Immune parameters affecting maternal tolerance towards the fetus in normal and aberrant pregnancies

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effect of seminal plasma on dendritic cell differentiation *in vitro* depends on the serum source in culture medium

Abstract

Dendritic cells (DC) are key in shaping immune responses and are recruited to the human cervix after coitus by seminal plasma (SP). SP has been shown to skew the differentiation of monocyte-derived DC towards an anti-inflammatory profile when cultured in medium containing fetal calf serum (FCS). However, DC cultured in FCS show phenotypical differences when compared to those cultured in medium containing human serum (HS). Therefore, to create a setting more similar to the *in vivo* situations in humans, we tested the immune regulatory effect of SP on DC in cell cultures containing HS. We confirmed that SP skewed FBS-DC towards a tolerogenic profile. HS-DC cultured in the presence of SP showed increased CD14 and decreased CD1a gene expression, accompanied by an increased percentage of CD14+CD1a- cells. Both TGF-β and IL-10 gene expression were elevated in LPS matured SP-DC, the latter accompanied by increased protein expression. Whereas no effect on the pro-inflammatory cytokines IL-12b and TNF-α mRNA levels was found, IL-12p70 protein levels were decreased compared to control DC. Co-cultures of SP-DC or control DC with allogeneic PBMC did not show an effect of SP on proliferation or inflammatory cytokine production. SP can skew the differentiation of monocyte-derived DC cultured in HS towards alternatively activated DC. This immune regulatory phenotype appears to be less pronounced when compared to SP-treated DC cultured in FCS containing medium. These findings highlight the importance of the serum source used in SP treated cell cultures *in vitro*.

Introduction

Semen contains various immunomodulatory factors, such as chemokines, cytokines and prostaglandins, but also soluble HLA antigens, which can be recognized as foreign and evoke an immune response [1-4]. The presence of seminal plasma (SP) in the female reproductive tract after coitus can lead to an influx of immune cells [5]. Many studies have demonstrated that factors in human SP can suppress the function of several components of the immune system including T-cells, B-cells, natural killer (NK) cells, and the complement system [6-11]. Furthermore, Lenicov showed that SP can redirect the differentiation of human dendritic cells (DC) toward a regulatory phenotype [12]. DC are professional antigen presenting cells that have the ability to capture and present antigens to T cells, in particular for the stimulation of naïve T cells. They play a key role in inducing an active immune response as well as maintaining tolerance. DC differentiated from human monocytes in the presence of SP expressed low levels of CD1a and high levels of CD14, which are hallmarks of tolerogenic DC [13]. While these SP-DC showed increased expression of maturation markers HLA-DR and CD86, they were unable to develop a fully mature phenotype in response to lipopolysaccharides (LPS). Upon LPS treatment, SP-DC produced low amounts of the inflammatory cytokines IL-12p70, IL-1β, TNF-α, and IL-6 and elevated levels of the regulatory cytokines IL-10 and TGF-β compared to control DC.

Potent suppression of NK and T cell responses by SP components *in vitro* has been shown to be dependent on the addition of bovine serum factors [14]. NK cell mediated cytotoxicity against K562 targets was suppressed when the effectors were treated with SP in the presence of 10% fetal calf serum (FCS), but suppression was considerably less when the effectors were treated with SP in the presence of 10% autologous human plasma [14]. Furthermore, human SP has been shown to induce cytotoxic effects on lymphocytes in cultures containing FCS [10]. So far, the immunomodulatory effect of SP on human DC has only been studied in *in*

vitro cultures containing FCS. Previous studies showed that human DC cultured in medium containing FCS are different from those cultured in medium containing HS. HS cultured DC are described to be more granular and heterogeneous and have a decreased CD1a expression compared to FCS cultured DC [15-18]. Thus, studies showing an effect of SP on human DC in cultures with FCS may have resulted in physiologically less relevant conclusions. We therefore examined the effect of SP on human DC in cultures containing fetal bovine serum (FBS) or human serum (HS). A less profound effect on DC biology in HS containing cultures was found compared to cultures containing FBS .

