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# Regulatory B10 Cells Restore Pregnancy Tolerance in a Mouse Model<sup>1</sup>

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## ABSTRACT

During mammalian pregnancy, the immune system defies a double challenge: to tolerate the foreign growing fetus and to fight off infections that could affect both mother and fetus. Minimal disturbances to the fine equilibrium between immune activation and tolerance would compromise fetal survival. Here, we show that regulatory B10 cells are important for pregnancy tolerance in mice. The frequency of these cells increases during normal murine pregnancies, while mice presenting spontaneous abortion do not show elevated levels of regulatory B10 cells. When B10 cells are transferred to the abortion-prone mice, dendritic cells are kept in an immature state, and regulatory T cells increase, thus avoiding immunological rejection of the fetuses. In vitro, we could identify IL-10 secreted by B10 cells as the main mediator of these salutary effects. Our data add an important piece of information to the complex immune crosstalk during pregnancy. This study opens novel lines of work to better understand how to help women who have trouble in maintaining a pregnancy.

*female reproductive tract, IL-10, pregnancy, regulatory B cells, reproductive immunology, rodents (rats, mice, guinea pigs, voles), spontaneous abortion, tolerance*

## INTRODUCTION

Mammalian pregnancy represents a unique process during a limited period of time, in which the maternal immune system defies a double challenge: to tolerate the foreign growing fetus and to fight off infections that could affect both mother and fetus [1]. This delicate balance is of special importance when the maternal blood is in direct contact with fetal structures, as it is in mammals. Minimal disturbances to the fine equilibrium between immune activation and tolerance would compromise fetal survival. Cytokines produced by immune and nonimmune cells play a central role in orchestrating the immune status [2].

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Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine that is related to pregnancy success; in fact, its application can prevent naturally occurring fetal loss in a mouse model of pregnancy disturbance [3]. Genetic deletion of IL-10, however, did not lead to abortions [4]. Hence, it was questioned whether it is essential or even important for pregnancy success. The concept of IL-10 being important for pregnancy reemerged after it was shown that it protects against lipopolysaccharide (LPS)-induced fetal death [5, 6]. Further, IL-10 deficiency is related to fetal growth restriction [6], which has serious consequences for the offspring. It had always been assumed that the cellular source of IL-10 was trophoblasts or T cells. However, a subset of B cells, the regulatory B10 cells, emerged as an important source of IL-10 [7–10].

B lymphocytes in general are defined as cells that express diverse cell-surface immunoglobulin receptors that recognize specific antigenic epitopes. Based on cellular markers, function, and localization, B cells can be subdivided into two main populations: B1 and B2 cells [11]. B1 and B2 cells also differ in their ontogeny. While B1 cells mainly originate during the embryonic life from precursors present in the embryonic liver, B2 cells are continuously formed during the postnatal life from precursors in the bone marrow [11]. Alongside their classic role as antibody producers, B cells have emerged as pleiotropic players and as central regulators of immune responses [12]. Regulatory B10 cells in particular have strong immunoregulatory properties, with their main hallmark being their ability to produce IL-10 [7–10]. Indeed, most, if not all, of the suppressive functions attributed to regulatory B cells are strictly related to their capacity to produce IL-10 [7, 9, 13–16]. The main function of IL-10-producing regulatory B10 cells is to maintain the fine immune balance that is required for tolerance; thus, it has been proposed that they prevent autoimmunity [14] and are important mediators of chronic infections [17]. Whether they are involved in this balance to support pregnancy is unknown.

Here, we addressed the participation of regulatory B10 cells in the establishment of pregnancy tolerance using a well-established model of spontaneous abortion and investigated the mechanisms behind their protective properties.

## MATERIALS AND METHODS

### *Animals and Animal Experiments Setup*

Eight-week-old CBA/J females as well as BALB/c and DBA/2J males were purchased from Charles River (Sulzfeld, Germany). C57BL/6 B cell-deficient M mice [18] were kindly provided by Dr. Annegret Reinhold (Magdeburg, Germany). Mice were kept in our animal facility under optimal conditions in a 12-h light cycle. Food and water were given ad libitum. Animal experiments were carried out according to institutional guidelines after approval (review board institution: Landesverwaltungsamt Sachsen-Anhalt, approval code: AZ2-868 provided to A.C.Z.). The experiments were conducted in conformity with the European Union Council Directive 86/609/EEC, which is in agreement with the National Research Council's *Guide for Care and Use of Laboratory Animals*. A well-established model was used in which the mating combination CBA/J × DBA/2J represents the abortion-prone group (AP), and CBA/J × BALB/c represents the normal pregnancy controls (NP) [19]. Two-month-old

