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Semen Promotes the Differentiation of Tolerogenic Dendritic Cells

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Seminal plasma is not just a carrier for spermatozoa. It contains high concentrations of cytokines, chemokines, and other biological compounds that are able to exert potent effects on the immune system of the receptive partner. Previous studies have shown that semen induces an acute inflammatory response at the female genital mucosa after coitus. Moreover, it induces regulatory mechanisms that allow the fetus (a semiallograft) to grow and develop in the uterus. The mechanisms underlying these regulatory mechanisms, however, are poorly understood. In this study, we show that seminal plasma redirects the differentiation of human dendritic cells (DCs) toward a regulatory profile. DCs differentiated from human monocytes in the presence of high dilutions of seminal plasma did not express CD1a but showed high levels of CD14. They were unable to develop a fully mature phenotype in response to LPS, TNF-α, CD40L, Pam2CSK4 (TLR2/6 agonist), or Pam3CSK4 (TLR1/2 agonist). Upon activation, they produced low amounts of the inflammatory cytokines IL-12p70, IL-1β, TNF-α, and IL-6, but expressed a high ability to produce IL-10 and TGF-β. Inhibition of the PG receptors E-prostanoid receptors 2 and 4 prevented the tolerogenic effect induced by seminal plasma on the phenotype and function of DCs, suggesting that E-series PGs play a major role. By promoting a tolerogenic profile in DCs, seminal plasma might favor fertility, but might also compromise the capacity of the receptive partner to mount an effective immune response against sexually transmitted pathogens. ***The Journal of Immunology*****, 2012, 189: 4777–4786.**

Semen contains a large and diverse array of components including carbohydrates, lipids, peptides, and proteins produced by different sources: the testis, the epididymis, and the accessory glands (1, 2). The concentration of proteins in the seminal plasma ranges from 35 to 55 g/l, and proteomic analysis leads to the identification of >900 proteins (3, 4). High levels of cytokines and chemokines have been described in seminal plasma, being the concentrations of TGF-β1, TGF-β2, TGF-β3, IL-7, stromal cell-derived factor-1α, MCP-1, and IL-8 >1000 pg/ml (4–7). Other cytokines and chemokines were detected at lower concentrations (~50–200 pg/ml), among them, IL-1β, IL-5, IL-6, IL-13, IL-17, RANTES, MIP-1β, IFN-α, and G-CSF (4, 6). The concentration of PGs in human seminal plasma is also extremely high, reaching levels >300 μg/ml (8).

Previous studies have analyzed the ability of seminal plasma to induce or modulate the course of the immune response. It has been shown that semen deposition in the human female reproductive mucosa results in the induction of a strong inflammatory response that includes the production of inflammatory cytokines and chemokines (7, 9, 10), the upregulation of cyclooxygenase-2 expression (11), the infiltration of the cervix by neutrophils, and the recruitment of dendritic cells (DCs) (9, 12, 13). This inflammatory response induced by semen has also been described in mice, pigs, rabbits, and other mammals, and appears to induce the adaptation of the female immune response to promote fertility (14, 15). Interestingly, recent observations reported by Robertson and coworkers showed that seminal fluid elicits the expression of proinflammatory cytokines and chemokines, and a robust recruitment of monocytes/macrophages, DCs, and memory T cells in the human cervix (7). This inflammatory response appears to be mediated, at least in part, by the three isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) that are found at high concentration in human semen (16). In contrast, in mouse models, the same group has shown that seminal plasma induces the expansion of regulatory T cells (Tregs) specific to seminal Ags in the receptive partner, leading to the expansion of Tregs, which subsequently migrate to the endometrium and promote tolerance to paternal alloantigens avoiding allogeneic fetal rejection (17, 18).

When thinking about the ability of semen to modulate the immune response in the receptive partner, it should be considered that seminal plasma might interact not only with epithelial cells lining the vagina and the cervix, but also with leukocytes that reside within or beneath the epithelium, among them monocytes and DCs that populate the female genital mucosa under steady-state conditions and are recruited in large numbers during the course of

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Abbreviations used in this article: DC, dendritic cell; EP, E-prostanoid receptor; pTreg, peripheral regulatory T cell; SFU, spot-forming unit; SP-DC, seminal plasma-dendritic cell; Treg, regulatory T cell.

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inflammatory processes (7, 16, 19, 20). Inflammatory reactions in the female genital mucosa such as those induced by semen deposition, as well as microabrasions of the epithelial surface induced either by mechanical stress during intercourse (21, 22) or by genital ulcer diseases (23, 24), may allow seminal fluid to directly access monocytes, DCs, and other leukocyte populations localized in close vicinity of the epithelium. Notably, this is not an unusual scenario; in fact, epithelial microabrasions in the vagina are detected in 60% of healthy women after consensual intercourse (21, 22).

Monocytes represent the most important reservoir of myeloid precursors for the renewal of tissue macrophages and DCs (25, 26). The differentiation of monocytes into DCs is mostly observed under inflammatory conditions (25, 26), and consistent with this view, it has been shown that inflammation triggers the massive recruitment of monocytes into the vaginal epithelium and their subsequent differentiation into DCs (20). Thus, we speculate that the deposition of semen in the female genital mucosa might be able not only to induce the local recruitment of monocytes (7, 20), but also to modulate the differentiation profile of monocyte-derived DCs.

In this study, we show that seminal plasma promotes the differentiation of tolerogenic DCs. DCs differentiated from human monocytes in the presence of seminal plasma showed no expression of CD1a, high levels of CD14, and failed to develop a mature phenotype in response to LPS, TNF-α, CD40L, Pam2CSK4, or Pam3CSK4. Upon stimulation by inflammatory stimuli, they produced low levels of the inflammatory cytokines IL-12p70, IL-1β, TNF-α, and IL-6, and high levels of IL-10 and TGF-β. PGs appear to play a major role in determining the tolerogenic profile of DCs induced by seminal plasma. This profile might promote fertility by inducing tolerance toward paternal alloantigens, but might also compromise the ability of the receptive partner to mount an effective immune response against sexually transmitted pathogens.

Materials and Methods

Reagents

LPS from *Escherichia coli*, recombinant human IL-4, recombinant human GM-CSF, trypsin, pronase, recombinant human TNF-α, PHA from *Phaseolus vulgaris*, PGE_2, AH6809 (E-prostanoid receptor 2 [EP2] antagonist), and AH23848 (EP4 antagonist) were obtained from Sigma-Aldrich. Pam2CSK4 and Pam3CSK4 were purchased from InvivoGen. Ficoll-Hypaque was obtained from GE Healthcare.

Semen samples

All protocols using human cells and semen samples were approved by the Ethical Committee of the National Academy of Medicine (Buenos Aires, Argentina). Semen samples were collected from healthy donors (aged 25–45 y). Informed consent was obtained from each patient before sperm collection. Ejaculates were obtained by masturbation under hygienic conditions, after a period of 2–4 d of sexual abstinence, and were collected in sterile containers as previously described (27). The samples were allowed to liquefy for 30 min at 37°C. Seminal plasma was separated from the cell fraction by centrifugation (1000 × *g*, 30 min); the supernatant was passed through a 0.22-μm syringe filter (Millipore, Amsterdam, the Netherlands) and stored at −80°C until use.