Material and Methods

Semen samples

All semen samples were obtained from men visiting the fertility clinic at the Leiden University Medical Center (LUMC). SP samples were collected via masturbation. Sperm quality (semen volume, sperm density, motility, morphology and viscosity) was assessed the same day. Normozoospermic samples were selected using the WHO guidelines [19]. Within four hours after collection, semen samples were centrifuged at 2,000 rpm for 10 min, sperm cells were discarded and aliquots of SP were stored at −80°C. For addition to cell cultures, samples were thawed at room temperature and centrifuged at 14,000 rpm for 4 min.

In vitro generation of human dendritic cells

Human peripheral blood mononuclear cells (PBMC) were isolated by means of density gradient centrifugation (Ficoll separation solution, pharmacy LUMC) from buffy coats obtained from anonymous healthy donors (Sanquin Blood Supply, Amsterdam, the Netherlands) after informed consent. PBMC were washed three times with PBS and monocytes were purified using CD14-MicroBeads according to the manufacturer's protocol (Miltenyi Biotech, Bergisch Gladbach, Germany).

CD14+ cells were seeded in 12-well tissue culture plates (Corning Costar, Merck KGaA, Darmstadt, Germany) at a density of 1.5 x 10^6 cells per well in 1.5 ml. SP was added at the beginning of the culture at a final concentration of 1:1,000 (unless stated otherwise). Multiple semen samples were used in parallel for each experiment. CD14+ cells were cultured for 6 days in RPMI-1640 (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, United States) supplemented with 1% L-glutamine (Gibco, Thermo Fisher Scientific), 1% penicillin/streptomycin (Lonza, Basel, Switzerland) containing 500 U/ml recombinant human IL-4 (Gentaur, Kampenhout, Belgium), 800 U/mL recombinant human GM-CSF (Gibco, Thermo Fisher Scientific) and either 8% heat inactivated FBS (Merck) or 8% pooled inactivated HS. Whereas in most experiments CD14+ cells from one source were cultured in either FBS or HS, in a few experiments CD14+ cells from one donor were cultured in parallel in both FBS and HS. On day 3, culture medium including supplements was refreshed. On day 6, the resulting immature DC were either harvested or treated with 100 ng/mL LPS (Merck) for maturation. After 48 h, culture supernatants were harvested and frozen until further use, and activated DC were harvested for further analysis. Culture conditions were at 37°C in a humidified atmosphere in the presence of 5% CO_2.

Quantitative PCR

RNA was extracted using NucleoSpin® RNA spin columns (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. RNA quantity and integrity were determined on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). First strand cDNA was synthesized from 200 ng total RNA using Superscript III RT (Invitrogen, RT, 200 U/µl), dNTP (10 mM each; Promega, Madison, Wisconsin, USA), RNase OUT (40 U/ul; Invitrogen, Thermo Fisher Scientific), DTT (0,1

M; Invitrogen, Thermo Fisher Scientific), oligodT (OligodT 15, 0,5 ug/ul; Promega) and random nucleotide hexamers (0,5 ug/ul; Promega). Real-time quantitative PCR was performed using the Real Time PCR machine ViiA7 (Life Technologies, Carlsbad, California, USA) based on specific primers and general fluorescence detection with SYBR Green (BioRad). To control for sample loading and to allow for normalization between samples, *β-actin* and GAPDH were analysed. Primer sequences can be found in Table 1.

Table 1. Primer sequences.

	5'primer	3'primer
β-actin	ACCACACCTTCTACAATGAG	TAGCACAGCCTGGATAGC
GAPDH	ACCCACTCCTCCACCTTTGAC	TCCACCACCCTGTTGCTGTAG
CD14	AGCCTAGACCTCAGCCACAA	CTTGGCTGGCAGTCCTTTAG
CD1a	ATGGTATCTCCGCGCAAC	AAGCCCACGGAACTGTGAT
CD80	GAAGCAAGGGGCTGAAAAG	GGAAGTTCCCAGAAGAGGTCA
CD86	CGAGCAATATGACCATCTTCTG	CGCTTCTTCTTCTTCCATTTCC
HLA-DR	AATGGAGAGCACGGTCTG	TGTCCTTTCTGATTCCTGAAG
TGF-β	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA
TNF-α	CCCCAGGGACCTCTCTCTAATC	TACAACATGGGCTACAGGCTTG
IL-10	GCGCTGTCATCGATTTCTTCC	GTAGATGCCTTTCTCTTGGAGCTTA
IL-12a	CCAGAGTCCCGGGAAAGTC	ACCAGGGTAGCCACAAGG
IL-12b	CCCTGACATTCTGCGTTCA	AGGTCTTGTCCGTGAAGACTCTA