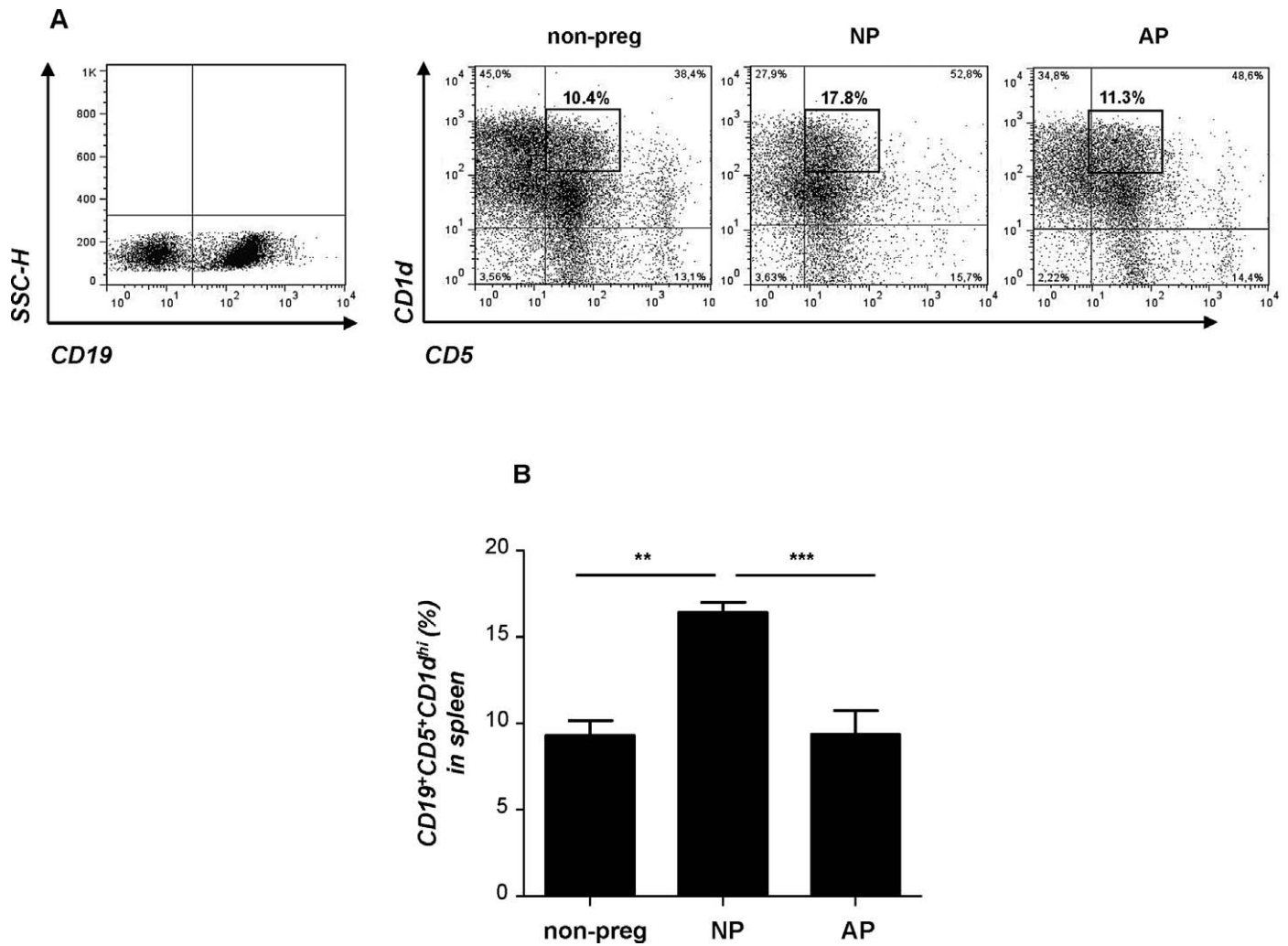


FIG. 1. Regulatory B10 cells are augmented in the spleen of NP but not AP mice. **A**) Representative dot-plots showing the percentage of CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>hi</sup> regulatory B10 cells in nonpregnant (non-preg), NP, and AP mice. **B**) Bar graph depicting the percentage of CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>hi</sup> regulatory B10 cells in non-preg, NP, and AP mice as analyzed by flow cytometry. Data are expressed as mean  $\pm$  SEM. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , as analyzed by one-way ANOVA, followed by Tukey multiple-comparison test.

CBA/J females were paired to BALB/c or DBA/2J males and separated from the males after the appearance of the vaginal plug indicating Day 0 of pregnancy. Pregnant females were euthanized on Day 14 of pregnancy, and the percentage of CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>hi</sup> IL-10-producing regulatory B10 cells was analyzed by flow cytometry.

For the experiments on the transfer of regulatory B10 cells, the following groups were made: group 1, BALB/c-mated CBA/J females (NP) that received treatment with PBS (i.v.; n = 7); group 2, DBA/2J-mated CBA/J females (AP) that received PBS treatment (i.v.; n = 8); group 3, AP females that received  $3 \times 10^5$  effector B cells (IL-10 negative; i.v.; n = 7); and group 4, AP females that received  $3 \times 10^5$  regulatory B10 cells (i.v.; n = 7). The effector and regulatory B10 cells were obtained from BALB/c-mated pregnant females (Day 14 of pregnancy). Cell transfer was performed intravenously on Day 0 of pregnancy. Animals were euthanized at Day 14 of pregnancy, the uteri were removed, and the implantation sites were documented. The abortion sites were identified by their smaller size and a necrotic, hemorrhagic appearance when compared to normal implantations. The percentage of abortions was calculated as the ratio of resorbed fetuses to total implantation sites [20].

