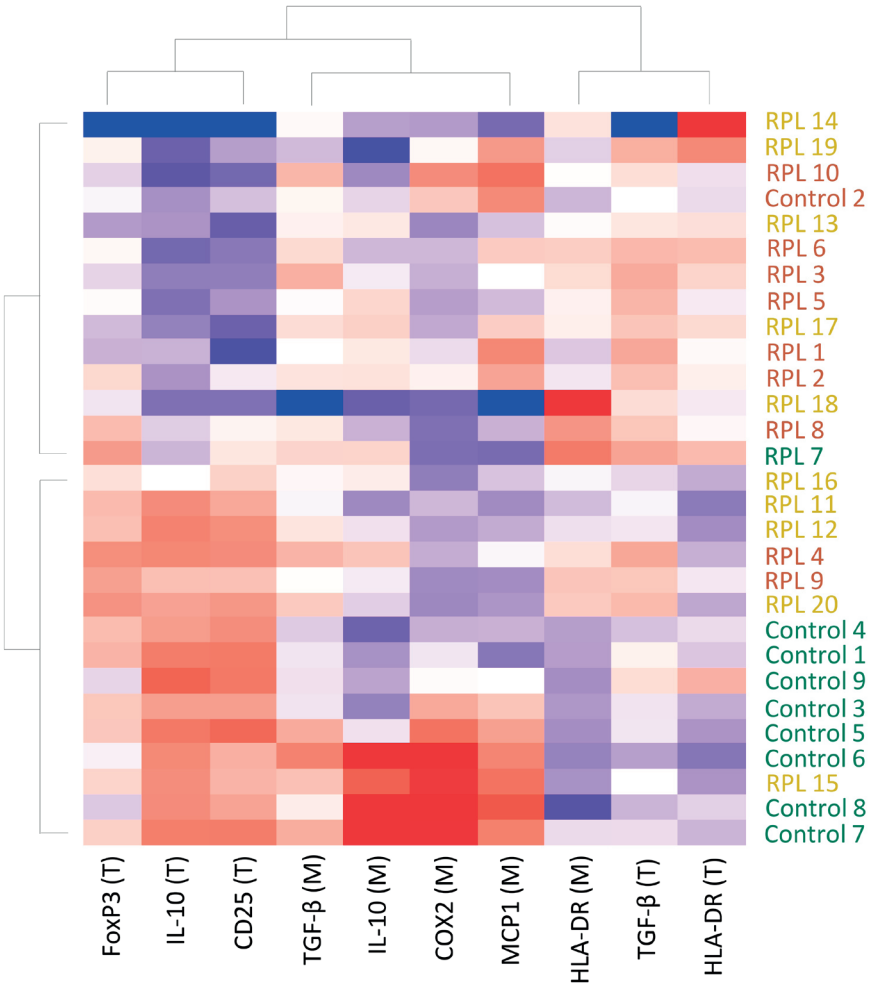


Figure 2. Messenger RNA expression of female T cells and monocytes after stimulation with seminal plasma of RPL males, seminal plasma of control males and without seminal plasma stimulation

Messenger RNA expression (standardized signals) was compared between three groups: after stimulation with seminal plasma of males in RPL couples (RPL males), after stimulation with seminal plasma of males whose partners had ongoing pregnancies (control males) and without seminal plasma stimulation (negative controls). For each sample, levels of mRNA expression were standardized to the average expression of beta-actin (ACTB) and glyceraldehyde-3-phoseminal plasmahate dehydrogenase (GADPH) reference genes using the ΔCq method and the formula $2^{-(Cq\ [transcript]\ -\ AVG\ Cq\ [references])}$. Significant differences in mRNA expression between groups are indicated with * (after Benjamini-Hochberg adjustment for multiple comparisons).

