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Pregnancy-specific glycoprotein 1a activates dendritic cells to provide signals for Th17-, Th2-, and Treg-cell polarization

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Because of their plasticity and central role in orchestrating immunity and tolerance, DCs can respond to pregnancy-specific signals, thus promoting the appropriate immune response in order to support pregnancy. Here, we show that pregnancy-specific glycoprotein (PSG1a), the major variant of PSG released into the circulation during pregnancy, targets DCs to differentiate into a subset with a unique phenotype and function. This semi-
mature phenotype is able to secrete IL-6 and TGF-β. PSG1a also affected the maturation of DCs, preventing the up-regulation of some costimulatory molecules, and inducing the secretion of TGF-β or IL-10 and the expression of programmed death ligand 1 (PD-L1) in response to TLR-9 or CD40 ligation. In addition, PSG1a-treated DCs promoted the enrichment of Th2-type cytokines, IL-17-producing cells, and Treg cells from $CD4^+$ T cells from DO11.10 Tg mice. Moreover, in vivo expression of PSG1a promoted the expansion of Ag-specific $CD4^+CD25^+Foxp3^+$ Treg cells and IL-17-, IL-4-, IL-5-, and IL-10-secreting cells able to protect against *Listeria monocytogenes* infection. Taken together, our data indicate that DCs can be targeted by PSG1a to generate the signals necessary to mount an appropriate, well-balanced, and effective immune response able to protect against invading pathogens while at the same time being compatible with a successful pregnancy.

Keywords: Pregnancy • Th1/Th2 cells • Th17 cells • Tolerance • Treg cells

Supporting Information available online

Introduction

DCs are a heterogeneous population of highly specialized APCs recognized for their crucial role as regulators of innate and adaptive immunity. They can also fine tune the context (and hence the outcome) of Ag presentation in response to a plethora of environmental signals that indicate the presence of pathogens or tissue damage. These signals generally boost DC maturation, which promotes their migration from peripheral tissues to secondary lymphoid organs and increases their capacity to induce and regulate effector T-cell responses. In addition, recent evidence has highlighted a critical role for DCs in promoting tolerance, limiting uncontrolled inflammation and maintaining immune cell homeostasis [1].

The DC-derived factors that determine the outcome of DC–T-cell interactions are the levels of Ag presentation, the display of costimulatory molecules, and the presence of

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immunomodulatory factors such as cytokines. While increased levels of Ag presentation and the expression of costimulatory molecules such as CD80, CD86, and CD40 on DCs are crucial for the expansion of Ag-specific T cells, the expression of coinhibitory molecules, such as programmed cell death-1 (PD-1) ligands, PD-L1 and PD-L2, can act synergistically to inhibit T-cell activation, proliferation, and cytokine production [2]. Regarding the role of cytokine in driving the type of cellular effector response, stimuli that induce IL-12 promote IFN-γ-producing Th1 cells, stimuli that induce IL-10 and TGF-β favor Treg-cells differentiation, and stimuli that induce TGF-β and IL-6 promote a Th17 response in the mouse [3, 4]. In addition, IL-4 and IL-10 are both candidates for a Th2-driving signal from DCs, however, it has been demonstrated that both IL-4- and IL-10-deficient DCs can still drive Th2 responses [5]. Of note, the specific anatomical compartment where the immature DCs (iDCs) resides and encounters a maturation stimuli, profoundly impacts the character of the immune response generated by the DCs after it has migrated to the lymph nodes [6, 7].

It is well known that, from an immunological viewpoint, pregnancy constitutes a paradoxical situation in which a foreign tissue is tolerated by the host. Many studies have reported a predominant Th2-type immunity and suppressed Th1-type immunity during pregnancy [8, 9]. Recently, the Th1/Th2 paradigm in pregnancy has been redefined to include Th17 and Treg cells in view of the facts that the Treg cells resident in decidual, blood, and lymph nodes are increased in mice and pregnant women and that the frequency of Th17 in the decidua is significantly higher compared to that in peripheral blood [10].

Pregnancy-specific glycoprotein (PSG), the major placental glycoprotein, is a group of highly similar proteins synthesized in large amounts by placental trophoblasts [11]. PSGs seem to be essential for a successful pregnancy, since low levels of these glycoproteins are associated with pathological conditions including spontaneous abortion and intrauterine growth retardation [12–14]. In addition, the administration of anti-PSG Ab can induce spontaneous abortion in primates [15]. In a previous work, we demonstrated that cultures of mammalian cells infected with a vaccinia (Vac)-based expression vector harboring the open reading frame of human PSG1a cDNA (Vac-PSG1a), release to the culture supernatant an N-glycosilated 72 kDa PSG1a protein similar to that secreted by the human placenta [16]. Also, recombinant PSG1a is able to modulate the monocyte (MΦ) metabolism to regulate T-cell activation and proliferation in vitro [16] and in vivo [17]. Furthermore, other groups have demonstrated the biological effect of murine PSGs on human MΦs or vice versa, with murine PSG17 and PSG18 being able to mimic the biological effects of human PSG by inducing IL-10, IL-6, and TGF-β expression in human MΦs [18], and, human PSG1, PSG6, and PSG11, inducing the secretion of anti-inflammatory cytokines by human and murine MΦs [19].

The recognition that DCs are uniquely able to initiate responses in naïve T cells and also participate in Th-cell education has prompted us to investigate whether PSG1a may have a role in regulating the DC function to modulate the adaptive immune response. In this study, we used murine bone marrow derived DCs to investigate the ability of PSG1a to modulate DC cytokine production, costimulatory molecules expression, and the T-cell polarizing function.

Results

PSG1a targets DCs to differentiate into a subset with a unique phenotype

We first investigated the effect of PSG1a on DC expression of costimulatory and coinhibitory molecules involved in T-cell activation and polarization. As shown in Fig. 1A, the treatment of iDCs with PSG1a did not affect significantly the CD40, ICAM-1, and MHC class II expression. Interestingly, PSG1a treatment induced a small up-regulation in PD-L2 expression (from 16.7%, MFI 9.4 to 23.0%, MFI 16.2) whereas no changes were observed in PD-L1 expression (Fig. 1A). As expected, LPS fully matured DCs showed a high expression of CD40, CD80, ICAM-1, MHC class II, and PD-L2 (Fig. 1A).

Then, to exclude the possible contamination of PSG1a by LPS, polymyxin B, an antibiotic known to inhibit activities induced by LPS, was used. Therefore, the observed effects of PSG1a found on PD-L2 expression were not caused by LPS contamination, with LPS-induced PD-L2 expression being inhibited (Fig. 1A).

Treatment of iDCs with PSG1a was accompanied by an increase in the levels of IL-6 and TGF-β, with no significant differences being found in the levels of IL-1β, TNF, IL-12, IL-23, or IL-10 (Fig. 1B and not shown). iDCs treated with LPS secreted high amounts of all the studied cytokines (Fig. 1B). The PSG1a-induced IL-6 secretion was observed to be dose dependent (Fig. 1C) and not inhibited by addition of polymyxin B (not shown). Moreover, concentrations of PSG1a as low as 2.5 μg/mL induced important levels of TGF-β secretion (Fig. 1C).