### Isolation of IL-10-Producing Regulatory B10 Cells

For the isolation of mouse regulatory B10 cells, we used the commercially available Regulatory B Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). CD19<sup>+</sup> B cells were magnetically purified from the spleen of NP females and further stimulated in vitro with 10  $\mu$ g/ml of LPS (serotype O111:B4; Sigma Aldrich Chemie GmbH, Munich, Germany) for 24 h. During the last 5 h of culturing, phorbol 12-myristate 13-acetate (PMA; 50 ng/ml)

and ionomycin (iono; 500 ng/ml) were added. Regulatory B10 cells were magnetically sorted. IL-10-negative B cells (referred to as effector B cells) were also collected. The production of IL-10 by regulatory B10 cells was confirmed by intracellular staining of IL-10 (Fig. 1A).

### Dendritic Cell Generation and Coculture System

CD19<sup>+</sup> B cells were magnetically isolated from the spleen of NP or AP mice at Day 14 of pregnancy and further stimulated in vitro with LPS for 24 h. PMA (50 ng/ml) and iono (500 ng/ml) were added for the last 5 h. Supernatants were collected, frozen at  $-80^\circ\text{C}$ , and later used to culture bone marrow-derived dendritic cells.

Mouse bone marrow-derived dendritic cells (mBMDCs) were generated from mouse bones cultured for 7 days as described by Soldati et al. [21]. Bone marrow cells from the femur and tibia of CBA/J females were centrifuged at 1200 rpm for 5 min at room temperature (RT) and resuspended in DC medium ( $1.5 \times 10^6$  cells per milliliter). The medium consisted of Roswell Park Memorial Institute medium supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, 50  $\mu$ M  $\beta$ -mercaptoethanol (PAA Laboratories GmbH, Pasching, Austria), and 20 ng/ml granulocyte macrophage colony-stimulating factor (PeproTech GmbH, Hamburg, Germany). Cells were cultured for 7 days (10 ml/75 cm<sup>2</sup> flask; 37°C, 5% CO<sub>2</sub>), changing the medium at Days 3 and 5 of the culture. On Day 7, mBMDCs were resuspended in serum-free condition medium ( $0.3 \times 10^6$  cells per milliliter) generated from 24-h supernatants from CD19<sup>+</sup> B cells isolated either from AP or NP mice and stimulated with LPS + iono + PMA. Cells were cultured with or without LPS (100 ng/ml) to induce

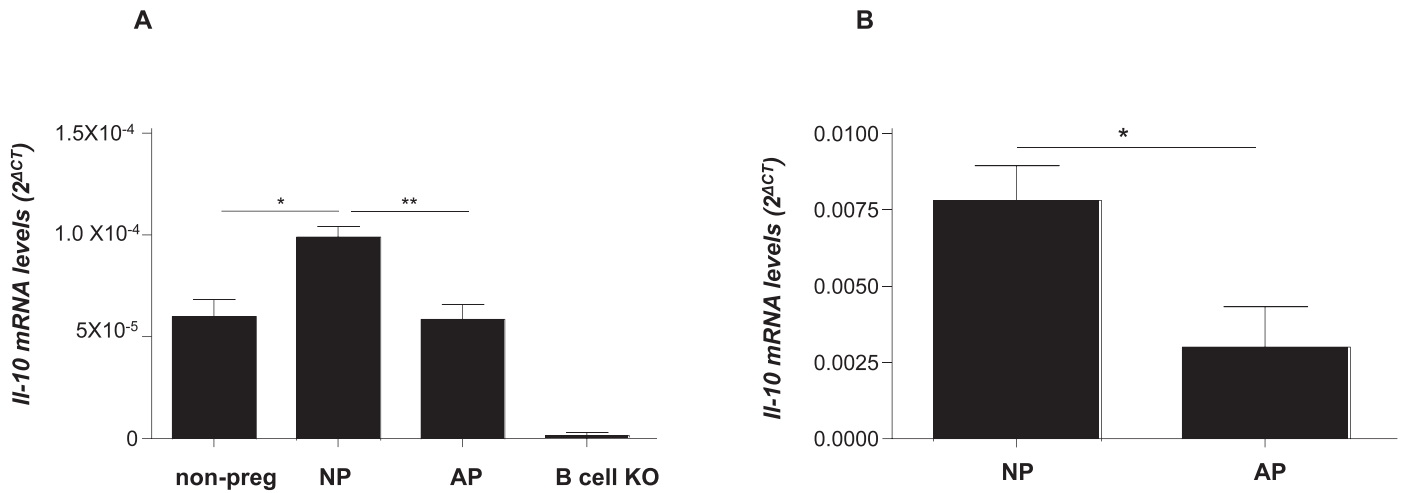


FIG. 2. IL-10 expression is augmented in normal pregnancies. **A)** IL-10 mRNA expression is augmented in the spleen of NP mice compared to that of AP and non-preg mice. As a further control, we analyzed the expression of IL-10 in the spleen of B cell knockout females (B cell KO). **B)** CD19<sup>+</sup> B cells were isolated either from NP or from AP mice and further stimulated in vitro with LPS + PMA + iono for 5 h. Stimulated CD19<sup>+</sup> B cells isolated from NP mice showed a significantly higher expression of IL-10 mRNA compared to B cells isolated from AP mice. Data are expressed as mean ± SEM. \**P* < 0.05, \*\**P* < 0.01, as analyzed by one-way ANOVA, followed by Tukey multiple-comparison test.