Antibodies and flow cytometry

The following fluorochrome-conjugated antibodies (clone) for flow cytometry were used: HLA-DR (L243), CD1a (HI-149), CD14 (M5E2), CD80 (L307.4), and CD45 (HI30). All antibodies were obtained from BD Pharmingen (Becton Dickinson, Franklin Lakes, New Jersey, United States). Flow cytometric data was acquired on an LSR-II flow cytometer (Becton Dickinson) and analysed using FACS DIVA 8.0.2 (Becton Dickinson) and FlowJo 10.0.8 (Ashland, Oregon, United States) software.

Cell proliferation assay (T Cell Stimulation in vitro)

Mixed lymphocyte reactions (MLR) were performed in an allogeneic setting: PBMC (1 x 10^6 cells/ml) were co-cultured in triplicate wells with activated DC at a 1:10 ratio in RPMI supplemented with 8% FBS or HS. HLA typing of PBMC and DC was performed by SSO PCR technique using a reverse dot-blot method at the National Reference Laboratory for Histocompatibility Testing (Leiden University Medical Center, the Netherlands). The responders and stimulators were two HLA-DR antigen mismatched, in order to induce an alloantigen-specific immune response. PBMC were activated with 1 μg/ml phytohaemagglutinin (PHA) (Remel, San Diego, California, United States) as positive control. Cells were cultured in a 96-well round-bottomed plate for 5 days after which culture supernatants were harvested and frozen until further use. Cells were exposed to [3H]-thymidine (Pelkin Elmer, Waltham, Massachusetts, United States) during the last 18 h of culture after which [3H]-thymidine uptake was measured by using a liquid scintillation counter (Micro Beta Trilux 1450; Pelkin Elmer).

Cytokine analysis

Supernatants of DC cultures and MLR were analysed for the presence of cytokines using the Luminex-based Bio-Plex Pro™ Human Cytokine Th1/Th2 Assay (Bio-Rad Laboratories, Veenendaal, the Netherlands) following the manufacturer's instructions. Samples were analysed using a Bio-Plex™ Array Reader with Bio-Plex software (Bio-Rad). All samples were measured in duplicate. Additionally, we measured the cytokine levels in culture conditions without cells. These baseline cytokine values in the medium and/or SP were subtracted from the cytokine

production in the wells with cells.

Statistical analysis

Data are expressed as medians ± SD, unless stated otherwise. Data were analysed using the Wilcoxon matched-pairs signed rank test or the Mann-Whitney U test. Data were considered statistically significant when $p<0.05$.

Results

SP alters the phenotype and function of FBS cultured DC

In a first set of experiments, we confirmed that SP induced a change in gene expression in immature DC cultured in FBS containing medium. In these culture conditions, CD14 mRNA levels were increased and CD1a mRNA levels were decreased in SP-DC compared to control DC. Also, we observed that mRNA levels of CD86 and HLA-DR were upregulated in SP-DC compared to control DC (Figure 1A). Additionally, we confirmed that DC incubated with SP in FBS containing medium for six days showed an alternative phenotype by analysis of protein expression of CD14 and CD1a (Figure 1B-C) and that this effect of SP was dose-dependent (Figure 1D). Furthermore, HLA-DR surface expression was upregulated in SP-DC compared to control DC (Figure 1E).

Upon LPS maturation, gene expression levels of the anti-inflammatory cytokines IL-10 and TGF-β1 were increased in SP-DC compared to control DC, whereas gene expression of the pro-inflammatory cytokines IL-12b and TNF-α was decreased (Figure 1F). In line with these results and with published data, SP-DC showed increased IL-10 and decreased IL-12p70 protein production compared to control-DC (Figure 1G-H). Whereas TNF-α gene expression was marginally affected, we