We next analyzed whether PSG1a could affect the DC-Ag capture machinery. As shown in Fig. 1D, incorporation of FITC-mannosylated BSA by untreated DCs was comparable with PSG1a-treated DCs. In contrast, on TLR-4 triggering (LPS), a decrease in FITC-mannosylated BSA uptake was observed (Fig. 1D).

In order to study the capacity of PSG1a-targeted DCs to be activated by TLR ligands or anti-CD40, PSG1a or untreated DCs were stimulated with TLR4-ligand (LPS), TLR9-ligand (CpG), or agonistic anti-CD40 Ab, and the phenotypic and functional changes induced by the stimulation analyzed after 24 h. Untreated DCs stimulated with LPS or CpG showed an up-regulated expression of CD40, CD80, and of MHC class II molecules (Fig. 2A). In contrast, TLR-ligands-induced phenotypic changes were considerably impaired in PSG1a-treated DCs that showed a down-regulated expression of CD40, CD80, and MHC class II molecules compared with untreated DCs (Fig. 2A). Agonistic anti-CD40 mAb induced a strong expression of CD80 and MHC class II molecules in untreated DCs, phenotypic changes that were not observed in PSG1a-treated DCs (Fig. 2A). In addition, PSG1a-treated DCs was more proficient than untreated DCs at expressing PD-L1 in response to CpG or CD40 ligation (Fig. 2B). Moreover, CpG and anti-CD40

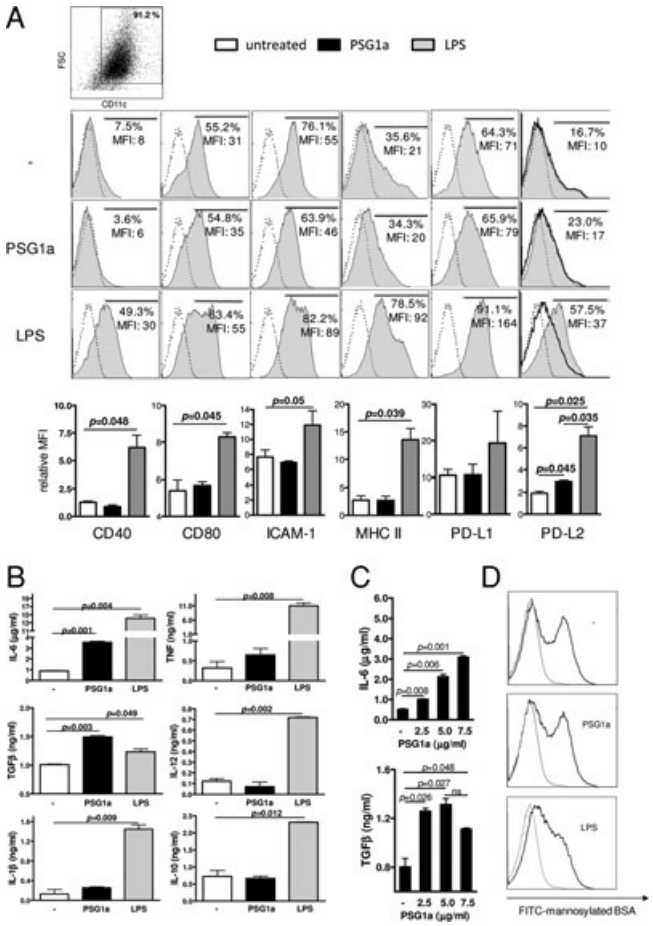

Figure 1. Effect of PSG1a on DC phenotype and function. DCs were treated for 18 h with PSG1a (5 μg/mL), LPS (50 ng/mL), or left untreated. (A) Flow cytometry plots on $CD11c^+$ gated population (top) show the expression of the indicated markers by gray-filled histograms, with thin-line empty histograms showing the cells stained with control Ab, and bold-line empty histograms showing cells treated with polymyxin B (only for PD-L2 staining, right). Values represent the percentage of positive cells and the MFI (middle plots). Average MFI of each marker normalized to MFI of control Ab. Each column represents the mean + SEM of $n = 6$ samples pooled from three independent experiments (bottom). Significance was determined by unpaired Student's *t*-test. (B, C) Cytokine concentrations were determined in culture supernatants by ELISA. Data are shown as means + SEM of data pooled from three cultures of the same experiment. Significance was determined by unpaired Student's *t*-test. (D) Cells were pulsed for 1 h with medium containing 1 mg/mL FITC-mannosylated BSA. Thin-line histograms show the background autofluorescence, and bold-line histograms show the specific uptake at 37°C. All data are from one experiment representative of three to five in total.

stimulation induced in PSG1a-treated DCs, but not in untreated DCs, a population of cells expressing high levels of surface PD-L1 (Fig. 2B). On the other hand, PSG1a treatment did not significantly modify PD-L2 expression after TLR or CD40 ligation (Fig. 2B).

After stimulation, PSG1a-treated DCs showed a stimulus-dependent capability to modulate the profile of secreted cytokines. PSG1a-targeted DCs retained a similar capacity as untreated DCs to secrete TNF in response to TLR-9 ligation, with concomitantly, lower levels of IL-12 and IL-1β but higher levels of IL-10 being secreted compared to untreated DCs. These cells also secreted lower levels of IL-12, TNF, and IL-1β along with higher levels of TGF-β than untreated DCs in response to TLR-4 ligation. Finally, PSG1a-treated DCs preserved a similar capacity as untreated DCs to secrete IL-12 in response to CD40 ligation, but secreted significantly higher amounts of IL-10 and IL-6 and lower amounts of IL-1-β cytokines (Fig. 2B).

PSG1a-treated DCs induce Treg cells and $CD4^+$ T cells that secrete Th2-type cytokines and IL-17

To study the ability of PSG1a-treated DCs to participate in Th-cell polarization in vivo, untreated or PSG1a-treated DCs were pulsed with $OVA_{323–339}$ for 4 h and then injected i.p. into naive BALB/c mice that had been transferred with CFSE-labeled-DO11.10 splenocytes (spleen mononuclear cells, SMCs) 2 days before. LPS-treated DCs was used as control of fully mature DCs able to address the Th1 differentiation. Five days later, the Ag-specific cytokine production was analyzed in SMCs after ex vivo restimulation. SMCs from mice injected with PSG1a-treated DCs secreted significantly higher amounts of IL-4, IL-5, IL-10, and IL-17 but similar amounts of IFN-γ or TGF-β compared with SMCs from mice injected with untreated DCs (Fig. 3A). As expected, SMCs from mice injected with LPS-treated DCs produced higher levels of IFN-γ and similar amounts of Th2-type cytokines compared with SMCs from mice injected with untreated DCs (Fig. 3A). In addition, the percentage and the absolute number of transferred ($CFSE^+$) $^+CD4^+$ T cells per spleen that produced IL-17 was significantly higher in mice immunized with PSG1a-treated than in those immunized with untreated DCs, whereas the expansion of spleen TCR Tg cells was similar between the two experimental groups (Fig. 3B–D).