Preparation of DCs

PBMCs were isolated from healthy volunteers by standard density gradient centrifugation on Ficoll-Hypaque. Monocytes were obtained using CD14 microbeads (Miltenyi Biotec). The purity was checked by FACS analysis using an anti-CD14 mAb and was found to be >95%. To obtain DCs, we cultured purified monocytes for 5 d alone or in the presence of the indicated dilutions of seminal plasma, in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.1 mM nonessential amino acids (complete culture medium; all from Life Technologies) at 1×10^6 cells/ml with 10 ng/ml IL-4 and 10 ng/ml GM-CSF, as previously described (28). On day 5, the cells were analyzed by FACS. Maturation of DCs was induced by LPS (100 ng/ml), TNF-α (10 ng/ml), Pam2CSK4 (1 μg/ml), or Pam3CSK4 (1 μg/ml) for 24 h. As a source of CD40L, we used murine fibroblasts stably transfected with a human CD40L cDNA (provided by Dr. Claire Hivroz from Institut Curie, Paris, France).

Quantitation of cellular apoptosis and viability by flow cytometry

Apoptosis and viability of cells was evaluated by flow cytometry using an Annexin V-FITC/propidium iodide kit, according to manufacturer's instructions (BD Biosciences).

Flow cytometry

FITC, PE, or allophycocyanin-conjugated mAbs directed to CD1a, CD14, CD80, CD86, CD40, HLA-A, -B, -C, -DR, CD83, CD206, CD11c, DC-SIGN, CD4, CCR7, CCR5, and CXCR4 were obtained from BD Pharmingen. In all cases, isotype-matched control Abs were used, and a gate (R1) was defined in the analysis to exclude nonviable cells and debris, based on size and propidium iodine staining. Analysis was performed by using a FACS flow cytometer and CellQuest software (Becton Dickinson). The results are expressed as the mean fluorescence intensity or as the percentage of positive cells.

Confocal microscopy

DCs were placed on poly-L-lysine–coated glass coverslips (12 mm) during 20 min at room temperature. Then cells were washed and fixed in 4% paraformaldehyde (10 min on ice) and washed twice with 0.1 mM glycine in PBS. Subsequently, cells were incubated with a mouse mAb directed to CD1a for 1 h and revealed with Alexa 488-conjugated anti-mouse mAb for 45 min. Then DCs were stained with propidium iodide for 10 min. Coverslips were mounted on glass slides using Fluoromount G. Immunofluorescence images were acquired with a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) using a Plapon 60-1.42 NA oil immersion objective, and images were analyzed using the Olympus FV10-ASW software.

Quantitative real-time RT-PCR

Total RNA was obtained from 1×10^6 cells lysed with TRIzol (Invitrogen, Carlsbad, CA). Reverse transcription was carried out using Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer's instructions. In brief, 0.5 μg RNA was incubated for 50 min at 37°C in the presence of 200 ng random decamer primers (Invitrogen), 0.01 M DTT, and 10 mM dNTP mix. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Invitrogen) in 20 μl reaction. All primers were used at a concentration of 250 nM. Primers for CD1a were 5′-TGTTAGCTGTTCTCCCAGGTGA-3′ (forward) and 5′-AGGATGCGATCCAGATGACAT-3′ (reverse); for COX-2 were 5′-TGGATGCTTCGTTAATTTGTTC-3′ (forward) and 5′-ACCCACAGTGCTTGACAC-3′ (reverse); for TGF-B were 5′-GGACACCAACTATTGCTTCAG-3′ (forward) and 5′- TCCAGGCTCCAAATGTAGG-3′ (reverse); and for GAPDH were 5′- GAGTCAACGGATTTGGTCGT-3′ (forward) and 5′-TTGATTTTGGAGGGATCTCG-3′ (reverse). Reactions were carried out in an ABI 7500 cycler (Applied Biosystems, Foster City, CA). The cycling program used was 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method using GAPDH as a reference gene (29).

Measurement of cytokine production

Supernatant harvested from DC cultures or MLCs were diluted with culture medium, and the presence of IL-10, IL-12p70, IL-6, IL-1β, IL-23, IFN-γ, and TNF-α was assessed by ELISA (BD Biosciences), according to the manufacturer's recommendations.

Cell proliferation assay

DCs were differentiated from human monocytes, in the absence or presence of the indicated dilutions of seminal plasma for 5 d. Freshly isolated lymphocytes were labeled with CFSE (Invitrogen) at 1 μM concentration, according to the manufacturer's instructions. Maturation of DCs was induced by treatment with LPS (100 ng/ml) for 24 h. DCs were then washed and cultured with CFSE-labeled allogeneic lymphocytes in U-bottom 96-well plates (Costar), using a DC/lymphocyte ratio of 1:5. After 4 d, lymphocytes were stained with an mAb directed to CD3 labeled with PerCP (BD Biosciences), and cellular proliferation was assayed by flow cytometry.

ELISPOT assays

DCs were differentiated from monocytes, in the absence or presence of the indicated dilutions of seminal plasma for 5 d. Monocytes were obtained

from adults vaccinated with bacillus Calmette-Guérin. Autologous lymphocytes were purified and cryopreserved. One day before starting the assay, lymphocytes were thawed and rested overnight in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Life Technologies BRL), 2 mM L-glutamine (Life Technologies BRL), 100 U/ml penicillin (Life Technologies BRL), and 50 μg/ml streptomycin (Life Technologies BRL) (>85% viability checked by trypan blue exclusion). Heat-killed *M. tuberculosis* H37Rv (Mycobacteria Research Laboratories) were sonicated and used at a final concentration of 10 μg/ml to prime DCs. IFN-γ–secreting cells were detected using an ELISPOT assay conducted as described previously (30) with minor modifications. Lymphocytes were placed on 96 flat-bottom plates (MultiScreen IP plates; Millipore), previously coated with mouse anti-human IFN-γ mAb (BD Biosciences) at 10^5 cells/well. *M. tuberculosis*-treated DCs were added to the lymphocyte cultures using a DC/lymphocyte ratio of 1:10. After 24 h, plates were developed using biotinylated anti-human IFN-γ mAb, streptavidin-peroxidase complex, and AEC (3-amino-9-ethylcarbazole) substrate reagent set (BD Biosciences). Plates were scanned using an ImmunoSpot reader (Cellular Technology), and spots were counted using the ImmunoSpot software. Results were expressed as spot-forming units (SFU)/10^6 lymphocytes after subtraction of the negative control values.

cAMP assay

Monocytes were suspended in RPMI 1640 medium at a density of 1×10^6 cells/ml. They were pretreated for 5 min with a mix of EP2 and EP4 receptor antagonists and exposed to the indicated dilutions of seminal plasma for 10 min. After centrifugation, pellets were resuspended in 1 ml ethanol. Ethanol was dried and residues were resuspended in 50 mM Tris-HCl, pH 7.4, 0.1% BSA, for cAMP determination. cAMP content was determined by competitive radio-binding assay for PKA using [^{3}H]cAMP, as described previously (31). The standard curve was generated using different concentrations of cAMP.

Statistics

All data are shown as mean values ± SEM. Student paired *t* test was used to determine the significance of differences between treatment groups. Multiple analyses were followed by Bonferroni's multiple-comparison posttest. The *p* values <0.05 were considered statistically significant.