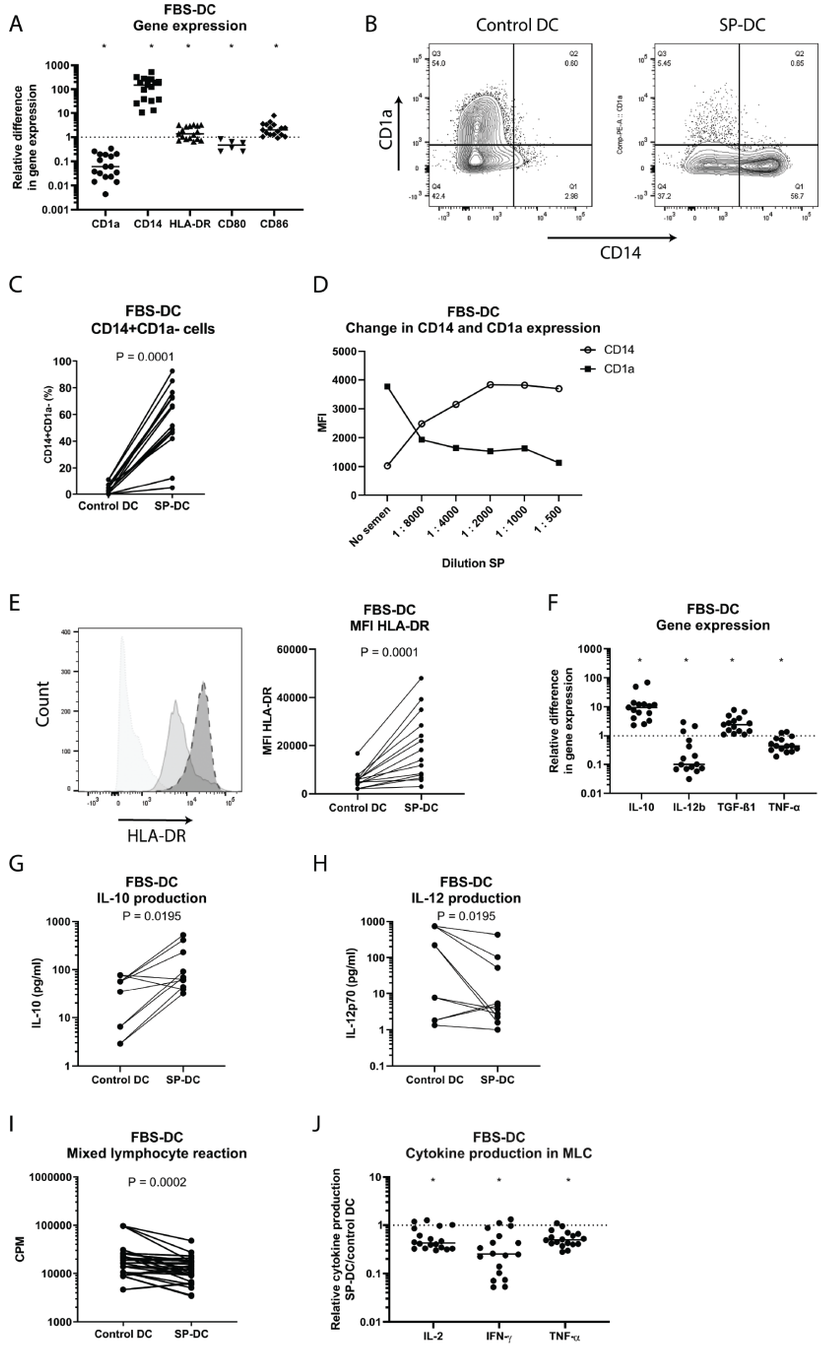

Figure 1. Phenotypical and functional characterization of dendritic cells (DC) cultured with fetal bovine serum (FBS) in the presence or absence of seminal plasma (SP).

(A) Difference in CD1a (median 0.06; P<0.0001), CD14 (median 150.80; P<0.0001), HLA-DR (median 1.40; P=0.0395), CD80 (median 0.47; P=0.0313) and CD86 (median 2.01; P<0.0001) mRNA expression between immature SP-DC and control DC (n=17; except CD80 n=6). Expression level in the control DC was set to 1 for each gene, and relative expression levels in SP-DC were compared with the control.

(B) CD14 and CD1a expression in immature DC cultured in the presence or absence of SP was analyzed by flow cytometry. Dot plots from a representative experiment (n=16) are shown.

(C) Percentage of CD14+CD1a- immature DC after culturing monocytes with or without SP (n=16).