The fact that PSG1a treatment induced DC resistance to the classical maturation, decreased the expression of some costimulatory and MHC class II molecules, and also increased expression of the coinhibitory receptors PD-L1 and also promoted IL-10 secretion (features that define the tolerogenic DC phenotype [1]), prompted us to explore whether DC-PSG1a were able to induce Treg cells. We studied the presence of $CD4^+$ $CD25^+Foxp3^+$ Treg cells 5 days after DC-PSG1a immunization and found that 11.2% (±0.8) of $CD4^+$ T cells of the spleen from untreated DC-immunized mice were $CD25^+$ $Foxp3^+$, whereas 13.8% (±0.59) of the $CD4^+$ spleen cells from DC-PSG1a-immunized mice were $CD25^+$ $Foxp3^+$ (Fig. 4). This reflected a small but significant ($p = 0.048$) expansion of the Treg-cell population in DC-PSG1a-immunized mice, taking in account that the total number of cells per spleen as well as the spleen $CD4^+$ population did not increase in size compared with untreated DC-immunized mice (Fig. 3D). In addition, the absolute number of Treg cells per spleen and the ratio of effector CD4 T cells ($CD4^+$ $CD25^-$) per Treg-cell ($CD4^+CD25^+Foxp3^+$) in PSG1a-treated DC-immunized mice was 6.3 to 1 compared to 8.1 to 1 for untreated DC-immunized mice ($p = 0.047$) (Fig. 4B). In addition, we did not find any significant differences in the percentage of $CD4^+CD25^+$ $GITR^+$ or

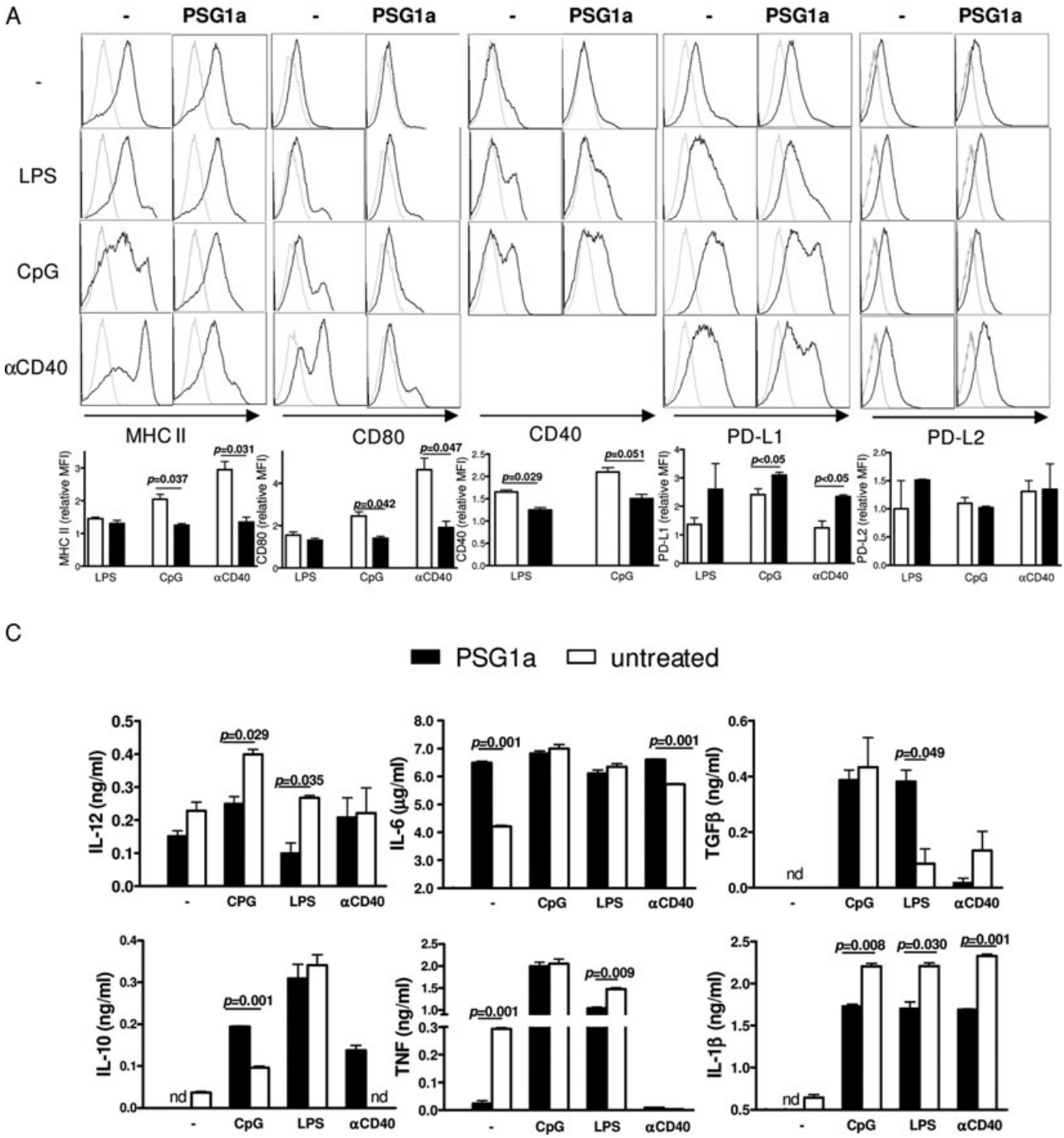

Figure 2. PSG1a modulates DC maturation induced by TLR or CD40 ligation. DCs were treated with PSG1a (5 μg/mL) or left untreated (-) for 18 h before being activated with LPS, CpG-ODN, anti-CD40, or left untreated (-) for another 18 h. (A) Representative histograms show the expression of the indicated markers on $CD11c^+$ gated population by the bold-line histograms, with thin-line empty histograms showing the cells stained with control Ab (top). Average MFI of each marker normalized to MFI of the same population (untreated or PSG1a-treated) without stimulation. Each column represents the mean + SEM of data pooled from three independent experiments. Significance was determined by unpaired Student's *t*-test. (B) Cytokine concentrations were determined in culture supernatants by ELISA. Data points represent means + SEM of data pooled from three cultures of the same experiment. Significance was determined by unpaired Student's *t*-test. All data are from one experiment representative of three in total. nd, not detectable.