Results

Seminal plasma promotes the differentiation of CD1a$^-$ CD14$^+$ DCs with a semimature phenotype

In a first set of experiments, we analyzed whether seminal plasma was able to modulate the differentiation profile of human DCs. Human monocytes were cultured with IL-4 and GM-CSF for 5 d in the absence (DCs) or presence of seminal plasma DCs (SP-DCs). Most experiments were performed using a seminal plasma dilution of 1:5000; otherwise, the seminal plasma dilutions were indicated. As expected, control DCs expressed high levels of CD1a and were mostly negative for the expression of the CD14. By contrast, resembling the phenotype of tolerogenic DCs (32, 33), SP-DCs expressed high levels of CD14 and were negative for the expression of CD1a (Fig. 1A, 1B). The expression of CD1a mRNA was significantly lower in SP-DCs compared with control DCs (Fig. 1C). Notably, a significant reduction in the expression of CD1a was observed using seminal plasma dilutions as high as $1:10^6$, and this effect was highly reproducible among different donors (Fig. 1D). Kinetic studies showing the expression of CD1a and CD14 in DCs differentiated in the absence or presence of seminal plasma (1:5000) are shown in Fig. 1E. Throughout the differentiation process, no transient expression of CD1a was observed for SP-DCs (Fig. 1E), whereas the levels of CD14 were comparable with those expressed by monocytes (Fig. 1E and data not shown).

The phenotype of SP-DCs is illustrated in Fig. 1F. Monocytes do not express the pattern recognition receptor DC-SIGN (34), and both control DCs and SP-DCs upregulated the expression of this receptor in a similar way. SP-DCs showed some changes typically associated with DC maturation, namely, increased expression of HLA-DR and CD86 (Fig. 1F). However, they express very low levels of CD80, CCR7, and CD83 (Fig. 1F and data not shown), suggesting that seminal plasma promotes the differentiation of semimature DCs. Moreover, we consistently observed that seminal plasma moderately increased the expression of CD4 and CCR5, whereas it markedly upregulated the expression of CXCR4. It is relevant to note that all these molecules are receptors for HIV.

Because previous observations have shown that seminal plasma is able to induce cytotoxic effects on human lymphocytes in long-term cultures (35), we analyzed whether treatment of monocytes with seminal plasma resulted in a loss of cell viability. Monocytes were cultured with IL-4 and GM-CSF in the absence or presence of seminal plasma (1:5000), and cell viability was analyzed after 2 and 5 d by flow cytometry (Annexin V/propidium iodide). Fig. 1G shows that seminal plasma did not induce deleterious effects on cell cultures, being cell viability >90% in all cases.

We then asked whether SP-DCs could acquire a mature phenotype in response to conventional inflammatory stimuli. Fig. 2A and 2B show that control DCs markedly upregulate the expression of CD83 in response to LPS, CD40L, TNF-α, Pam2CSK4 (TLR2/6 agonist), or Pam3CSK4 (TLR1/2 agonist). By contrast, only a low-to-moderate increase in the expression of CD83 was observed in SP-DCs. Similar results were found when the expression of CCR7 was analyzed (not shown). We conclude that SP-DCs are deficient in terms of acquisition of a fully mature phenotype upon stimulation by inflammatory stimuli.

Seminal plasma promotes the differentiation of tolerogenic DCs

We then analyzed the profile of cytokines produced by SP-DCs. As expected, control DCs produced high amounts of IL-12p70 and IL-10 in response to LPS and CD40L. SP-DCs produced markedly lower amounts of IL-12p70 and higher amounts of IL-10 compared with control DCs (Fig. 3A). Moreover, the production of IL-1β, TNF-α, and IL-6 induced by LPS was shown to be significantly lower for SP-DCs compared with control DCs (Fig. 3B). Finally, when analyzing the production of mRNA for TGF-β by quantitative RT-PCR, we found that SP-DCs spontaneously produced higher levels of TGF-β mRNA compared with control DCs (Fig. 3C).

We next analyzed the ability of SP-DCs to induce the activation of T cells in mixed lymphocytes cultures, using allogeneic lymphocytes labeled with CFSE. In these experiments, DCs and SP-DCs were treated with LPS (100 ng/ml, 24 h), washed, and cultured with CFSE-labeled lymphocytes, using a DC/lymphocyte ratio of 1:5. Proliferation of T cells was analyzed at day 4 by measuring the dilution of CFSE staining in the gate of CD4$^+$ T cells by flow cytometry. Lymphocytes cultured alone in the absence and presence of PHA (10 μg/ml) were used as negative and positive controls, respectively. We found that control DCs and SP-DCs showed a similar ability to induce the proliferation of CD4$^+$ T cells (Fig. 4A). Similar results were observed when lymphocyte proliferation was analyzed by measuring the expression of the cellular proliferation marker Ki67 (Fig. 4B).

To gain insight into the ability of SP-DCs to determine the differentiation profile of activated CD4$^+$ T cells, we quantified the concentrations of IFN-γ, IL-10, and TGF-β in the supernatants of the mixed lymphocytes cultures. In these experiments, DCs and SP-DCs were treated with LPS (100 ng/ml, 24 h), washed, and cultured with lymphocytes using a DC/lymphocyte ratio of 1:5. Supernatants were harvested at day 3, and the concentration of IFN-γ, IL-10, and TGF-β was evaluated by ELISA. Results in Fig. 4C show that IFN-γ was produced at very low levels, and no differences were found between control DCs and SP-DCs (Fig. 4C). By contrast, the production of both IL-10 and TGF-β was shown to be markedly higher for the cultures performed with SP-