DC maturation. After 24 h, the expression of CD80 in CD11c<sup>+</sup> DCs was analyzed by flow cytometry.

*Flow Cytometry*

Mononuclear cells were isolated from the spleen and para-aortic lymph nodes. 1 × 10<sup>6</sup> cells were stained for extracellular markers for 30 min at 4°C with monoclonal antibodies against CD19 PE-Cy7 (1D3), CD5 APC (53-7.3), CD1d PE (1B1), CD11c APC (HL3), CD80 PE (B7-1), and CD4 FITC (RM4-4) or their specific isotype controls (Becton Dickinson GmbH, Heidelberg, Germany). For the intracellular staining of IL-10, cells were first fixed in paraformaldehyde (4% in PBS) and permeabilized with a 10% saponin solution. Cells were then stained with IL-10 PE antibody (Becton Dickinson GmbH). For intracellular Foxp3 staining, a commercially available solution was used for permeabilization (eBioscience, Frankfurt, Germany), and a PE-labeled anti-mouse Foxp3 (clone FJK-16s) antibody was applied following the instructions of the manufacturer (eBioscience). Data were acquired by the FACSCalibur flow cytometer (Becton Dickinson GmbH) and analyzed with FlowJo software (TreeStar, Inc., Ashland, Oregon).

*Tissue Collection and Processing*

Animals were euthanized by cervical dislocation at Day 14; spleen and para-aortic lymph nodes were removed and either frozen in liquid nitrogen and stored at -80°C for RNA extraction or freshly used for flow cytometry analysis.

*Real-Time RT-PCR*

Frozen spleen tissue (100 mg) was treated with 1 ml of TRIzol (Life Technologies GmbH, Darmstadt, Germany) and disaggregated with a homogenizer (T25 digital Ultra-Turrax; IKA-Werke GmbH & Co. KG, Staufen, Germany). Pellets from CD19<sup>+</sup> B cells were treated with TRIzol as well. Isolation of RNA, cDNA synthesis, and real-time PCR were performed as described in a previous publication [20]. RNA concentration and purity was evaluated spectrophotometrically. First-strand cDNA synthesis was carried out using 2 µg of RNA. All samples were amplified in duplicate. Nontemplate controls were included to check for potential reagent contamination. Primer pairs for IL-10 and β-actin were designed using Primer3Plus software (bioinformatics developer Dr. Andreas Untergasser, who used the initial Primer3 program [22] as platform) with the following parameters: primer dimer, self-priming formation, and primer melting temperature. All primer pairs were chosen to span an exon-intron boundary to exclude amplification of genomic DNA. Real-time PCR for murine *IL-10* was performed using SYBR Green PCR master mix (Life Technologies) in an iCycler (Bio-Rad Laboratories GmbH, Munich, Germany) for the detection of PCR products. β-actin amplification was included and used as a housekeeping gene. Forward/reverse primer sequences were as follows: for *IL-10* (GAA GAC CCT CAG

GAT GCG G/CCT GCT CCA CTG CCT TGC T); for β-actin (GCT TCT TTG CAG CTC CTT CGT T/GTT GTC GAC GAC CGC).

*Statistics*

Normality was assessed by Kolmogorov-Smirnov tests. Abortion rate data were not normally distributed and was, therefore, analyzed by the Kruskal-Wallis test followed by the Mann-Whitney *U*-test between two particular groups. For the rest of the data sets, ANOVA and Tukey multiple post *t*-test were applied to evaluate the differences of means of multiple groups. Significant differences between groups were indicated with asterisks as follows: \**P* < 0.05; \*\**P* < 0.01; and \*\*\**P* < 0.001.

**RESULTS**

*The Frequency of Regulatory B10 Cells Is Augmented in Normally Developing Pregnancies, but Not in Failing Murine Pregnancies*

To address the question of whether the frequency of regulatory B10 cells is relevant for pregnancy outcome, we took advantage of a very well-described model of immunological spontaneous abortion [19]. In this model, an abnormal maternal immune response leads to the rejection of some of the fetuses [20] in DBA/2J-mated CBA/J females, but not in BALB/c-mated CBA/J females presenting the latter normal pregnancies. We began by analyzing the frequency of CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>+</sup> regulatory B10 cells in the spleen of NP females at Day 14 of pregnancy as compared to virgin animals. The percentage of regulatory B10 cells was significantly higher than that observed in nonpregnant CBA/J females (Fig. 1, A and B). However, increased levels of B10 cells were not observed in pregnant AP females undergoing immunological abortions at Day 14 of pregnancy (Fig. 1, A and B). AP females did not experience an augmentation in CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>+</sup> regulatory B10 cells; the frequency of B10 cells in these animals was comparable with the ones observed in nonpregnant females (Fig. 1, A and B).