(D) The effect of different concentrations of SP on CD14 and CD1a expression in immature DC. The more seminal plasma was added to the culture the more distinct the phenotype from control DC.

(E) Histogram from a representative experiment (n=14) is shown. HLA-DR mean fluorescence intensity (MFI) values in DC cultured with SP are compared to controls.

(F) Difference in IL-10 (median: 9.46; P<0.0001), IL-12b (median: 0.10; P=0.0015), TGF-β1 (median: 2.43; P<0.0001) and TNF-α (median: 0.43; P=0.0034) mRNA expression between mature SP-DC and control DC (n=15; except CD80 n=5). Expression level in the control DC was set as 1 for each gene, and relative expression levels in SP-DC were compared with the control.

(G) Cytokine production in DC culture upon stimulation with LPS (n=10). Mature SP-DC produced high levels of IL-10 (P=0.0195) compared to mature control DC (in pg/ml).

(H) Cytokine production in DC culture upon stimulation with LPS (n=10). Mature SP-DC produced low levels of IL-12p70 (P=0.0195) compared to mature control DC (in pg/ml).

(I) Proliferation of T cells stimulated in co-cultures with either control DC or SP-DC in counts per minute (CPM)(n=31). SP-DC had low stimulatory capacity compared to control DC (P=0.0002).

(J) Relative IL-2, IFN-γ and TNF-α production in the co-culture with SP-DC compared to the co-culture with control DC (n=28). Low amounts of IL-2 (median 0.51; P<0.0001), IFN-γ (median 0.42; P=0.0001) and TNF-α (median 0.54; P<0.0001) were found in co-cultures of PBMC with SP-DC compared to co-cultures with control DC. Cytokine level in the control DC was set to 1 for each cytokine, and relative cytokine levels in SP-DC were compared with the control. Absolute numbers of cytokine levels are depicted in Table 2.

did not observe a decreased TNF-α protein production in SP-DC compared to control DC (data not shown).

In line with these phenotypic and functional characteristics, SP-DC showed a decreased stimulatory capacity in co-cultures with allogeneic PBMCs, significantly affecting the proliferative potential op the responder cells compared to cultures with control DC (Figure 1I). Concomitantly, the production of pro-inflammatory cytokines IFN-γ, TNF-α, and IL-2 during co-culture was significantly decreased compared to control DC (Table 2; Figure 1J).

Table 2. IL-2, IFN-γ, and TNF-α production in co-cultures of PBMC with either SP-DC or control DC.

	IL-2			IFN-γ			TNF-α		
	Control DC	SP-DC		Control DC	SP-DC		Control DC	SP-DC	
FCS									
Median (pg/ml)	195	88	P<0.0001	311	76	P=0.0006	184	89	P<0.0001
Minimum (pg/ml)	44	38		29	12		43	25	
Maximum (pg/ml)	535	369		9975	12018		3381	1655	
Nr of values	28	28		28	28		28	28	
HS									
Median (pg/ml)	46	38	P=0.9697	83	63	P=0.2036	1154	1209	P=0.4697
Minimum (pg/ml)	19	21		18	9		262	199	
Maximum (pg/ml)	63	99		504	404		1724	1918	
Nr of values	12	12		12	12		12	12	

SP alters the phenotype of HS cultured immature DC

To determine whether SP had similar effects on DC in HS containing cultures, we analysed CD14, CD1a, CD86, and HLA-DR gene expression in the treated cells. Similar to FBS cultured SP-DC, CD14 and HLA-DR mRNA expression was increased and CD1a mRNA expression was decreased in immature SP-DC compared to control DC, albeit to a lesser extent. In contrast, SP did not affect CD80 or CD86 mRNA in this culture condition (Figure 2A).