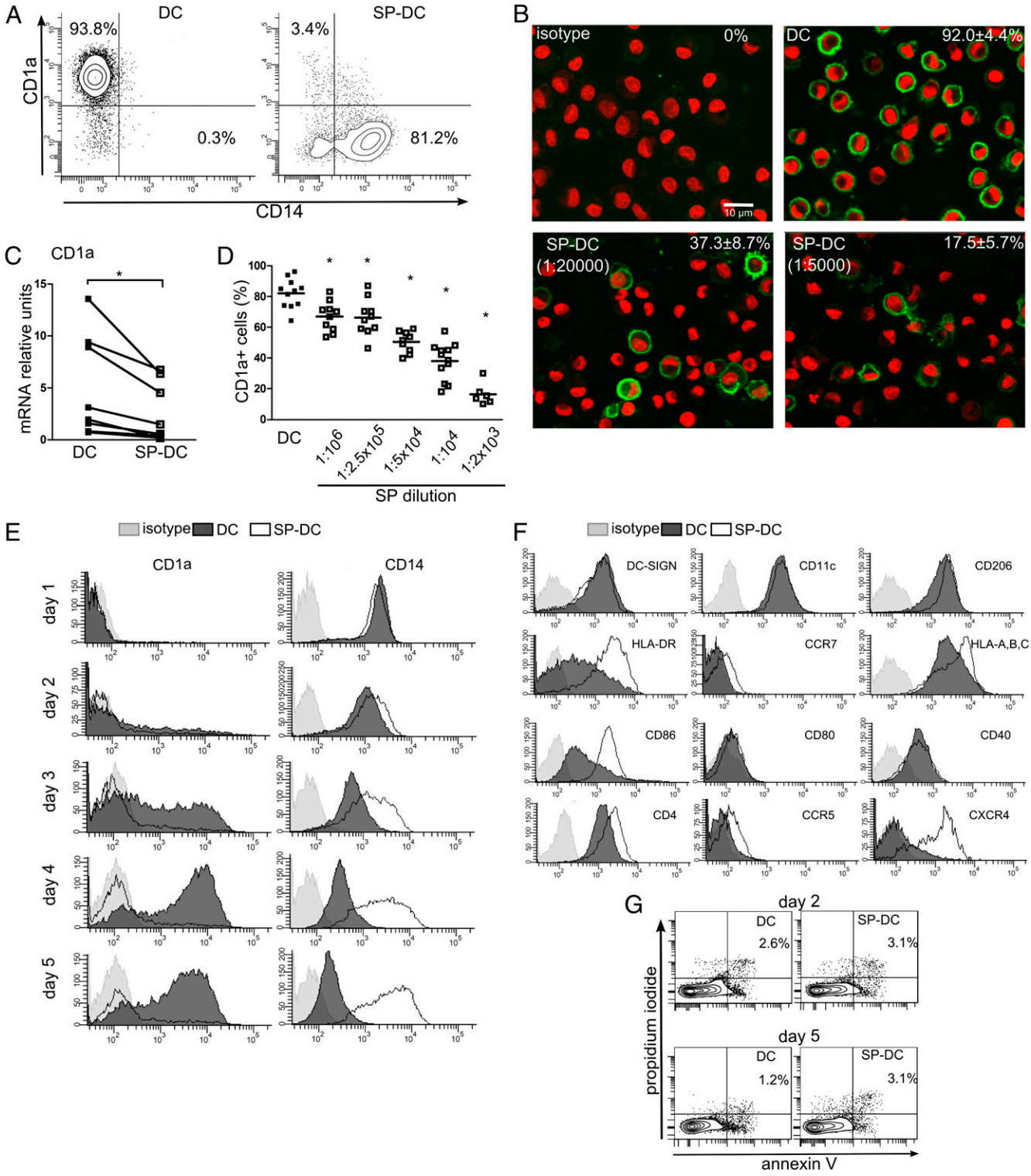

FIGURE 1. Seminal plasma promotes the differentiation of $CD1a^{-}$ $CD14^{+}$ DCs with a semimature phenotype. (**A**–**C**) DCs were obtained from monocytes cultured for 5 d with IL-4 and GM-CSF in the absence (DC) or presence of seminal plasma (SP-DC). Experiments were performed using a seminal plasma dilution of 1:5000; otherwise, the dilutions used were indicated inside the figure. (A) The expression of CD1a and CD14 was analyzed by flow cytometry. Dot plots from a representative experiment ($n = 10$) are shown. (B) DCs and SP-DCs were fixed and stained with FITC–anti-CD1a Abs and propidium iodide, and analyzed by confocal microscopy. A representative experiment is shown. The numbers inside the figure represent the percentages of $CD1a^{+}$ cells (mean ± SEM, $n = 3$). Scale bar, 10µm. (C) Total RNA from DCs and SP-DCs was obtained and CD1a mRNA was studied by quantitative RT-PCR ($n = 8$). (**D**) Monocytes were cultured with IL-4 and GM-CSF for 5 d under increasing dilutions of seminal plasma, and the percentages of $CD1a^{+}$ cells was determined by flow cytometry. Experiments were performed using 12 distinct seminal plasma donors and 7 distinct monocyte donors. (**E**) The expression of CD1a and CD14 was analyzed by flow cytometry every day during the differentiation of DCs and SP-DCs. Histograms from a representative experiment ($n = 6$) are shown. (**F**) The phenotype of DCs and SP-DCs (seminal plasma dilution 1:5000, 5 d of culture) were analyzed by flow cytometry. Representative histograms from at least three experiments are shown. (**G**) The viability of DCs and SP-DCs (seminal plasma dilution 1:5000) were analyzed by annexin V and propidium iodide staining after 2 and 5 d of differentiation. Dot plots from a representative experiment are shown. $*p < 0.05$ versus DCs.

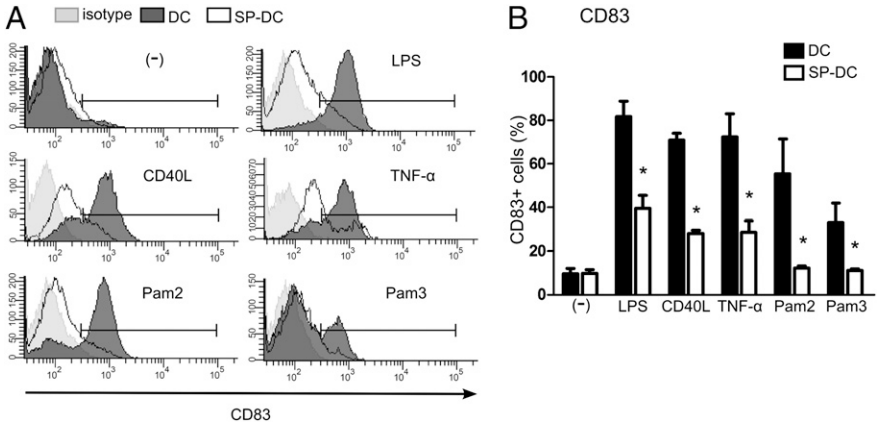

FIGURE 2. DCs differentiated in the presence of seminal plasma do not upregulate the expression of CD83 upon activation by inflammatory stimuli. (**A** and **B**) Monocytes were cultured with IL-4 and GM-CSF for 5 d in the absence (DC) or presence of seminal plasma (SP-DC; dilution of 1:5000). Then cells were washed and cultured for 24 h with LPS (100 ng/ml), CD40L-transfected fibroblasts (DC/fibroblast ratio of 1:2, 18 h), TNF-α (10 ng/ml), Pam2CSK4 (1 μg/ml), or Pam3CSK4 (1 μg/ml). The expression of CD83 was then analyzed by flow cytometry. Histograms from a representative experiment ($n = 4–6$) are showed in (A). Graph bars indicating the percentages of $CD83^+$ cells (mean ± SEM, $n = 4–6$) are shown in (B). Percentages were calculated using the gates defined in (A). $^*p < 0.05$ versus DCs.

DCs compared with those carried out with control DCs (Fig. 4D, 4E). Considering the low amounts of IFN-γ produced in mixed lymphocytes cultures, we analyzed the ability of SP-DCs to stimulate the production of IFN-γ by syngeneic memory T cells. In these experiments, monocytes were obtained from adults vaccinated with bacillus Calmette-Guérin. Control DCs and SP-DCs, obtained as described in the legend for Fig. 4, were pulsed overnight with heat-killed *M. tuberculosis* strain H37Rv. Then they were cultured for 24 h with syngeneic lymphocytes at a DC/lymphocyte ratio of 1:5, and IFN-γ–producing cells were quantified by ELISPOT, as described in *Materials and Methods*. Fig. 4F shows that the number of SFU was significantly lower for the cultures performed with SP-DCs compared with those carried out with control DCs.