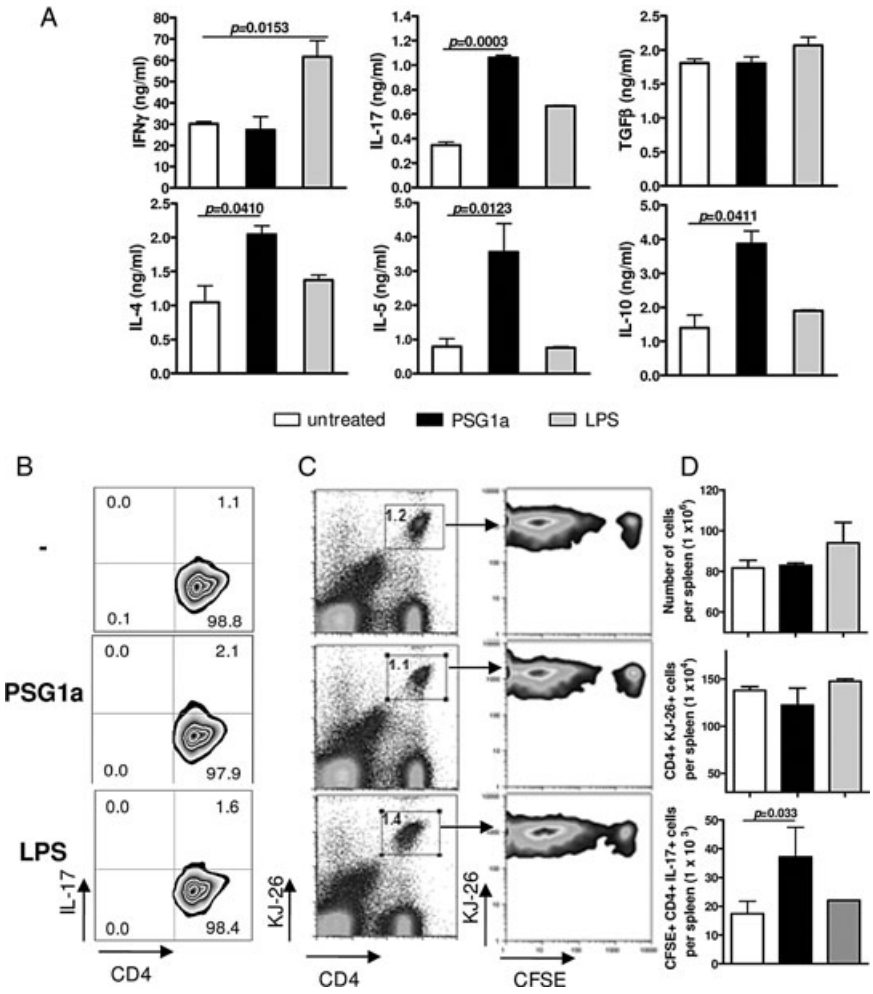

Figure 3. Effect of the immunization with PSG1a-treated DCs on Th differentiation. Untreated, PSG1a-, or LPS-treated DCs pulsed with $OVA_{323–339}$ for the last 4 h of culture were i.p. injected in BALB/c mice that had been transferred with CFSE-labeled SMCs containing 5×10^6 OVA-specific KJ1–26$^+$ CD4$^+$ T cells 2 days before. Five days later, the Ag-specific cytokine production was determined in SMCs. (A) SMCs were cultured with $OVA_{323–339}$ peptide and the cytokine levels were determined after 72 h in the culture supernatants by ELISA. Data are shown as mean + SEM of $n = 4–5$/group and are representative of four independent experiments. (B) Intracellular IL-17 was determined after 4 h stimulation with PMA and ionomycin. A representative plot showing the percentage of CD4$^+$ T cells expressing IL-17 on the CFSE$^+$ gated population in SMCs from one mouse representative of each group (OVA-pulsed untreated, PSG1a-treated, or LPS-treated DCs). (C) Representative plots of the percentage of CD4$^+$ KJ-26$^+$ cells in spleen from mice immunized with OVA-pulsed untreated, PSG1a-, or LPS-treated DCs are shown on the left. CFSE dilution plots of the CD4$^+$ KJ-26$^+$ gated population are shown on the right. (D) The number of total cells (top), of CD4$^+$ KJ-26$^+$ cells (middle), and of CD4$^+$ T cells expressing IL-17 on the CFSE$^+$ gated population (bottom) per spleen are shown as mean ± SEM of $n = 5$ mice per group from one experiment representative of four. Significance was determined by unpaired Student's *t*-test.

CD4$^+$CD25$^+$CTLA-4$^+$ Treg cells in the spleen from DC-PSG1a-immunized mice, compared with untreated DC-immunized mice (data not shown).

In agreement with the results obtained in vivo, CD4$^+$ T cells cultured with OVA-pulsed-PSG1a-treated DCs produced significantly greater amounts of IL-4, IL-5, IL-10, and IL-17 than CD4$^+$ T cells primed with untreated DCs (Supporting Information Fig. 1A). Moreover, CD4$^+$ T cells cocultured with LPS-treated DCs promoted the development of Th1 cells that produced more than eight times the amount of IFN-γ than that arising from cells primed with DC-PSG1a (Supporting Information Fig. 1A), and also showed increased IFN-γ/IL-4 and IFN-γ/IL-17 ratios. Neutralization of IL-6 or TGF-β from the beginning of the DC–T cell coculture resulted in less secretion of IL-17 (Supporting Information Fig. 1B). Notably, PD-L1 blockade also reduced IL-17 production (Supporting Information Fig. 1B). In addition, neutralization of IL-6 or blockade of IL-10 receptor resulted in less IL-4 secretion (Supporting Information Fig. 1B).

In addition, CD4$^+$ T cells cocultured with PSG1a-treated DCs showed an expansion of CD4$^+$CD25$^+$Foxp3$^+$ cells compared with those cocultured with untreated DCs ($14.1 \times 10^3 \pm 4.6$ cells per well; $9.7 \pm 0.5\%$ versus $32.7 \times 10^3 \pm 2.8$ cells per well; $5.3 \pm 0.6\%$ $p < 0.05$ and < 0.01, respectively) (Supporting Information Fig. 1C). We also observed that PSG1a was as able as LPS to condition DCs to expand a population of T cells, with a phenotype of Treg cells (CD4$^+$CD25$^+$Foxp3$^+$). Finally, the blockade of PD-L1 and the neutralization of IL-6 or TGF-β from the beginning of the DC-T cell coculture reduced the development of Treg cells (Supporting Information Fig. 1C).

Effect of in vivo expression of PSG1a on the T-cell response during *L. monocytogenes* infection

To investigate whether in vivo circulating PSG1a could be able to modulate the Ag-specific T-cell response during an infection, *L. monocytogenes,* a facultative intracellular bacterium able to induce a strong protective Th1 response [20], was used as a model. DO11.10 transgenic mice that were injected with a Vac-PSG1a 4 days earlier were infected i.v. with *L. monocytogenes* expressing soluble OVA (rLm-OVA) to allow the detection of Ag-specific T-cell responses. Age-matched DO11.10 mice injected with Vac-WT were used as controls. As we previously demonstrated [17], a single i.p. injection of Vac-PSG1a at a