Next, we studied IL-10 levels in the spleen of nonpregnant, NP, and AP female mice. We observed an up-regulation of *IL-10* mRNA levels in the spleen of NP versus nonpregnant females, an augmentation that was not observed in AP animals (Fig. 2A). This goes along with the augmentation of CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>hi</sup> regulatory B10 cells in NP but not in AP, as analyzed by flow cytometry (Fig. 1). As controls, we

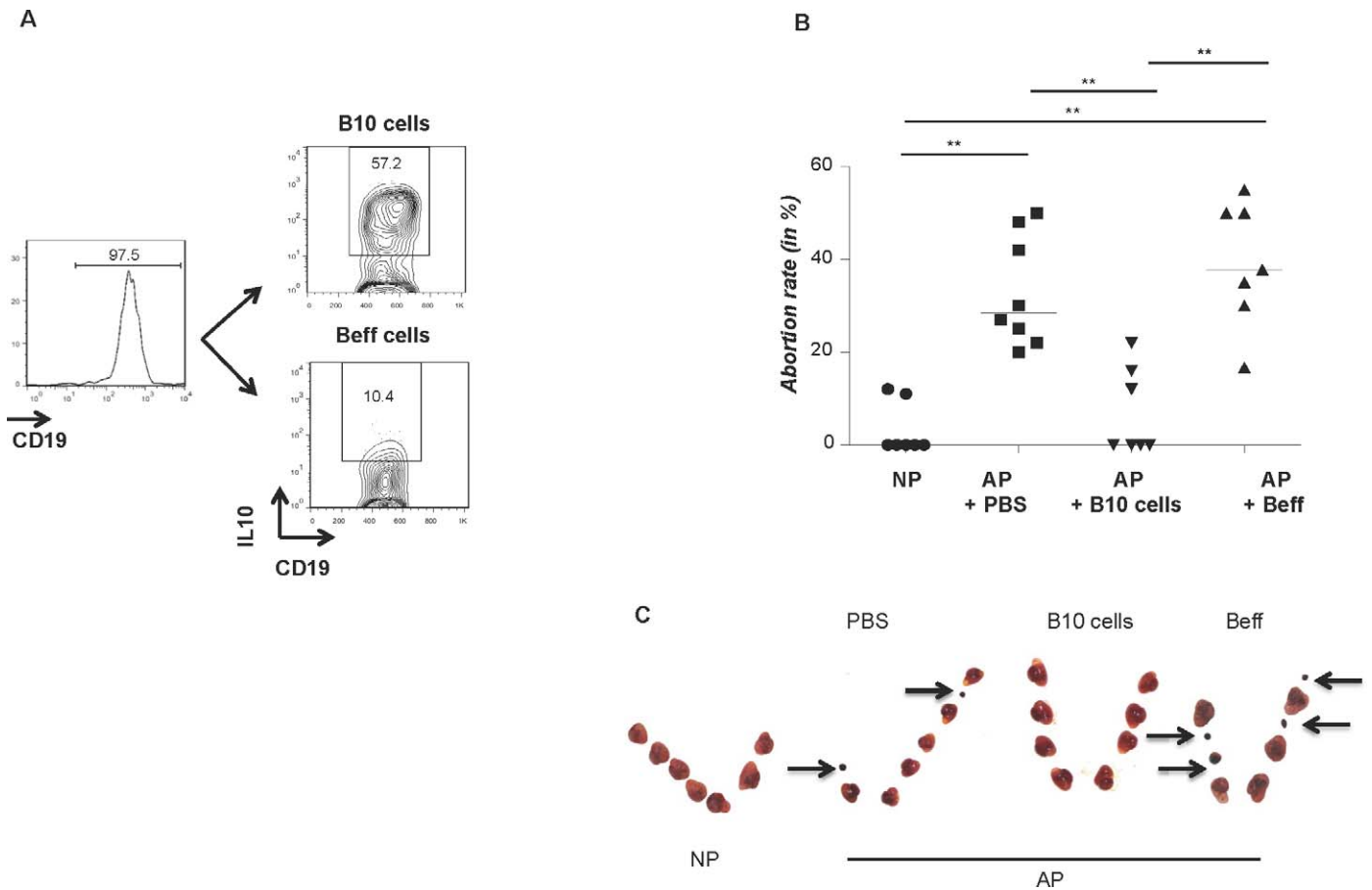


FIG. 3. Transfer of regulatory B10 cells prevents immunological abortions. **A)** Representative histogram and contour plots depicting the characterization of isolated regulatory B10 cells as well as B effector cells (Beff cells). **B)** Abortion rate of NP mice (median, 0%; min, 0; max, 15) and AP mice that received PBS (mean, 30%; min, 20; max, 50), regulatory B10 cells (mean, 0%; min, 0; max, 22), or B effector cells (mean, 37.8%; min, 30; max, 55). Data are shown as single dots with medians. **\*\*P** < 0.01, as analyzed by Kruskal-Wallis followed by Mann-Whitney *U*-test. **C)** Representative pictures showing the uterus of NP mice carrying healthy fetuses treated with PBS, as well as AP mice treated with PBS, regulatory B10 cells, or B effector cells (Beff). PBS-treated AP animals and AP animals that received Beff cells show both healthy and resorbed embryos, the latter highlighted by arrows. The control (NP) animal and the AP mouse treated with regulatory B10 cells presented no abortions.

used spleen samples from animals deficient in B cells (uMT animals [18]) that barely expressed *Il-10*. This further highlights that the main source of IL-10 in the spleen is the B cells.