By means of flow cytometry, we confirmed the generation of predominantly CD1a negative DC that were cultured in the presence of HS, as was shown in previous

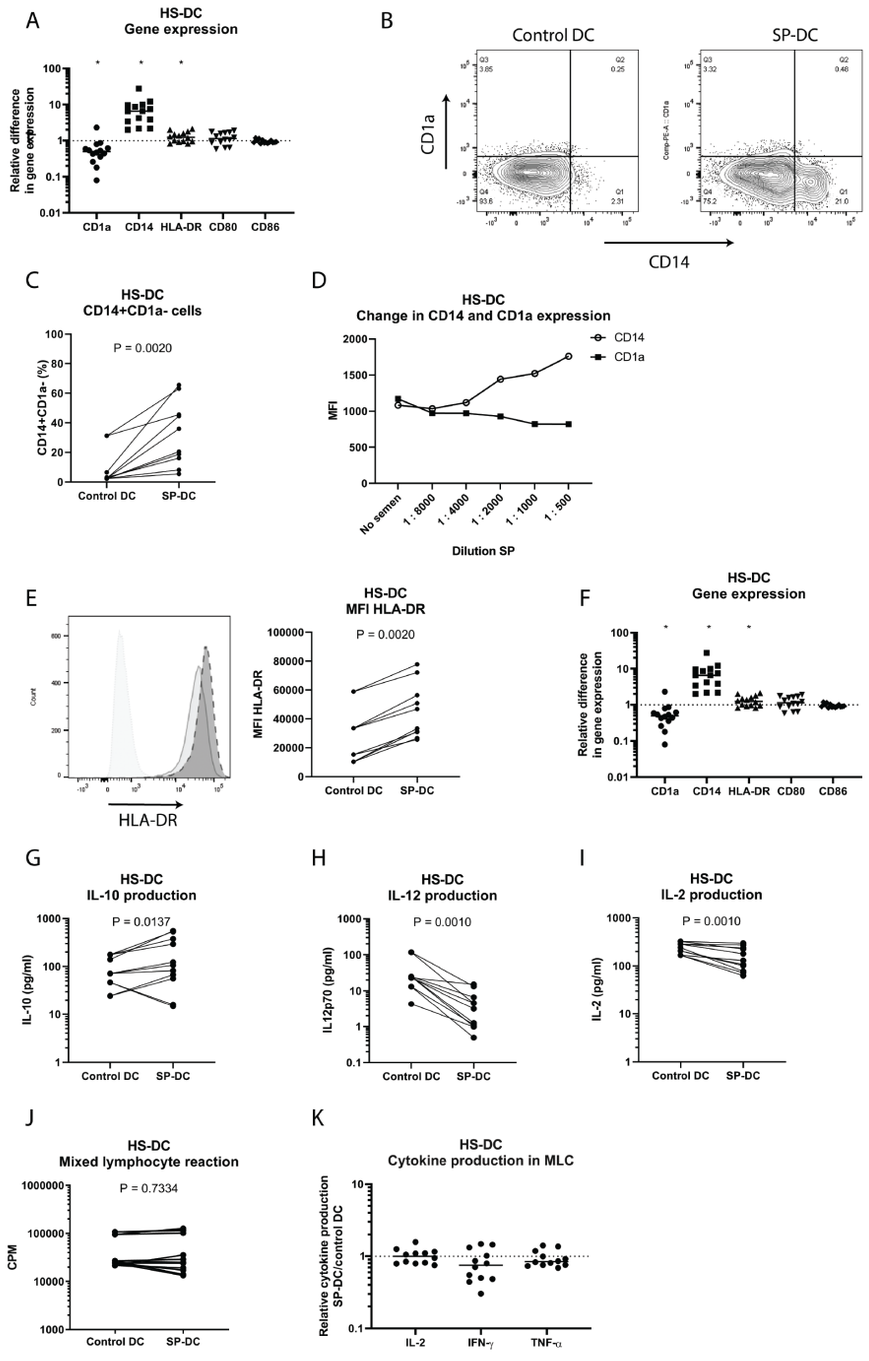

Figure 2. Phenotypical and functional characterization of dendritic cells (DC) cultured with human serum (HS) in the presence or absence of seminal plasma (SP).

(A) Difference in CD1a (median 0.50; P=0.0023), CD14 (median 6.61; P=0.0001), HLA-DR (median 1.25; P=0.0353), CD80 (median 1.14; P=0.0906), CD86 (median 0.90; P=0.2412) mRNA expression between immature SP-DC and control DC (n=14). Expression level in the control DC was set as 1 for each gene, and relative expression levels in SP-DC were compared with the control.

(B) CD14 and CD1a expression in immature DC cultured in the presence or absence of SP was analyzed by flow cytometry. Dot plots from a representative experiment (n=12) are shown.