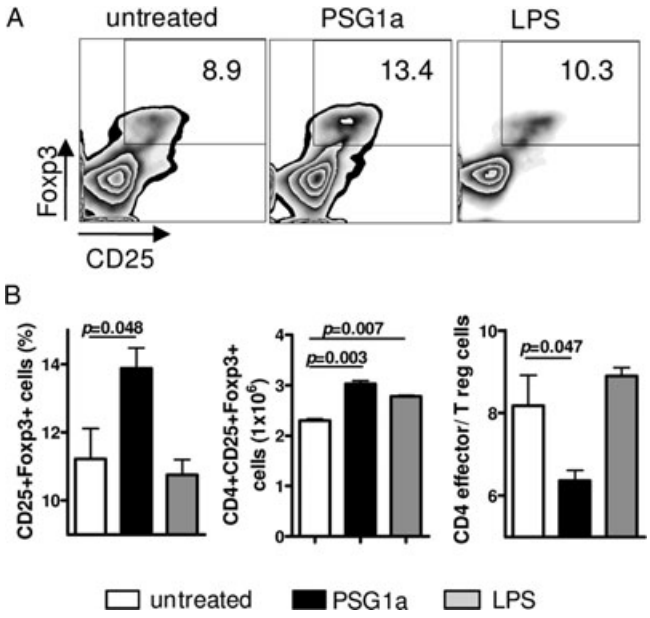

Figure 4. Effect of the immunization with PSG1a-treated DCs on Treg-cell differentiation. Untreated, PSG1a-, or LPS-treated DCs pulsed with $OVA_{323–339}$ for the last 4 h of culture were i.p. injected in BALB/c mice that had been transferred with CFSE-labeled SMCs containing 5×10^6 OVA-specific KJ1–26$^+$ CD4$^+$ T cells 2 days before. Five days later, the presence of Treg cells was determined in SMCs. (A) A representative plot showing CD25 and Foxp3 expression on the CD4$^+$ gated population in SMCs from one mouse representative of each group (OVA-pulsed untreated, PSG1a-treated, or LPS-treated DCs). The values represent the percentage of $^+$CD4$^+$ T cells expressing CD25 and Foxp3 in the live SMC population. (B) Percentage of CD4$^+$ T cells expressing CD25 and Foxp3, the absolute number of CD4$^+$CD25$^+$Foxp3$^+$ cells per spleen and the relative ratio of effector T cells (CD4$^+$CD25$^-$) per Treg-cell (CD4$^+$CD25$^+$Foxp3$^+$). Data are shown as mean ± SEM of $n = 5$ mice per group and are representative of three experiments, all of which gave similar data. Significance was determined by unpaired Student's *t*-test.

dose of 10^7 PFU was able to induce in vivo expression of PSG1a that could be detected in sera since 24 h postinfection (p.i.) (not shown). Seven days later, the OVA-specific cytokine production and the presence of Treg cells were determined in splenocytes. SMCs from infected mice injected with Vac-PSG1a produced, in response to OVA, significantly higher amounts of IL-17, IL-4, and IL-10 cytokines and similar amounts of IFN-γ compared with SMCs from control mice (Fig. 5A). Moreover, SMCs from control mice produced IFN-γ but little or no IL-17, IL-10, or IL-4.

Next, we examined the magnitude of pathogen-specific T effectors and Treg cells primed after acute infection during in vivo PSG1a expression. Noninfected (NI) DO11.10 mice contained a small population of KJ-26 TCR Tg$^+$ cells (~3% of total spleen cells and between 4 and 5×10^6 cells per spleen) (Fig. 5B, C). Among this TCR Tg$^+$ population present in NI mice, ~5% were CD25$^+$Foxp3$^+$ Treg cells (between 200 and 300×10^3 cells per spleen) (Fig. 5B, C). During the peak of T-cell response (day 7 after rLm-OVA infection), Vac-WT treated mice showed a selective expansion of OVA-specific T cells (from 4.97 ± 0.39 to $9.30 \pm 4.26 \times 10^6$ cells per spleen) (Fig. 5B, C), while the percentage and number of Foxp3$^+$CD25$^+$ Treg cells among TCR Tg$^+$ cells was reduced tenfold from $5.15 \pm 0.75\%$ to $0.26 \pm 0.09\%$ (from 252.8 ± 15.4 to $26.9 \pm 18.1 \times 10^3$ cells per spleen) (Fig. 5B, C). Interestingly, in vivo expression of PSG1a induced a similar growth of OVA-specific T cells, but concomitantly significantly expanded the percentage and absolute number of Foxp3$^+$CD25$^+$ Treg cells among the TCR Tg$^+$ population (approximately tenfold, from $0.26 \pm 0.09\%$ to $2.1 \pm 0.9\%$ and from 26.9 ± 18.1 to $243.8 \pm 50.2 \times 10^3$ cells per spleen, $p < 0.05$ (Fig. 5B,C). In addition, neither the percentage nor the absolute number of CTLA-4$^+$ or GITR$^+$ cells among the TCR Tg$^+$ population was significantly expanded. Finally, to determine whether the PSG1a-induced Th1^{low}/Th-17/Th2-type cell response compromised the protective immunity to Lm infection, BALB/c mice that had been injected with Vac-PSG1a or Vac-WT 4 days before were primed with Lm-OVA (day 0) and challenged with Lm-OVA on day 7. Two days later (day 9), CFU were enumerated in liver and spleen. Compared with naïve BALB/c mice, both groups of mice that had been primed with Lm-OVA (Vac-PSG1a and Vac-WT) were equally protected (Fig. 5D), indicating that the immune response developed in the context of circulating PSG1a was as effective as the Th1 response alone in controlling the Lm infection. In addition, although not significant, the Vac-PSG1a treated mice showed a tendency to present lower bacterial load than control mice in liver or in the spleen.

Discussion

Among several explanations for the acceptance of the fetus, the one that suggests that the maternal immune system is suppressed or modified has been the subject of many studies. Thus, it has been proposed that the innate immunity might be able to distinguish the pregnant from the nonpregnant state producing a signal, the so-called "signal P" [21]. We have previously proposed that PSG1a may have a "signal P" role thus modulating the activation of the innate immune system and actively contributing to the T-cell shift of the maternal cell immunity toward a less harmful phenotype [17].

Herein, we demonstrated that PSG1a programmed iDCs to differentiate into a unique subset of DCs, with a distinctive cytokine profile and phenotype. In addition, this particular DC phenotype has the capacity to activate Ag-specific T cells and drive the acquisition of mixed phenotypes of Th2-type-/IL17-secreting cells and Treg cells.