We isolated CD19<sup>+</sup> B cells from NP or AP females, stimulated them in vitro with LPS + iono + PMA, and analyzed their *Il-10* mRNA expression by real-time PCR. We observed that B cells isolated from AP animals expressed significantly lower levels of *Il-10* mRNA than did B cells isolated from AP females (Fig. 2, A and B). Our data indicate that both the frequency of regulatory B10 cells and their capacity to produce *Il-10* mRNA are augmented in normal pregnant animals. We also confirmed that B cells from AP animals are impaired in their ability to produce IL-10.

#### Adoptive Transfer of Regulatory B10 Cells Prevents Immunological Abortions

Having demonstrated that regulatory B10 cells and IL-10 are augmented in NP but not in AP mice, we next tested the functional ability of regulatory B10 cells to prevent immunological rejection of the fetuses in AP females. For this, we transferred regulatory B10 cells isolated from NP females into AP animals at Day 0 of pregnancy. It is of note that regulatory B10 cells were isolated by their capacity to produce IL-10

(Regulatory B Cell Isolation Kit; Fig. 3A). As supported by Figure 3B, solely transferring  $5 \times 10^5$  regulatory B10 cells from NP females could completely prevent the abortive phenotype of AP animals. The abortion rate after the B10 cell transfer was comparable to the one observed in the PBS-treated NP animals, which had augmented levels of regulatory B10 cells and IL-10. As a further control, we transferred effector B cells (Beff), i.e., B cells that do not produce IL-10 (Fig. 3A). The transfer of Beff did not modify the elevated abortion rate of AP animals. Thus, only B cells that are capable of producing IL-10 can prevent abortion in this model. Our data clearly confirm that regulatory B10 cells but not Beff cells are important for immune tolerance during pregnancy.

#### Transfer of Regulatory B10 Cells into AP Mice Keeps DCs in an Immature State and Induces an Expansion of Regulatory T Cells

Next, we intended to gain some insight into the mechanisms triggered by the transfer of regulatory B10 cells into AP mice. We observed that the positive effect of B10 cells was associated with a significant diminution in the percentage of mature CD11c<sup>+</sup>CD80<sup>+</sup> DCs in the spleen of AP animals (Fig. 4A). This was not observed in AP animals to which PBS or Beff had been transferred (Fig. 4A). Thus, the transfer of