Figure 3 shows a cluster analysis of mRNA expression profiles of T cells and monocytes after stimulation with seminal plasma of either RPL males or control males. Based on the mRNA expression profiles, the clustering algorithm was able to separate the two groups to a great extent. The cluster analysis showed high correlation between expression of Foxp3, IL-10 and CD25 by T cells and between expression of IL-10, PTGS2 and CCL2 by monocytes. Also the mRNA expression of TGF-β by T cells and HLA-DR by monocytes and T cells were correlated. Furthermore, the linkage between seminal plasma expression of individual samples (Figure 1) and induction of T cell and monocyte responses (Figure 3) was visualized by the corresponding sample numbers and colour labels used in both Figures. Five out of ten RPL seminal plasma samples with an expression profile more similar to controls (in yellow) belong to the lower cluster of Figure 3, representing mRNA expression of immune cells more similar to controls. Seven out of nine RPL seminal plasma samples with a pro-inflammatory profile (in orange) belong to the upper cluster of Figure 3, with deviating responses of immune cells.



mRNA expression of T cells and monocytes after stimulation with seminal plasma

Figure 3. Messenger RNA expression profiles of female T cells and monocytes after stimulation with seminal plasma of RPL males and of control males

Rows represent stimulation with either seminal plasma of RPL males or control males. Columns represent mRNA expression of activation markers in T cells (T) and monocytes (M) after stimulation with seminal plasma. Standardized signals of mRNA expression are indicated in colours ranging from blue (low) to red (high). Both rows and columns were clustered with 1-Spearman's rank correlation distance and Ward's aggregation method. Dendrograms generated with this clustering method are shown in grey. Rows were labelled with colours orange, yellow and green based on their clustering in Figure 1. During the experiment, wells containing seminal plasma of two control males and monocytes were coincidentally mixed and could not be included in this part of the analysis. For this reason, nine instead of 11 controls are shown in this heatmap.

Messenger RNA expression of female T cells and monocytes after stimulation with seminal plasma is correlated with immunological factors present in the seminal plasma

Figure 4 shows correlations between seminal plasma factors and mRNA expression of T cells and monocytes. Messenger RNA expression of IL-10 and CD25 by T cells was positively correlated with TGF-β2 and VEGF. Messenger RNA expression of TGF-β by T cells was positively correlated with the amount of pro-inflammatory cytokines IL-1β, IFN-γ and TNF-α and negatively correlated with TGF-β1, TGF-β2 and sHLA class I in the seminal plasma. PTGS2 expression by monocytes was positively correlated with TGF-β2 and VEGF. Messenger RNA expression of HLA-DR by monocytes was negatively correlated with the amounts of TGF-β1, TGF-β2 and VEGF in the seminal plasma. The correlations shown were calculated within the total group of males. Correlations calculated within each group (RPL males and control males) separately were not significant.