The PSG1a-induced secretion of IL-6 and TGF-β has previously been reported in different cell types for human PSG1 and some murine PSGs [18, 22]. Taking into account the known roles of TGF-β and IL-6 in implantation, trophoblast differentiation and angiogenesis [23], and the direct participation of DCs in angiogenic responses [24], the PSG1a-induced secretion of these cytokines by decidual DCs could have a possible role beyond the regulation of the maternal immune system to support the pregnancy [25, 26]. Thus, the DCs accumulated in the uterus during the peri-implantation period may be targeted by PSG1a to provide

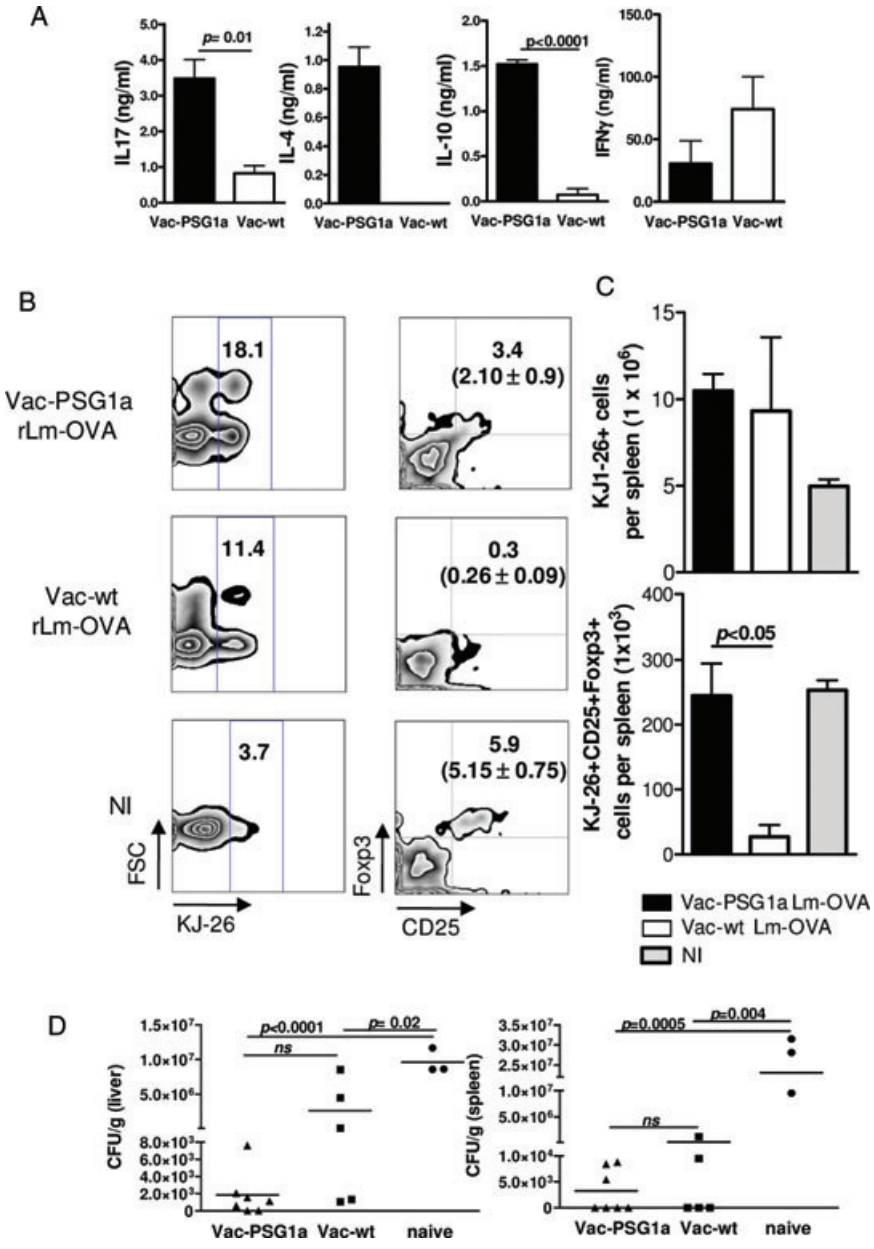

Figure 5. Effect of in vivo expression of PSG1a on the T-cell response against *L. monocytogenes*. DO11.10 mice were injected with Vac-PSG1a 4 days before i.v. infection with ~ 3 × 10^3 CFU *L. monocytogenes* expressing soluble OVA (Lm-OVA). Age-matched DO11.10 mice injected with Vac-WT were used as controls. Seven days later, OVA-specific cytokine production and the presence of Treg cells were determined in SMCs. (A) SMCs were cultured with $OVA_{323–339}$ peptide and the cytokine levels in culture supernatants were determined after 72 h by ELISA. Data are shown as mean + SEM of $n = 5$ mice per group and are representative of four independent experiments. (B) Representative flow cytometry profiles indicate the percentage of TCR-Tg cells (KJ-26^+) of total splenocytes (left), or the percentages of $CD25^+$ $Foxp3^+$ cells among the TCR-Tg population (right) before (NI) or 7 days after infection. The values in parentheses indicate the mean percentages + SEM of 5 mice per group. (C) Number of TCR-Tg cells and TCR $Tg^+CD25^+Foxp3^+$ cells per spleen are shown as mean + SEM of 5 mice per group. These results are from one of three similar experiments, all of which gave similar data. (D) Mice that had been infected with 3 × 10^3 CFU of Lm-OVA were challenged with 10^4 CFU of Lm-OVA 7 days later. Viable bacteria in the spleen and liver were enumerated 2 days after the challenge; each symbol represents a single mouse. One representative experiment of three is shown. Significance was determined by unpaired Student's *t*-test.

DCs with a role in processes such as decidualization and placentation and the associated vascular adaptations. In addition, the up-regulation of PD-L1 expression on PSG1a-treated DCs induced by TLR or CD40 ligation is particularly interesting since the syncytiotrophoblast, tissue where the PSG1a is synthesized, express TLR [27] and constitutively express PD-L1 [28], and the loss of PD-1:PD-L1 signaling has been reported to impair fetomaternal tolerance [29]. However, additional studies are required to establish whether a link exists between PSG1a and PD-L1 expression on this tissue.

Moreover, in agreement with the reported expression of IL-10 by uterine DCs [30], it also was reported that some PSGs can induce IL-10 secretion together with TGF-β secretion [18, 22]. In addition, we have previously reported that spleen macrophage populations from Vac-PSG1a-treated mice drive Th2 differentiation by an IL-10-dependent mechanism [17]. However, although in our experimental setting iDCs treated with PSG1a did not secrete IL-10; they would be able to secrete this cytokine on interaction with T cells (anti-CD40 treatment). Thus, PSG1a-treated DCs was able to prime $CD4^+$ T cells to produce IL-4 by a mechanism that involved IL-6 and IL-10 (as we have previously demonstrated for spleen MΦs targeted in vivo by PSG1a [17]). In addition, in line with these results, it was demonstrated that IL-6 suppresses IL-12-mediated T-cell polarization and is able to direct Th2 differentiation of naïve T cells into IL-4-secreting cells [31]. Moreover, it was shown that IL-6 is the key factor used by pulmonary DCs to polarize naive T cells toward Th2 [32].