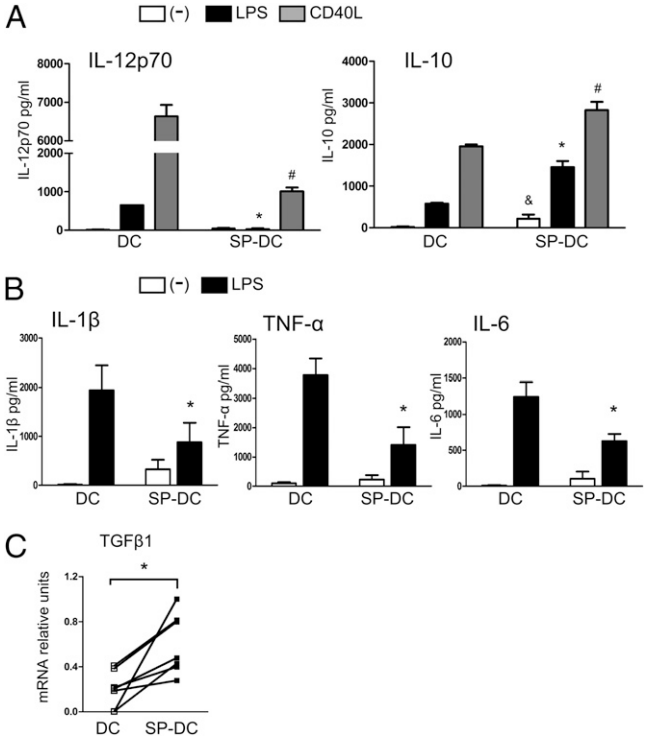

FIGURE 3. DCs differentiated in the presence of seminal plasma produce low levels of IL-12p70 and high levels of IL-10 upon activation by inflammatory stimuli. (**A** and **B**) DCs were obtained from monocytes cultured for 5 d with IL-4 and GM-CSF in the absence (DC) or presence of seminal plasma (SP-DC; dilution 1:5000). Then cells were washed and cultured with LPS (100 ng/ml, 24 h) or CD40L-transfected fibroblasts (DC/fibroblast ratio of 1:2, 18 h). Culture supernatants were collected and the concentrations of IL-12p70, IL-10, IL-1β, TNF-α, and IL-6 were measured by ELISA ($n = 5$, mean ± SEM). (**C**) DCs were obtained from monocytes cultured for 5 d with IL-4 and GM-CSF in the absence (DC) or presence of seminal plasma (SP-DC; dilution 1:5000). Total RNA was then collected from DCs and SP-DCs, and TGF-β mRNA was analyzed by quantitative RT-PCR ($n = 7$). $^*p < 0.05$ versus LPS-treated DCs, $^{\#}p < 0.05$ versus CD40L-treated DCs, $^{\&}p < 0.05$ versus untreated DCs.

PGs are involved in the differentiation of tolerogenic DCs induced by seminal plasma

In a preliminary set of experiments, we observed that seminal plasma fully retained its ability to promote the differentiation of $CD1a^-$ $CD14^+$ DCs after treatment with trypsin (1000 U/ml, 18 h at 37°C) or pronase (20 ng/ml, 18 h at 37°C), as well as after filtration through a 3-kDa cutoff membrane (data not shown). This suggested the involvement of a nonprotein low-m.w. compound.

Previous studies performed by Kaliński and coworkers (36) have shown that PGE_2 promotes the differentiation of $CD1a^-$ $CD14^+$ DCs from human monocytes. Moreover, they demonstrated that DCs differentiated in the presence of PGE_2 are IL-12 deficient but produce increased levels of IL-10 upon stimulation. Because seminal plasma contains high concentrations of PGE_2, PGE_1, and 19-OH-PGE_2 (8), we hypothesized that these lipid mediators would be responsible for the ability of seminal plasma to induce the differentiation of tolerogenic DCs. PGE_2, PGE_1 and 19-OH-PGE_2 are known to signal through EP2 and/or EP4 (37, 38), the two EPs expressed by human monocytes (39). Thus, to analyze the role of seminal PGs, we used a mix of EP2 (AH6809, 50 μM) and EP4 (AH23848, 90 μM) receptor antagonists. DCs were differentiated in the absence or presence of seminal plasma and EP2/EP4 antagonists. Then the phenotype of DCs and their ability to upregulate the expression of CD83 and to produce IL-12p70 upon stimulation by LPS was analyzed. Results in Fig. 5A–D show that blocking of EP2 and EP4 receptors significantly restored the ability of monocytes to differentiate into $CD1a^+$ $CD14^-$ DCs, as well as their ability to upregulate the expression of CD83 and to produce IL-12p70 upon stimulation by LPS. Moreover, considering recent observations indicating that the development of tolerogenic DCs stimulated by PGE_2 involves the upregulation of