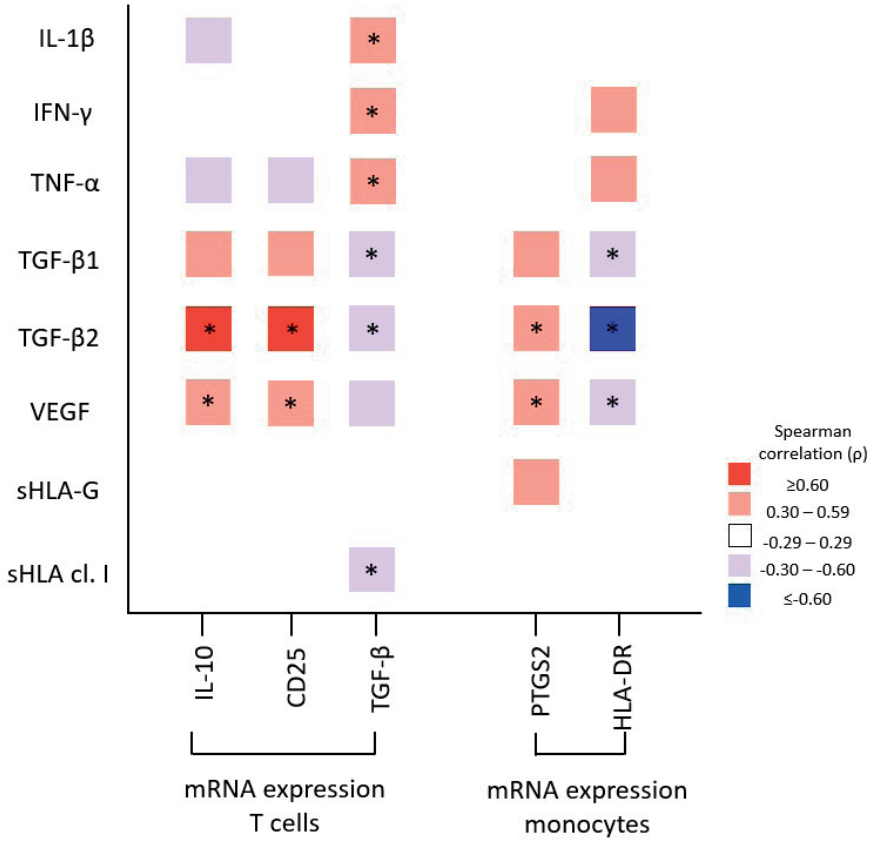


Figure 4. Correlations between mRNA expression of T cells and monocytes after seminal plasma stimulation and individual factors in seminal plasma

Only Spearman's rank correlation coefficients (ρ) ≥0.30 or ≤-0.30 are shown. Shown correlations were calculated within the total group of males. Significant correlations (after Benjamini-Hochberg adjustment for multiple comparisons) are indicated with *.

DISCUSSION

In this study we demonstrated that contact with seminal plasma leads to a change in gene expression of female T cells and monocytes. We showed for the first time that the stimulatory capacity of seminal plasma of males in RPL couples deviate from that of seminal plasma of control males whose partners had ongoing pregnancies. Our findings clearly suggest that impaired immunomodulatory effects due to disbalances in seminal plasma content play a role in the pathophysiology of unexplained RPL.

Previous *in vitro* studies showed that seminal plasma exposure has impact on female T cells and monocytes, compatible with the differentiation toward a more immune regulatory phenotype.(3, 15) We observed increased mRNA expression of IL-10, CD25 and Foxp3 by T cells after interaction with seminal plasma. IL-10 is known for its properties to sustain and amplify a suppressive immune response, while CD25 and Foxp3 may be indicators of the induction or development of a Tregs subset, which is considered vital for immunotolerance toward the semi-allogeneic fetus.(20, 21) Remarkably, Meuleman et al. only found changes in IL-10 and CD25 mRNA expression by purified T cells in the presence of antigen presenting cells (APCs), while they observed increased mRNA expression of Foxp3 also in absence of APCs. Differences with our study may be attributable to the fact that Meuleman et al. only used seminal plasma samples collected at an infertility clinic and did not include a healthy fertile control group. In our study, we only found significant increases in IL-10 and CD25 expression after stimulation with seminal plasma of control males and not after stimulation with samples of RPL males. Possibly, certain effects of seminal plasma on T cells do not essentially depend on the presence of APCs but also on the composition of the seminal plasma. Nevertheless, it is likely that the effects that we found would be amplified in the presence of APCs.

Differences were observed between the stimulatory capacity of seminal plasma of RPL males and seminal plasma of control males. After incubation with seminal plasma of RPL males, we observed no significant change in mRNA expression of CD25 and IL-10 by T cells. Prior studies that investigated the prevalence of CD25+ T cell subsets in normal pregnancy and in unexplained (recurrent) pregnancy loss found significantly lower proportions in the peripheral blood and decidua of females with pregnancy loss, suggesting that these cells might be important for maintenance of the pregnancy.(22-26) In contrast, we found relatively higher mRNA expression of HLA-DR by monocytes after incubation with seminal plasma samples of the RPL group. Multiple previous studies showed upregulation of HLA-DR on CD3+ and CD8+ T cells in females with unexplained RPL and another study found significantly increased HLA-DR+ monocyte subsets in females with preeclampsia.(25, 27-29) HLA-DR, which is a surface activation marker involved in antigen presentation, is capable of both inducing and intensifying an

immune reaction.(30) It has been postulated that an excess of HLA-DR+ cells may lead to a reduced immune regulatory environment, ultimately resulting in pregnancy failure. (29)