Simultaneously, PSG1a-treated DCs were able to drive the development of $^{+}CD4^{+}$ T cells to Treg P cells and IL-17-secreting cells by a mechanism that involved IL-6, TGF-β, PD-L1, and IL-10 signaling with IL-6; TGF-β and PD-L1 signaling promoting both cell populations; and IL-10 signaling partially suppressing the IL-17 secretion. Thus, in agreement with the requirement of TGF-β for both Treg- and Th17-cell development [3], when TGF-β was neutralized in PSG1a-treated DC-T cell cocultures, a significant decrease in IL-17 production and in the percentage of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells were observed. In addition, the key role of PD-L1 in promoting inducible Treg-cell development and function, with PD-L1 and TGF-β having synergistic roles has been demonstrated recently [33]. Moreover, it is known that TGF-β acting in the presence of IL-6 is sufficient to induce naïve T-cell differentiation into Th17 cells [3, 4], and recently it was demonstrated that IL-10 signaling can directly suppress Th17 cells [34, 35]. Furthermore, PD-L1–PD-1 signaling is able to negatively regulate IL-17 differentiation during EAE [36, 37].

It has been well demonstrated that Treg and Th17 cell populations are reciprocally regulated during differentiation with TGF-β inducing Foxp3 expression and IL-6 inhibiting this regulation, and both driving Th17 differentiation at the expense of Treg-cell development [3, 4, 38]. However, when IL-6 was neutralized in the PSG1a-treated DC-T-cell cocultures, a significant decrease of both Treg-cell percentages and IL-17 production were observed, indicating that PSG1a-induced IL-6 accounts for the ability of the PSG1a-treated DCs to promote Treg, IL-17-, and IL-4-secreting cells. In agreement with this IL-6-dependent DC phenotype, a new type of tolerogenic DCs that is induced by activation with TLR2 or TLR4 ligands at low concentrations and including an autocrine/paracrine loop via IL-6 have been recently described [39]. This particular DC phenotype, like PSG1a-treated DCs, shows a semimature phenotype and is resistant to TLR ligand-induced maturation. In addition, Wolfe et al. [40] have recently reported the pivotal role of IL-6-STAT-3 pathway in regulating PD-L1 expression on tolerogenic human DCs that are able to expand $CD4^{+}$ $Foxp3^{+}$ T cells.

During the acute phase of *Listeria monocytogenes* infection, the priming and expansion of Ag-specific Foxp3-$CD4^{+}$ effector T cells without changes in the number of Ag-specific $CD4^{+}$ $Foxp3^{+}$ Treg cells have been clearly demonstrated [41]. Interestingly, we demonstrated that the infection with rLm-OVA in the context of circulating PSG1a (in vivo expression of PSG1a) promoted the expansion of Ag-specific $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells and Ag-specific IL-17-, IL-4-, and IL-10-secreting cells, which were as able as the known effective Th1 response to protect against *L. monocytogenes* infection.

During the early phase of pregnancy, a successful implantation occurs in a proinflammatory microenvironment and Th1-type response that is followed by a shift to a less deleterious response that is induced in part by some hormones and placental products. Therefore, this process could be seen as being a "controlled inflammatory process" that depends on the development of a Th1 response being modulated early by PSG1a-targeted DCs in order to restrict the initial Th1-effector response and thereby contribute to establishing a uniquely regulated immune response to allow antimicrobial defense and tissue repair, while at the same time preventing damage to developing fetal organs or the triggering of preterm labor. In agreement with this hypothesis, pregnant women are not significantly more susceptible to infections than nonpregnant ones, with their adaptative immune system being prepared to mount an effective immune response to invading pathogens [42].

This study provides the first evidence for the existence of placental products able to activate DCs to promote IL-17-producing cells. However, the Th17 response is known to play a pathogenic role in autoimmune diseases and in tissue rejection [38, 43], and therefore may be harmful to the maintenance of pregnancy. Nevertheless, the PSG1a-driven response induce IL17-secreting cells and also leads to induction of Treg cells and IL-10-secreting cells and recently was demonstrated that this cytokine is able to endow Treg cells with the ability to suppress pathogenic Th17 cell responses [34, 35]. In addition, since it has been demonstrated that the stimulation of Th17-effector cells with TGF-β plus IL-6 but not with IL-23 is able to completely abrogate the Th17 pathogenic function (despite up-regulation on IL-17 production [44]), and that PSG1a-treated DCs are able to secrete both these cytokines (but not IL-23), then PSG1a-targeted DCs may be responsible for the maintenance of this nonpathogenic phenotype once it is acquired.

In our experiments, we observed Ag-specific $CD4^{+}IL\text{-}17^{+}$ cells in spleen from Vac-PSG1a-treated mice that were infected with *L. monocytogenes*. However, it is possible that during pregnancy the Th17-mediated response might be restricted to the decidua, as suggested by the report showing that decidual formation prevents the DCs stationed at the maternal/fetal interface from migrating to the lymphatic vessels of the uterus [45], and the fact that the frequency of Th17 cells in $CD4^{+}$ T cells during all stages of pregnancy period is similar to that in nonpregnant women while being significantly higher in the decidua compared to that in peripheral blood [10]. Finally, recent reports showing that IL-17 increases progesterone secretion by the JEG-3 human choriocarcinoma cell line and induces the invasive capacity of these cells [46], suggest another role for IL-17, in addition to inducing protective immunity during the pregnancy.

Materials and Methods

Animals

Female BALB/c mice, 6–8 weeks old, were obtained from Comisión Nacional de Energía Atómica (CNEA; Argentina) and maintained according to the National Research Council's guide for the care and use of laboratory animals. OVA TCR-transgenic DO11.10 mice were kindly provided by Dr. Oscar Campetella, USAM, Argentina. The studies were approved by the Institutional Animal care and Use Committee of the Faculty of Chemical Sciences, National University of Cordoba (Res 459/09).

Antibodies

Abs used in cultures was from eBioscience, including anti-CD3, anti-CD28, anti-CD40, anti-PD-L1, neutralizing Ab against murine IL-6. Neutralizing Ab against TGFβ (1, 2, and 3) was from R&D Systems and anti-IL-10R from Biolegend. Ab for flow cytometry included PE-anti-PD-L2, PE-anti-PD-L1, FITC-anti-CD80, PE-anti-CD40, FITC-anti-CD4, PerCP.Cy 5.5-anti-CD4, anti-FITC/PE-anti-DO11.10 TCR (KJ1.26), PE-anti-IL-17A, PE-anti-Foxp3, allophycocyanin-anti-CD25, FITC-anti-IL-4, and FITC-anti-ICAM, all were from eBioscience. PE-anti IA^d and PE-anti-CTLA4 were from BD PharMingen.

PSG1a production and purification

The recombinant vaccinia virus vector (Vac-PSG1a) construction has been previously described [16]. To scale up the PSG1a production, J774 cells were infected with Vac-PSG1a at a multiplicity of 30 PFU/cell in serum-free medium, and the level of the PSG1a protein secreted to the culture supernatant was analyzed at different times p.i. by Western blot using specific polyclonal antisera (Dako A/S Denmark). The medium used as the starting material for PSG1a purification was harvested 20 h p.i. when no significant alterations in cell morphology were observed. Portions of 200 mL of medium were concentrated to 20 mL by ultrafiltration on a 30 kDa membrane (Amicon). Aliquots of 0.5 mL of the concentrated material were loaded onto a Superdex-200 column (Hi-Load, Amersham Pharmacia Biotech). This column was equilibrated with filtered and degassed PBS. The fractions (fractions 76–80; 1 mL each) containing PSG1a (determined by western blot, Supporting Information Fig. 2) were pooled, filtered down through the 0.1 μm filter (Millipore) and the protein concentration quantified by the Bradford assay. Culture supernatants from J774 cells infected with the wild-type vaccinia virus (Vac-WT) were processed as described above, and the presence of protein in the pooled fractions (76–80) was not detected by UV or after silver staining of 12% SDS-PAGE (Supporting Information Fig. 2).