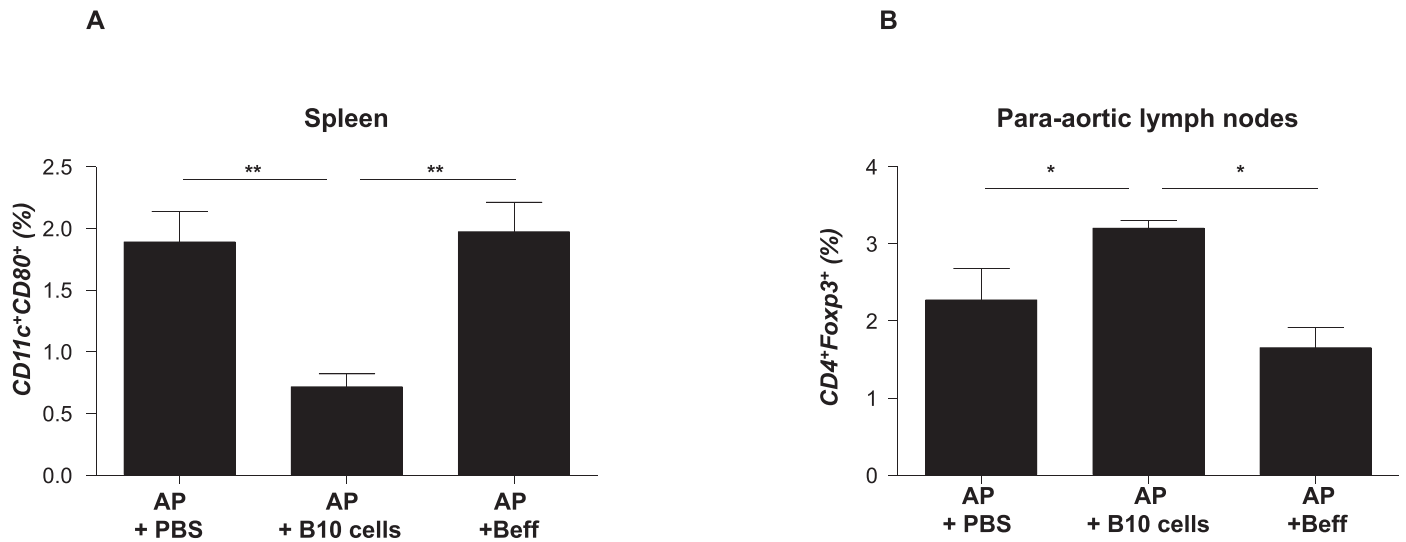


FIG. 4. Transfer of regulatory B10 cells keeps DCs in an immature state and induces an augmentation of Tregs. **A**) The percentage of mature CD11c<sup>+</sup>CD80<sup>+</sup> DCs was significantly reduced in the spleen of AP mice that received regulatory B10 cells as compared to PBS-treated AP or AP mice receiving B effector cells (Beff), as analyzed by flow cytometry. **B**) The transfer of regulatory B10 cells induced an augmentation in the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in the lymph node draining the uterus as compared to PBS-treated AP mice or animals receiving Beff. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , as analyzed by one-way ANOVA, followed by Tukey multiple-comparison test.

regulatory B10 cells is related to a pregnancy-supportive DC phenotype [23, 24] in the spleen. As regulatory B10 cells reside in the spleen, they may prime migratory DCs to stay in an immature state before leaving the organ. Because immature DCs were shown to be important in expanding regulatory T cells (Tregs) [24], we next studied the frequency of Tregs in the lymph nodes draining the uterus (para-aortic lymph nodes). AP mice treated with regulatory B10 cells showed elevated levels of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs as compared to control animals treated with PBS or Beff cells (Fig. 4B). Thus, the transfer of regulatory B10 cells into AP animals normalizes the abortion rate, and this is associated with DCs kept in an immature state and with an expansion of Tregs, both of importance for tolerance maintenance [24].

#### *IL-10 Produced by B Cells from NP Mice Inhibits the Maturation of DC In Vitro*

The in vivo transfer of regulatory B10 cells was related to a diminution in the percentage of CD11c<sup>+</sup>CD80<sup>+</sup> mature DC in the spleen of AP mice. Therefore, we aimed to confirm that the IL-10 produced by regulatory B10 cells keeps DCs in an immature, and thus pregnancy-supportive, state. To do so, we established an in vitro culture system consisting of DCs matured by LPS and treated with or without supernatant from activated B cells (LPS + iono + PMA). To confirm the participation of IL-10, an anti-IL-10 antibody was employed. As expected, when mBMDCs derived from CBA/J females were treated with LPS, an augmentation in the expression of CD80 on CD11c<sup>+</sup> DCs was observed (Fig. 5). The addition of supernatant from activated CD19<sup>+</sup> B cells of NP mice to the DC culture was enough to keep DCs in an immature state as compared to the LPS-treated cells. Remarkably, the inhibitory effect on maturation achieved by the addition of supernatant from B cells was abolished in the presence of anti-IL-10 antibody. Hence, the immature phenotype of DCs was driven by IL-10-produced by B cells (Fig. 5). Interestingly, the sole addition of supernatant from CD19<sup>+</sup> B cells isolated from AP mice (that secrete much less IL-10 than cells from NP mice, Fig. 2B) to mBMDCs was enough to induce an up-regulation

of CD80 on CD11c<sup>+</sup> DCs, even in the absence of LPS (Fig. 5). Neither the treatment with LPS nor the addition of anti-IL-10 antibody changed the maturation state of mBMDCs cultured with supernatant from CD19<sup>+</sup> B cells isolated from AP mice (Fig. 5). Hence, our in vitro data clearly demonstrate that IL-10 produced by regulatory B10 cells during normal pregnancy is fundamental for retaining DCs in an immature state.

## DISCUSSION

In this manuscript, we introduce regulatory B10 cells as important players in the immune balance needed for the survival of the fetus within the uterus of an immune-competent mother. By using a mouse model, we confirmed the expansion of regulatory B10 cells in normal but not in failing pregnancies. The adoptive transfer of regulatory B10 cells could prevent fetal rejection in otherwise abortion-prone females. This confirms the functional importance of these cells. The present work highlights a novel pathway by which the immune system contributes to pregnancy tolerance toward the fetus.

The plasticity of B cells has been recently uncovered. B cells have emerged as pleiotropic cells with diverse functions beyond antigen presentation and antibody production [8–10]. B cells have arisen as modulators of the adaptive immune response because of their capacity to secrete cytokines. A new subpopulation of B cells, the regulatory B10 cells, has been described as having multiple effects on health and disease [16]. Because of their ability to produce IL-10, regulatory B10 cells were also suggested to play a critical role in the regulation of the alloimmune response in transplantation tolerance. Renal transplant patients who did not require immunosuppressive therapy showed higher levels of regulatory B10 cells when compared to patients who required immunosuppressive therapy for a stable graft function [25]. We are not aware of any reports on the supposed function of regulatory B10 cells in pregnancy tolerance; it is, however, known that the total numbers of CD19<sup>+</sup> B cells remain unchanged during pregnancy [26, 27].