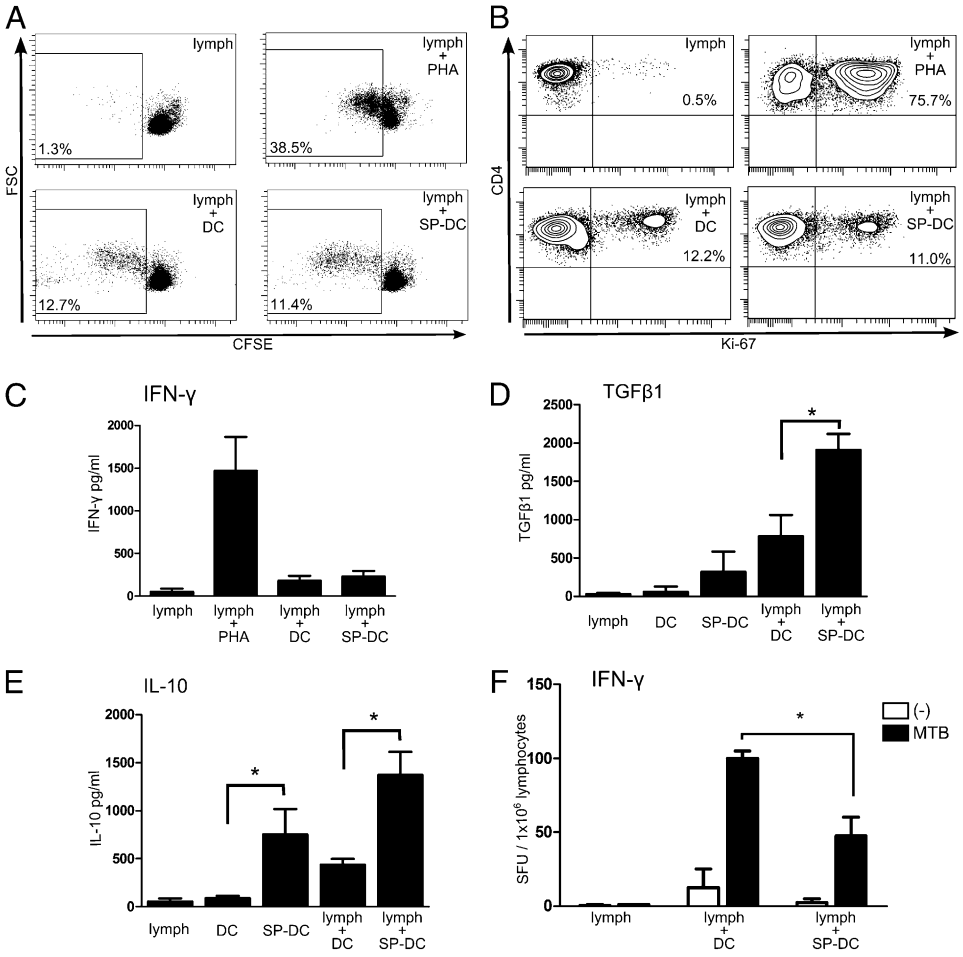

FIGURE 4. DCs differentiated in the presence of seminal plasma stimulate the production of IL-10 and TGF-β by lymphocytes. Monocytes were cultured with IL-4 and GM-CSF for 5 d in the absence (DC) or presence of seminal plasma (SP-DC; dilution 1:5000). (**A**) DCs and SP-DCs were treated with LPS (100 ng/ml, 24 h), washed, and incubated with CFSE-labeled allogeneic lymphocytes, using a DC/lymphocyte ratio of 1:5. The proliferative response of T cells was analyzed at day 4 by measuring the dilution of CFSE staining in the gate of $CD4^+$ T cells by flow cytometry. Lymphocytes cultured alone with or without PHA (10 μg/ml) were used as positive and negative controls, respectively. Dot plots from a representative experiment ($n = 7$) are shown. (**B**) DCs and SP-DCs were cultured with allogeneic lymphocytes using a DC/lymphocyte ratio of 1:5. At day 3, cells were harvested and the expression of Ki-67 was analyzed by intracellular staining and flow cytometry in the gate of $CD4^+$ T cells. Dot plots from a representative experiment ($n = 4$) are shown. (**C**–**E**) DCs and SP-DCs were treated with LPS (100 ng/ml, 24 h), washed, and incubated with allogeneic lymphocytes using a DC/lymphocyte ratio of 1:5. At day 3, supernatants were harvested and the concentration of IFN-γ, TGF-β, and IL-10 was measured by ELISA ($n = 5$, mean ± SEM). (C) the production of IFN-γ by lymphocytes cultured with PHA (10 μg/ml) during 3 d is also shown. (D and E) the production of TGF-β1 and IL-10 by DCs and SP-DCs cultured without allogeneic lymphocytes is also shown. (**F**) DCs and SP-DCs were pulsed overnight with heat-killed *M. tuberculosis* strain H37Rv, as described in *Materials and Methods*. Then they were cultured for 24 h with syngeneic lymphocytes at a DC/lymphocyte ratio of 1:5, and IFN-γ–producing cells were quantified by ELISPOT. Results are expressed as the number of SFU/10^6 lymphocytes ($n = 5$, mean ± SEM). *$p < 0.05$.

COX2 expression (40), we analyzed the expression of COX2 in SP-DCs. Fig. 5E shows that seminal plasma significantly induced the expression of COX-2.

Signaling through EP2 and EP4 receptors is mediated by the adenylate cyclase-triggered cAMP/PKA/CREB pathway, responsible for the most important anti-inflammatory and suppressive actions of PGE_2 (41). Fig. 5F shows that treatment of monocytes with seminal plasma (1:5000) elicited a 2-fold increase in the levels of cAMP that could be prevented by EP2/4 antagonists. Moreover, Fig. 5G shows that the activator of the adenylate cyclase forskolin mimicked the effect of seminal plasma in terms of CD1a expression. Taken together, our results suggest that the ability of seminal plasma to promote the differentiation of tolerogenic DCs is mediated, at least in part, by PGE_2 or other E-series PGs, which, acting through EP2 and/or EP4 receptors, lead to an increase in cAMP concentrations.

Challenging the notion that PGE_2 promotes the differentiation of tolerogenic DCs, it has been shown that differentiation of mouse bone marrow-derived DCs in the presence of PGE_2 alters the IL-12/IL-23 balance favoring the production of IL-23, and thus enabling DCs to efficiently promote the differentiation of $CD4^+$ T cells into Th17 cells (42). Taking this into account, we performed another set of experiments to analyze whether SP-DCs were able to produce IL-23 and to drive the differentiation of $CD4^+$ T cells into a Th17 profile. Fig. 6A shows that upon stimulation by LPS, control DCs, but not SP-DCs, produced significant amounts of IL-23. Moreover, Fig. 6B illustrates that both control DCs and SP-DCs failed to stimulate the production of IL-17A by $CD4^+$ T cells in mixed lymphocytes cultures. These results further support the notion that under the influence of seminal plasma, DCs acquire a tolerogenic phenotype.

Discussion

The function of seminal plasma is usually thought to be restricted to transport and protection of the spermatozoa until one of them can fertilize the egg. Challenging this view, different studies have