(C) Percentage of CD14+CD1a- immature DC after culturing monocytes with or without SP (n=12).

(D) The effect of different concentrations SP on CD14 and CD1a expression in immature DC. The more seminal plasma was added to the culture the more distinct the phenotype from control DC.

(E) Difference in IL-10 (median 4.57; P=0.0002), TGF- β1 (median 1.36; P=0.0017), COX-2 (median 2.82; P=0.0105), S100A8 (median 3.54; P=0.0005) and S100A9 (median 4.56; P=0.0002) mRNA expression between mature SP-DC and control DC (n=13). Expression level in the control DC was set as 1 for each gene, and relative expression levels in SP-DC were compared with the control.

(F) Histogram from a representative experiment (n=12) is shown. HLA-DR mean fluorescence intensity (MFI) values in DC cultured with SP are compared to controls.

(G) Cytokine production in DC culture upon stimulation with LPS (n=11). Mature SP-DC produced high levels of IL-10 (P=0.0137) compared to mature control DC (in pg/ml).

(H) Cytokine production in DC culture upon stimulation with LPS (n=11). Mature SP-DC produced low levels of IL-12p70 (P=0.0010) compared to mature control DC (in pg/ml).

(I) Cytokine production in DC culture upon stimulation with LPS (n=11). Mature SP-DC produced low levels of IL-2 (P=0.0010) compared to mature control DC (in pg/ml).

(J) Proliferation of T cells stimulated in co-cultures with either control DC or SP-DC in counts per minute (CPM) (n=12). SP-DC had similar stimulatory capacity compared to control DC (P=0.733).

(K) Relative IL-2, IFN-γ and TNF-α production in the co-culture with SP-DC compared to the co-culture with control DC (n=12). Similar amounts of IL-2 (median 1.00; P=0.970), IFN-γ (median 0.75; P=0.204) and TNF-α (median 0.84; P=0.470) were found in co-cultures of PBMC with SP-DC compared to co-cultures with control DC. Cytokine level in the control DC was set to 1 for each cytokine, and relative cytokine levels in SP-DC were compared with the control. Absolute numbers of cytokine levels are depicted in Table 2.

studies [15, 16]. A representative example of this CD1a negative population is shown in Figure 2B. Whereas HS cultured DC did not upregulate CD1a, we did observe that SP led to a higher percentage of CD14+CD1a- cells, and that this change in CD14/CD1a was dose dependent (Figure 2C-D). Additionally, we analysed the expression of HLA-DR in immature DC, but we did not observe a difference between SP-DC and control DC (data not shown).

SP leads to an increased IL-10 and decreased IL-12 production, but does not affect the stimulatory capacity of HS cultured DC

On day 6 we added LPS to the culture for DC maturation and 48 hours later we analysed expression levels of several genes. CD14 and HLA-DR mRNA expression was still increased in SP-DC compared to control DC, whereas CD1a mRNA expression was still decreased (data not shown). No difference was found for mRNA expression of pro-inflammatory markers IL-12b and TNF-α (data not shown). On the other hand, mRNA expression of anti-inflammatory markers IL-10, TGF-β, COX-2, S100A8, and S100A9 was increased in mature SP-DC compared to control DC (Figure 2E).

Next, we analysed surface marker expression. The increase of CD14+CD1a- cells we observed in immature SP-DC compared to control DC persisted, although not as strong as before LPS maturation (data not shown). Additionally, we observed an upregulated HLA-DR expression in mature SP-DC compared to control DC (Figure 2F).

To determine whether the changes in gene expression and phenotype of DC cultured in the presence of SP also resulted in an altered cytokine production, we analysed the cytokine profile produced by mature SP-DC and control DC. We observed higher IL-10 and lower IL-12 cytokine levels in supernatants of LPS activated SP-DC compared to control-DC (Figure 2G-H), consistent with the data obtained in FBS containing cultures. Additionally, we observed a lower IL-2 production for SP-DC compared to control-DC (Figure 2I).

In a next set of experiments, we analysed the stimulatory capacity of mature DC and the profile of cytokines produced in co-cultures of these cells with allogeneic PBMC. These co-cultures did not show a decreased T cell stimulatory capacity toward SP-DC compared to control DC (Figure 2J). Additionally, we did not find any differences in the production of pro-inflammatory cytokines IFN-γ, TNF-α, and IL-2 between co-cultures of PBMC with SP-DC and co-cultures with control DC (Table 2; Figure 2K).