The endotoxin levels in the PSG1a preparation were <0.5 IU/mg, and were detected using the Limulus amebocyte lysate test (EndosafeTimes, Charles River Laboratories, Wilmington, Delaware, USA).

Differentiation and activation of DCs

DCs were prepared as described by Lutz et al. [47] with slight modifications. Briefly, BM cells collected from femurs were cultured at a 2×10^6 live cells in 10 mL of RPMI 1640 (Gibco, Invitrogen) containing 10% heat-inactivated endotoxin-free FCSy (Hi Clone, Invitrogen), 2 mM L-glutamine (Life Technologies, Gaithersburg, MD, USA), 50 μg/mL Gentamicin (Gibco), and 50 μM 2-mercaptoethanol (Sigma-Aldrich) supplemented with 1% of supernatant from GM-CSF producing J558 cells (20 ng/mL). At day 3, 10 mL of medium containing GM-CSF was added. At day 6, 10 mL of the medium containing GM-CSF was refreshed. Then, at day 8, 10 mL of medium containing only 5 ng/mL of GM-CSF was refreshed and the cells were harvested 18 h later (day 9). After this time, ~90% of the harvested cells were iDCs (MHC class II^+, $CD11c^+$). iDCs were transferred into 96-well U-bottom plates, 5×10^5 DC/well, and incubated with medium alone or PSG1a 5 μg/mL of purified PSG1a was used (a PSG1a concentration able to mimic the previously published results on modulation of the MΦ metabolism [16]) for 18 h. Alternatively, iDCs were stimulated with 50 ng/mL of LPS (from *Escherichia coli* serotype 055:B5, Sigma-Aldrich) or incubated with PSG1a for 18 h, before being stimulated for 18 h with LPS (50 ng/mL), CpG-ODN (1 μg/mL, Operon, Technologies, Alameda, CA, USA), or 10 μg/mL of agonistic anti-CD40 Ab. For all culture settings, cell viability was assessed by using trypan blue assay and the annexin V-FITC apoptosis detection kit (BD, Biosciences, San Diego, CA, USA) with the dead-cell dye 7-AAD (Santa Cruz Biotechnology; San Diego, CA, USA). The viability of DCs with all treatments varied from 80 to 90%.

DC flow cytometry

Expression of surface molecules on DCs was quantified by FACS. Using the FACScanto II flow cytometer (Becton Dickinson), 50,000 events were acquired. Analyses of the acquired data were performed using FlowJo software (Tree Star, Inc).

Antigen uptake experiments

DCs treated as described in the figures was resuspended in serum-free culture medium. FITC-mannosylated BSA (Sigma) was added at a 1 μg/mL final concentration, and the cells were incubated at 37°C. After 3 h, the DCs were extensively washed with ice-cold PBS, fixed in PBS containing 2% formaldehyde, and analyzed by FACS.

Cytokine measurement

Cytokines were measured in cell culture supernatants by capture ELISA using Abs and protocols suggested by the manufacturer (eBiociences). The detection limits of the ELISA determinations were 125 pg/mL for IFN-γ, 31 pg/mL for IL-12, 62 pg/mL for IL-6, 15 pg/mL for TNF, 31 pg/mL for IL-4, 31 pg/mL for IL-5, 31 pg/mL for IL-10, 31 pg/mL for IL-17, 62 pg/mL for IL-1β, 62 pg/mL for TGF-β, and 31 pg/mL for IL-23.

In vivo T-cell priming assay

Lymph nodes and spleen were harvested from DO11.10 TCR transgenic mice that were age and sex matched to the adoptive transfer recipients. Single-cell suspensions were prepared and the number of $KJ1–26^+$ cells injected was calculated based on multiplying the

percentage of KJ1–26$^+$ CD4$^+$ T cells measured by FACS and the number of live cells obtained. CFSE (Invitrogen) labeled SMCs containing 5×10^6 OVA-specific KJ1–26$^+$ CD4$^+$ T cells were injected i.v. into recipient mice 2 days before i.p. injection with 5×10^5 DCs that had been treated overnight with PSG1a and pulsed or not with OVA peptide (500 nM) for the previous 4 h. After 5 days, SMCs (1×10^6/mL) were cultured in 24-well plates at 37°C in 5% CO_2 with media or $OVA_{323–339}$ peptide (500 nM). After 72 h, the cytokine levels in supernatants were measured by ELISA. For Treg analysis, the cells were stained with FITC-labeled mAb against CD4 combined with APC-labeled mAb against CD25 and PE-labeled antimouse Foxp3. Intracellular Foxp3 staining was performed using an eBioscience Foxp3 permealibization kit. Intracellular cytokine detection was performed with a Cytofix/Cytoperm kit (BD Biosciences) per manufacturer's recommendation after 4 h stimulation with phorbol myristate acetate (PMA, 50 ng/mL) and ionomycin (500 ng/mL; Sigma-Aldrich) in the presence of GolgiStop (BD Bioscience).

In vivo PSG1a expression and *L. monocytogenes* infection

Eight- to 10-week-old DO11.10 transgenic mice were injected i.p. with 10^7 PFU of Vac-PSG1a ($n = 7$), and age-matched mice were injected with 10^7 PFU of Vac-WT as controls ($n = 5$). After 4 days all mice were infected i.v. with $\sim 5 \times 10^3$ CFU virulent recombinant *L. monocytogenes* expressing secreted OVA protein (Lm-OVA) [48], provided by Dr. Leo Lefrançois (University of Connecticut Health Center, USA). At day 7 (primary response), p.i. splenocytes were collected for analysis of the specific cytokine production and FACS. In a challenge experiment, mice were immunized with 3×10^3 CFU of bacteria and then challenged with $\sim 10^4$ CFU of Lm-OVA, 7 days after the immunizing infection. Viable bacteria in the spleen and liver were enumerated 2 days after this challenge, and the difference of CFU per gram of tissue between each group and nonimmune (naïve) control mice ($n = 3$) was taken as the level of protection. Livers and spleens were weighed and homogenized in 10 mL 0.2% Igepal in H_2O. Organ homogenates were serially diluted and plated on streptomycin agar plates to determine the CFU of Lm-OVA.

Statistical analysis

The Student's *t*-test was used to determine the statistical significance between groups. A p value <0.05 was considered to be statistically significant.

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Abbreviations: **PD-1**: programmed cell death-1 · **PD-L**: PD-1 ligand · **PSG1a**: preganacy-specific glycoprotein 1a · **rLm-OVA**: *Listeria monocytogenes* expressing soluble OVA · **SMC**: spleen mononuclear cell · **Vac**: vaccinia virus

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