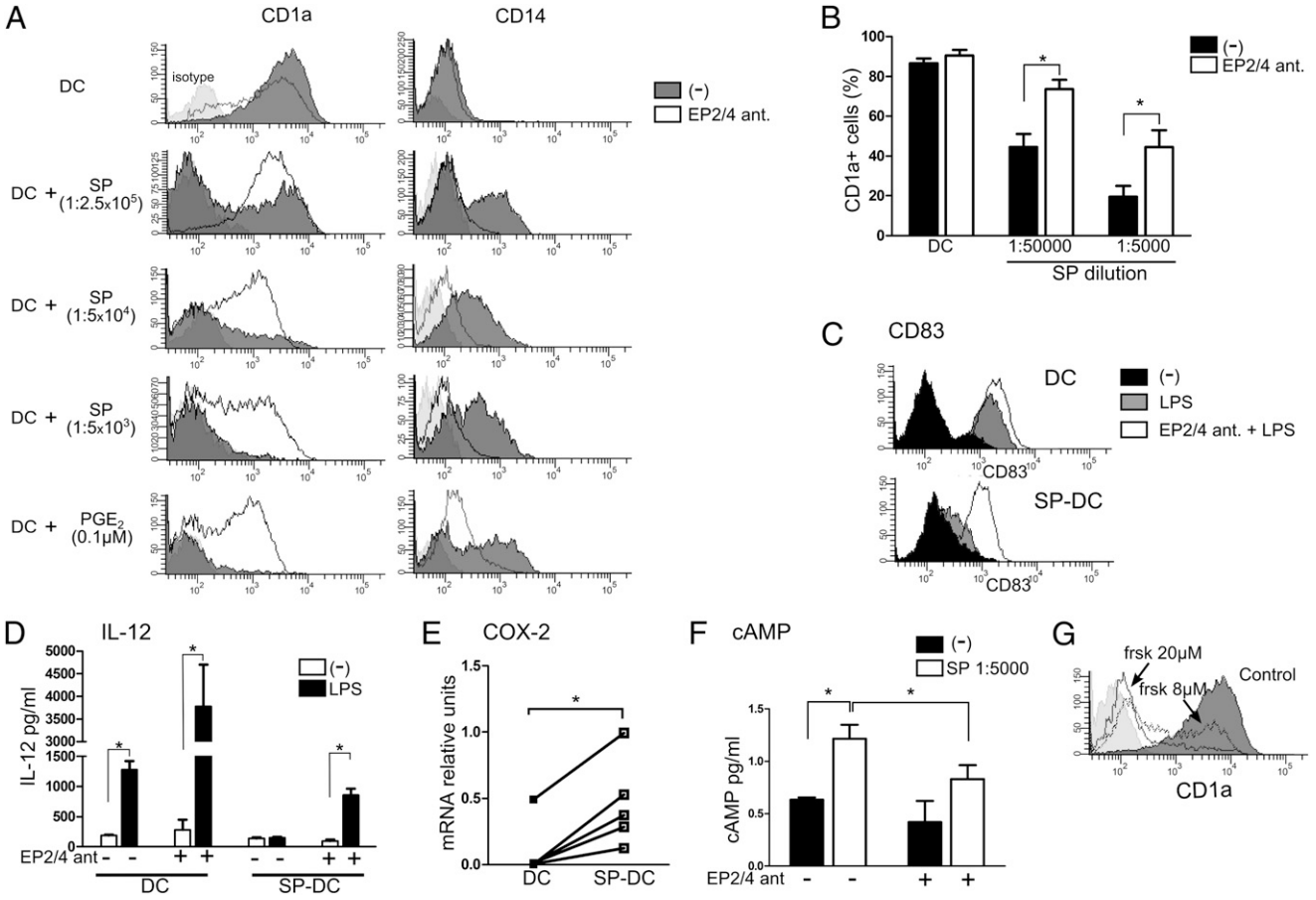

FIGURE 5. PGs are involved in the differentiation of tolerogenic DCs induced by seminal plasma. (**A** and **B**) Monocytes were preincubated with a mix of EP2 (AH6809, 50 μM) and EP4 (AH23848, 90 μM) receptor antagonists for 30 min, and cultured for 5 d in the absence (DC) or presence (SP-DC) of the indicated dilutions of seminal plasma. Then the expression of CD1a and CD14 was analyzed by flow cytometry. (A) The expression of CD1a and CD14 by DCs differentiated in the presence of PGE_2 (0.1 μM), previously treated or not, with the mix of EP2/EP4 antagonists is also shown. Histograms from a representative experiment ($n = 4$) and bars showing the percentages of $CD1a^+$ cells ($n = 4$, mean ± SEM) are shown in (A) and (B), respectively. (**C** and **D**) Monocytes were preincubated with the mix of EP2/EP4 antagonists for 30 min and cultured for 5 d in the absence (DC) or presence (SP-DC) of seminal plasma (dilution 1:5000). Then cells were washed and treated with LPS (100 ng/ml) for 24 h. (C) The expression of CD83 was analyzed by flow cytometry. Histograms from a representative experiment ($n = 7$) are shown. (D) The production of IL-12 p70 was analyzed in cell supernatants by ELISA. Data represent the mean ± SEM of four experiments. (**E**) Monocytes were cultured with IL-4 and GM-CSF for 5 d in the absence (DC) or presence of seminal plasma (SP-DC, dilution 1:5000). Total RNA from DCs and SP-DCs was collected, and COX2 mRNA was analyzed by quantitative RT-PCR ($n = 5$). (**F**) Monocytes were pretreated or not for 5 min with the mix of EP2/EP4 antagonists. Then monocytes were stimulated with seminal plasma (dilution 1:5000) for 10 min, and cAMP levels were measured by RIA, as described in *Materials and Methods*. Data represent the mean ± SEM of four experiments. (**G**) Monocytes were cultured with IL-4 and GM-CSF for 5 d in the absence (control) or presence of forskolin (frsk) at the concentrations indicated inside the figure. Then the expression of CD1a was analyzed by flow cytometry. Histograms from a representative experiment ($n = 3$) are shown. $^*p < 0.05$.

shown that seminal plasma is more than just a spermatozoa carrier. Acting at the female genital tract, it induces the activation of both inflammatory responses and regulatory mechanisms mediated by T cells (18). Semen has been shown to induce the expression of inflammatory cytokines and chemokines, and a massive recruitment of macrophages, DCs, and memory T cells to the female genital mucosa after coitus (7, 10, 43). A similar response has been described in mice, pigs, rabbits, and other mammals (14, 15). In humans, inflammation appears to be mediated through the stimulation of cervix epithelial cells by TGF-β, a cytokine found at very high concentrations in the human seminal plasma (16), long recognized by its ability to induce anti-inflammatory and immunosuppressive effects (44). Exposure to seminal plasma not only induces an inflammatory response in the female genital mucosa, but also induces regulatory mechanisms that allow the fetus (a semiallograft) to grow and develop in the uterus. In this regard, it has been clearly demonstrated in mouse models that exposure of the female genital mucosa to seminal plasma induces the expansion of $CD4^+CD25^+FOXP3^+$ Tregs in the lymph nodes draining the uterus, promoting tolerance to paternal alloantigens (17, 18). This occurs even in the absence of conception, thus suggesting a critical role for seminal fluid as an inducer of Treg (45).

By inducing tolerance to paternal alloantigens, Treg have been shown to play a critical role in pregnancy (46). Increased numbers of Treg are found in pregnant mice and humans (47). Depletion of Treg induces resorption of the embryos in allogeneic matings in mice (46, 48), whereas women with repeated spontaneous abortion show a decreased numbers of Treg (49, 50). Tregs are identified as $CD4^+CD25^+$ T cells expressing the signature transcription factor FOXP3 (51). They are generated in the thymus and extrathymically (peripheral Tregs [pTregs]) (52). Generation of pTregs is dependent on the activation of conventional naive $CD4^+$ T cells by tolerogenic DCs, which usually express a semimature phenotype and drive the differentiation of pTreg in a microenvironment characterized by the absence of inflammatory cytokines and the presence of high concentrations of TGF-β and IL-10 (52–54).

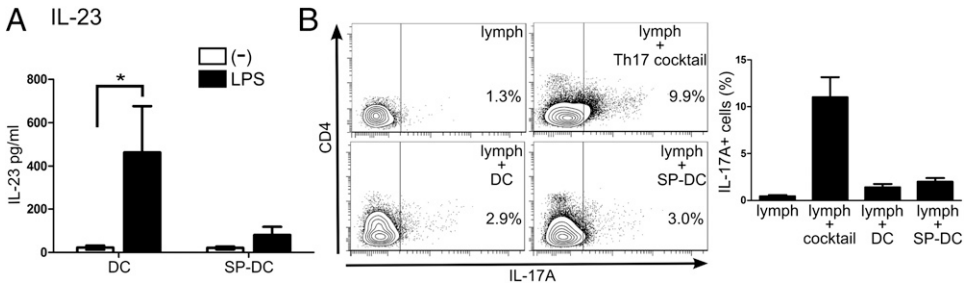

FIGURE 6. DCs differentiated in the presence of seminal plasma do not produce IL-23 upon stimulation by LPS. (**A** and **B**) DCs were obtained from monocytes cultured for 5 d with IL-4 and GM-CSF in the absence (DC) or presence of seminal plasma (SP-DC, dilution 1:5000). Then cells were washed and cultured with LPS (100 ng/ml) for 24 h. (A) The production of IL-23 was analyzed in cell supernatants by ELISA. Data represent the mean ± SEM of six experiments. (B) Cells were washed and cultured with allogeneic lymphocytes using a DC/lymphocyte ratio of 1:5. At day 5, cells were harvested and the expression of IL-17A was analyzed by intracellular staining and flow cytometry in the gate of $CD4^+$ T cells. Lymphocytes cultured alone were used as a negative control, and lymphocytes cultured on anti-CD3 immobilized mAbs in medium supplemented with a TH17-promoting mixture containing an mAb directed to CD28, neutralizing mAbs directed to IL-12p70, IFN-γ, and IL-4, and the cytokines TGF-β1 (10 ng/ml) and IL-6 (20 ng/ml) were used as positive control. Dot plot from a representative experiment ($n = 4$), and bars showing the mean ± SEM of four experiments are shown. *$p < 0.05$.

Interestingly, Samstein and coworkers (55) recently showed that pTregs play a critical and nonredundant role in maternal–fetal tolerance, thus reinforcing the notion that tolerogenic DCs are involved in the generation of tolerance to the embryos. These tolerogenic DCs might capture paternal alloantigens either in the cervix after coitus or when placental trophoblasts invade maternal tissues during embryo implantation, thus activating alloreactive $CD4^+$ T cells and promoting the acquisition of a regulatory phenotype.