Our results suggest that the observed altered maternal immune response towards seminal plasma in RPL cases may be related to perturbations in seminal plasma content. Most striking were the highly positive correlations between seminal plasma TGF-β and mRNA expression of IL-10 and CD25 by T cells, and the negative correlation between seminal plasma TGF-β and mRNA expression of HLA-DR by monocytes. Normally, the seminal plasma is a rich source of TGF-β, which is known for its ability to induce anti-inflammatory and immunosuppressive effects.(12) We found significantly lower concentrations of TGF-β in the seminal plasma of males in RPL couples compared to seminal plasma samples of the control group. No significant correlations were found between seminal plasma factors and mRNA expression of immune cells when calculated within each group (RPL males or control males) separately. This may be explained by the moderate sizes of the individual groups as well as by the fact that differences in seminal plasma concentrations were much larger between groups than within groups.

Our study holds several strengths. First, we compared the immunomodulating effects of seminal plasma between cases with a pathological condition (RPL) and a carefully selected control group, while previous studies were limited to effects of seminal plasma stimulation in general. In addition, we included only couples with unexplained RPL as case group. These cases were diagnosed after a complete diagnostic work-up following recommendations of the clinical ESHRE guideline (which mainly focusses on maternal risk factors for RPL).(1) This makes it more plausible that seminal plasma factors may contribute to the pathophysiology of RPL in this selection of patients. However, RPL is a multifactorial condition and it is unlikely that insufficient immune suppression due to seminal plasma disbalances was involved in all of the included cases. This was reflected in our cluster analysis performed on seminal plasma samples, showing one subgroup consisting of RPL samples with expression profiles deviating from the control group and one subgroup resembling the expression profiles of the control samples. We showed that, in some but not all RPL males, the presence of a seminal plasma expression profile more similar to control males was related to more a normal induction pattern of immune cells. However, the current data are not yet sufficient to accurately predict individuals who may have male contributions to RPL and those who do not, which is an important goal for the future. As we showed in a previous study, seminal plasma expression profiles may be linked to male age and lifestyle characteristics.(17) As these factors are assumed to be part of the causal pathway instead of being confounders, we did not adjust for or matched on these factors in this study.(31) In larger studies, it would be interesting to evaluate associations between male characteristics and the immunomodulating

effect of seminal plasma. Furthermore, as a limitation it should be mentioned that this exploratory study used an *in vitro* model with peripheral immune cells. Several studies showed the existence of substantial T cell and monocyte populations in the human female reproductive tract of premenopausal women, which may come into direct contact with (soluble components of) seminal plasma after ejaculation(32-35). Modest numbers of CD4+ T cells have been shown to be present in the normal vaginal and ectocervical epithelium, while the lamina propria and the luminal and glandular epithelium of the endocervix contain higher numbers of CD4+ T cells.(35) It is possible that, *in vivo*, these cells are directly primed by seminal plasma to expand into Treg cells. However, we acknowledge that this study could not fully capture the complex interactions between all cells and signalling molecules present in the female reproductive tract. Although direct interactions between T cells and seminal plasma components seem possible *in vivo*, APCs are probably also a major contributor to seminal plasma mediated induction of T cells, and these were not included in the current model. Furthermore, it would be an interesting next step to investigate the effect of seminal plasma on female epithelial tract cells. Another point to consider is that gene expression of immune cells was investigated at a specific, short-term moment in time (after 24 hours of incubation with seminal plasma). However, we expected the first signs of cell activation to be visible by then, since a quick induction of tolerance towards the fetus at the implantation site is crucial.(12)

To conclude, our findings support the immunoregulatory potential of seminal plasma constituents and indicate that perturbations in seminal plasma priming may be involved in cases of unexplained RPL, advocating a male contribution to this condition. Our study serves as an important starting point for future studies to examine interactions between seminal plasma and the immune environment in the female reproductive tract in greater detail. Ultimately, defining the pathways and mechanisms underlying a state of active immune tolerance in pregnancy could lead to novel therapeutic strategies for couples with RPL.

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