In addition to cellular markers, regulatory B10 cells are defined by their ability to secrete IL-10 upon activation. The in vivo role of B cell-derived IL-10 was first demonstrated in a



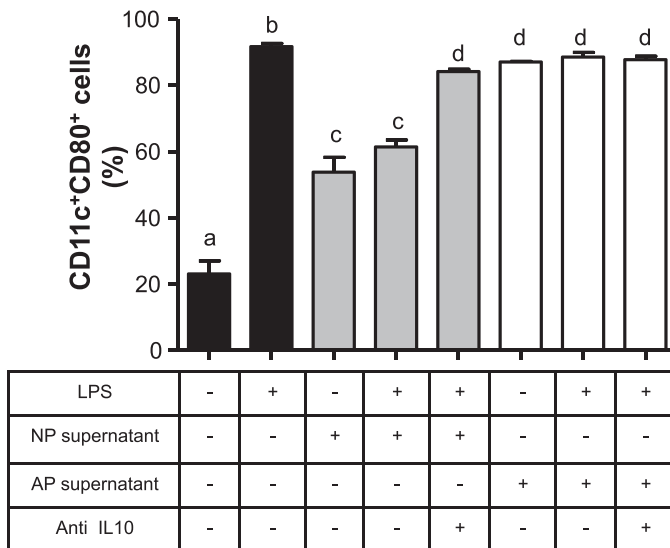


FIG. 5. Supernatant from CD19<sup>+</sup> cells of NP animals hinders DC maturation. Mouse BMDCs were generated from NP CBA/J females and further cultured with supernatant obtained from in vitro-stimulated CD19<sup>+</sup> B cells isolated from either NP or AP mice. Mouse BMDC maturation was induced with LPS. As a maturation indicator, we analyzed the expression of CD80 by flow cytometry. As shown in the graph, the addition of conditional supernatant from activated B cells isolated from NP mice significantly reduced the expression of CD80 in pure CD11c<sup>+</sup>, LPS-treated mBMDCs as compared to LPS-treated mBMDCs cultured in medium or with conditional supernatant from activated B cells isolated from AP mice. Notably, the addition of an anti-IL-10 antibody completely blocked the inhibitory effect on DC maturation. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA, followed by Tukey multiple-comparison test. All (c) are comparable, all (d) are comparable: a < b, a < c, a < d, b > c, b and d are comparable, and c < d. In all cases, P < 0.001.

murine model of experimental autoimmune encephalomyelitis, in which the missing IL-10 production by B cells was proposed to cause the disease [13]. The protective role of IL-10 produced by B cells was confirmed later in several human autoimmune diseases, including ulcerative colitis, lupus, and arthritis [16]. To characterize the participation of regulatory B10 cells in the process of pregnancy tolerance of the fetus, we employed a model of pregnancy loss the involved a deregulated immune response to paternal antigens [20]. With this model, we analyzed pregnancy outcome at Day 14 by determining whether the animals presented healthy fetuses or fetuses that were resorbed because they died in utero. Mice developing normal pregnancies showed an augmentation in the percentage of CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>high</sup> IL-10-producing regulatory B10 cells in the spleen as compared to NP and AP females. The transfer of regulatory B10 cells into AP animals rescued fetuses from immune rejection. Similar to the application of IL-10 [3], regulatory B10 cells were able to restore tolerance of the fetus. This supports the idea that IL-10 is an important factor in preventing pregnancy failures. We did not analyze pup viability or postnatal effects, as the main aim of this work was to study the ability of regulatory B10 cells to prevent abortions. The fact that B cells, unlike other immune cells, are barely present at the fetal-maternal unit suggests that their salutary effect is most likely systemic. Similarly, the transfer of regulatory B10 cells could successfully inhibit the onset of several diseases or ameliorate diseased organisms caused by excessive inflammation without the cells migrating to the site of inflammation [8, 14, 15, 28]. The expression of the IL-10 receptor (IL-10R) is heterogeneous [29]. DCs have the greatest

expression of IL-10R among immune cells [28], rendering them sensitive to the regulatory effects of IL-10.

Consistently, it has been demonstrated that IL-10 produced by regulatory B10 cells maintains DCs in an immature state, inhibiting their capacity to present antigens to T cells and consequently activate them [28]. We observed that in the spleen, where regulatory B10 cells reside, DCs are rather immature after the transfer of regulatory B10 cells. Thus, IL-10 secreted by regulatory B10 cells is likely responsible for keeping circulating DCs in an immature state. Besides, it is well known that immature DCs characterized by low expression levels of co-stimulatory molecules (e.g., CD80, as observed here) are efficient inducers of Tregs upon antigen presentation [30]. We also observed that pregnancy-induced tolerogenic DCs are able to induce the expansion of Tregs [24, 31]. Here, we observed an augmented frequency of Tregs in the para-aortic lymph nodes in those animals transferred with regulatory B10 cells. This reinforces the hypothesis of regulatory B10 cells priming DCs into an immature phenotype and expanding Tregs.

In conclusion, we introduce new cellular players in the immune balance during pregnancy. We show that B10 cells are important for tolerance acquisition. B10 cell-derived IL-10 maintains DCs in an immature state and supports Treg expansion. The transfer of B10 cells can normalize abortion rates in a model of disturbed tolerance. This novel concept warrants further investigation in other experimental models, with the final aim to create strategies for restoring immune balance in patients with spontaneous abortions due to an incomplete immune tolerance.

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