The mechanisms through which seminal plasma induces an inflammatory response at the female genital mucosa have been recently clarified (16). By contrast, the mechanisms underlying the ability of semen to induce a Treg response remain unknown. Our results suggest that these regulatory mechanisms could be related, at least in part, to the ability of semen to drive the differentiation of tolerogenic DCs.

In this study, we show that seminal plasma redirects the differentiation of human DCs toward a regulatory phenotype. DCs differentiated from peripheral blood monocytes in the presence of seminal plasma (SP-DCs) were shown to be $CD1a^-$ $CD14^+$ and expressed high levels of CD86 and HLA-DR but very low levels of CD80, CD83, and CCR7. Upon stimulation by inflammatory stimuli, they failed to develop a fully mature phenotype and produced low levels of IL-12p70, IL-1β, TNF-α, and IL-6, but high levels of IL-10 and TGF-β. A limitation of our study is that all the experiments were performed using peripheral blood monocytes instead of reproductive tract-derived cells. It should be noted, however, that the inflammatory response induced by semen in the human cervix after coitus leads to the local recruitment of large numbers of monocytes from the blood (7).

A large array of different biological compounds can induce the acquisition of a tolerogenic phenotype by DCs: TGF-β, IL-10, TSLP, hepatocyte growth factor, vascular endothelial growth factor, GM-CSF, G-CSF, CCL18, retinoic acid, vasoactive intestinal peptide, vitamin D_3, galectin-1, DC-SIGN ligands, estrogens, and PGE_2 (40, 53). PGE_2 and its derivates are present at very high concentrations in human seminal plasma: the primary forms PGE_1 and PGE_2 are found at concentrations >70 μg/ml, whereas the hydroxylated form 19-OH-PGE_2 reaches values >250 μg/ml (8). In fact, PGE_2 is present at ~10,000-fold higher concentration in seminal plasma than that produced locally at inflammation sites (56). Although PGE_2 and PGE_1 act through EP1, EP2, EP3, and EP4 receptors, 19-OH-PGE_2 is specific for the EP2 receptor (37, 38). Our results suggest that the acquisition of a tolerogenic phenotype by DCs induced by seminal plasma is mostly mediated by PGs. In fact, we found that blocking of EP2 and EP4, the two receptors for E-series PGs expressed by monocytes (39), largely restored the ability of monocytes to differentiate into $CD1a^+$ $CD14^-$ DCs, as well as their ability to upregulate the expression of CD83 and to produce IL-12p70 upon stimulation by LPS.

These results are consistent with previous observations reported by Kaliński and coworkers (36), showing that the addition of PGE_2 during the differentiation of monocytes into DCs induced by IL-4 plus GM-CSF leads to the differentiation of $CD1a^-$ $CD14^+$ tolerogenic DCs. Upon activation by maturation stimuli, these DCs produced very low levels of IL-12p70 and high levels of IL-10 (36). In a more recent study, the same group extended their original observations showing that exposure of monocytes to PGE_2 redirects the GM-CSF– and IL-4–driven differentiation of DCs toward a stable myeloid-derived suppressor cell, which expresses a set of immunosuppressive factors such as IDO, arginase 1, IL-4Rα (CD124), inducible NO synthase, and IL-10 (40). Moreover, they demonstrated that PGE_2 induces COX2, the key enzyme regulating PGE_2 synthesis, leading to autocrine production of PGE_2 and the establishment of a positive feedback loop that further promotes the development and the phenotypic stability of myeloid-derived suppressor cells (40). Interestingly, we found that seminal plasma induces the expression of COX2 during the differentiation of monocytes into DCs. The induction of COX2 might promote the stability of the tolerogenic phenotype of SP-DCs via the autocrine production of PGE_2. Moreover, because PGE_2 was shown to stimulate the migratory profile of DCs (57), we speculate that the induction of COX2 might also favor the migration of DCs from the female genital mucosa to the draining lymph nodes, the place where DCs prime the adaptive immune response (45).

The fact that PGE antagonists did not fully prevent the tolerogenic effect induced by seminal plasma on the phenotype and function of DCs suggests that factors other than PGs might also be involved. TGF-β is present at very high concentrations in semen (6). Its ability to induce immunological tolerance has been demonstrated in a variety of experimental settings (44). However, previous studies analyzing the effect of TGF-β on the differentiation of human monocytes into DCs induced by IL-4 plus GM-CSF revealed that TGF-β promotes the differentiation of Langerhans-like cells with a phenotype characterized by the expression of high levels of CD1a and E-cadherin, and the absence of CD14 (58). Moreover, upon activation by inflammatory stimuli such as CD40L, these Langerhans-like cells upregulated the expression of the maturation marker CD83 and produced higher levels of the inflammatory cytokines IL-12p70 and IL-23, and lower levels of IL-10 (59, 60) compared with those DCs differentiated by IL-4 plus GM-CSF in the

absence of TGF-β. Together, these observations suggest that the tolerogenic profile of DCs induced by seminal plasma does not involve the participation of TGF-β acting alone. However, it would be of interest to examine the interaction between TGF-β and PGE in regulating DC phenotype.

Recent observations demonstrated that DC-SIGN ligation during the differentiation of monocytes into DCs also induces the development of $CD1a^-$ $CD14^+$ tolerogenic DCs (61). Interestingly, we have recently identified a novel DC-SIGN ligand in the semen: clusterin (62). It is present at high concentrations in seminal plasma (~1 mg/ml) and interacts with high affinity with DC-SIGN. In fact, we observed that dilutions of seminal plasma as high as $1:10^4$ completely prevented the ability of HIV to bind to DC-SIGN expressed by DCs (63). Studies are currently being conducted in our laboratory to analyze the ability of seminal clusterin to promote the development of tolerogenic DCs.

Notably, semen might induce a tolerogenic effect by acting, not only on DC precursors, but also on other immune cells. A number of classical studies link seminal plasma to increased IL-10 production by monocytes and suppression of both lymphocyte proliferation and NK-mediated cytotoxicity (64, 65). It was also reported that human semen promotes the differentiation of $CD4^+$ T cells into a $FOXP3^-$ regulatory profile (66). Moreover, the indirect effects of seminal fluid on DCs could also be mediated by cytokines produced by epithelial cells. In fact, Sharkey and coworkers (10, 16) have shown that seminal plasma induces the production of a number of cytokines by cervical epithelial cells, among them GM-CSF and G-CSF, two inducers of a tolerogenic phenotype in DCs (40, 53).

In conclusion, our results show that human seminal plasma promotes the acquisition of a tolerogenic phenotype by DCs, and that this effect is mainly mediated by PGs. It should be relevant to test in vivo whether semen deposition after vaginal coitus actually results in the modulation of the phenotype and function of DCs in the cervix, which represents the most important inductive site of the female genital mucosa (67). This is a challenge that will require the use of nonmurine models because, contrasting with the huge concentrations of PGs found in human semen (>300 μg/ml) (8), the mouse semen contains only very few amounts of PGs (~1 μg/ml) (68). Finally, it should be considered that a tolerogenic environment induced by semen at the female genital tract would not only promote fertility, it might also be able to modulate the early events of the immune response against sexually transmitted pathogens, favoring the establishment of infectious diseases. Surprisingly, this matter has not been investigated yet.

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Disclosures

The authors have no financial conflicts of interest.

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