Discussion

In this study, we showed that SP-DC cultured in HS containing medium were resembling anti-inflammatory DC with regards to phenotype, gene expression patterns, and cytokine production. However, the effect of SP on DC differentiation was less pronounced in HS cultured DC than in FBS cultured DC, and it did not result in an altered T cell stimulatory capacity.

It has been widely reported that factors in human SP are capable of affecting lymphocyte function *in vitro* [6-11]. However, the majority of studies demonstrating SP suppression have been carried out using culture medium containing bovine serum in the assays, and its presence may have influenced the immune responses, as was shown in studies that compared different serum sources [10, 14]. It was previously shown that SP promotes the differentiation of tolerogenic DC [12], but these *in vitro* experiments were performed with SP in the presence of FCS. Previous studies showed that human DC cultured in medium containing FCS are different from those cultured in medium containing HS. Therefore, to create a setting that is more similar to the *in vivo* situation in humans, we set out to study the effect of seminal plasma on DC differentiation in the absence of xenoproteins.

Culturing monocytes in the presence of SP in HS containing medium led to a change in gene expression. CD14, IL-10, and TGF-β mRNA levels were upregulated, whereas CD1a levels were downregulated in mature SP-DC, all of these are hallmarks of tolerogenic DC [13, 20]. Additionally, we found higher mRNA levels for anti-inflammatory markers S100A8, S100A9, and COX-2. Previous studies have shown that S100A8 and S100A9 were upregulated in IL-10 treated tolerogenic

DC [21].S100A9 deficient DC have pro-inflammatory characteristics, such as an increase in IL-12p40 secretion and T-cell proliferation [22], and the addition of exogenous S100A8/S100A9 to the culture reduced T-cell proliferation [22]. COX2 can enhance the production of IL-10 in DC and has cytotoxic T cell suppressive function, which can be reversed by COX-2 inhibition. The induction of COX2 in SP-DC may promote a stable tolerogenic phenotype of SP-DC via a positive feedback loop between prostaglandin E2 (PGE2) and COX2 [23].

Analysis of surface markers showed an increase in CD14+CD1a- cells in cultures with SP. This is a similar phenotype as that of other modulated DC, such as those cultured in the presence of vitamin D3, dexamethasone or IL-10, which all have reduced T cell stimulatory capacity [24-27]. Upon LPS maturation, SP-DC showed an increased HLA-DR expression, a molecule typically associated with DC maturation, and necessary for binding the T cell receptor [28]. In addition, SP-DC produced higher levels of IL-10 and lower levels of IL-12 than control DC, which is characteristic for tolerogenic DC [20]. All these results hint towards the induction of tolerogenic DC by SP in the presence of HS. However, culturing of SP-treated DC in HS did not translate to a functional effect of these cells on T-cell stimulation. In contrast to what we observed in co-cultures with FBS cultured DC, we showed that there was no difference in stimulatory capacity between SP-DC and control-DC, nor in the production of Th1 cytokines in co-cultures with allogeneic PBMC. The MLR is classically affected when using tolerogenic DC instead of mature DC [29, 30], but it is possible that the effect of SP in HS cultured DC is not prominent enough to have a functional effect.

In mice, within hours after mating, macrophages, DC, and granulocytes are recruited into the reproductive tract [31-33]. SP antigens are presented by female DC in lymph nodes draining the genital tract, thereby activating and expanding inducible regulatory T cell populations [1, 34]. Subsequently, these populations migrate to the implantation site and facilitate maternal immune tolerance towards the semi-allogeneic conceptus [35]. In humans, it was shown that the presence of SP in the female reproductive tract after coitus can lead to an influx of immune cells [5] and possibly modulate the local immune response. Extrapolation of *in vitro* data to the situation *in vivo* in humans remains a challenge, and in this study we showed that even the serum source can influence the results of an *in vitro* experiment.

Acknowledgments

We thank the fertility clinic of the Leiden University Medical Center for providing the SP samples and the National Reference Laboratory for Histocompatibility Testing for HLA typing. Furthermore, we thank Mieke Versluis-Lops, Merve Uyar-Mercankaya and Els van Beelen for Luminex tests.

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