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Human pregnancy levels of estrogen and progesterone contribute to humoral immunity by activating T_{FH}/B cell axis

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Circulating T_{FH} (cT_{FH}) cells express CXCR5, PD-1, and, when activated, ICOS, and release IL-21. According to the production of IFN-γ, IL-4, and IL-17 and expression of FoxP3, these cells are also classified as $cT_{FH}1$, $cT_{FH}2$, $cT_{FH}17$, and cT_{FR} cells, respectively. This $CD4^+$T-cell subset is pivotal to efficient humoral immunity, and pregnancy appears to favor IgG production. Here, not only pregnancy amplified the in vivo production of anti-HBsAg IgG in HBV immunized women, but the frequency of cT_{FH} cells was directly correlated with estradiol levels. In vitro, pregnancy-related dose of 17-β-estradiol (E2) directly increased the percentage of different cT_{FH} subsets. While E2 and progesterone (P4) increased the proportion of differentiated T_{FH} cells derived from naïve $CD4^+$T-cells, only E2 amplified the release of IL-21 in those cell cultures. In addition, E2 and P4 increased the proportion of memory B cells and plasma cells, respectively. In SEB-activated B/T_{FH} cell co-cultures, E2, in the presence of P4, increased the production of total IgG. Finally, among the hormones, P4 was stronger in upregulating the percentage of IL-10^+T_{FR} cells. Collectively, our findings suggested that E2 and P4 cooperate in the humoral immune response by favoring the expansion of different cT_{FH} and B cell subsets.

Keywords: anti-HBsAg IgG · B cells · estrogen · progesterone · T_{FH} cells

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Human follicular helper T (T_{FH}) cells represent a distinct subset of $CD4^+$ T cells found in secondary lymphoid organs. They are identified by a high expression of the transcription factor B cell lymphoma-6 (Bcl-6), programmed cell death receptor-1 (PD-1), inducible T-cell co-stimulator (ICOS), and chemokine receptor CXCR5, and associated with the production of their

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signature cytokine, the IL-21 [1–4]. The interaction of T_{FH} with B cells is facilitated by the production of CXCR5 ligand, the CXCL13 chemokine, by follicular cells associated with reduced expression of CCR7 [5]. In addition to ICOS/ICOSL, at the T_{FH}/B cell level, positive signals are also triggered through CD154/CD40 and IL-21/IL-21R interactions [6–8]. Furthermore, T_{FH} cell-derived IL-4 promotes B cell survival through inhibition of cell apoptosis [9–11], and IL-10 and IL-6 that promote plasma cell differentiation [12–14] contribute to GC reaction. Functionally, mature T_{FH} cells upregulate humoral immunity by supporting proliferation, survival, affinity maturation, and differentiation of B lymphocytes into antibody-producing plasma cells and long-lived memory B cells [1–4]. Additionally, GC-T_{FH} cells are important for the process of heavy chain class switching of antibodies [1–4]. Another GC-T_{FH} cell subset, called natural follicular regulatory T (T_{FR}) cells ($CXCR5^+Bcl\text{-}6^+FoxP3^+CD4^+$), has been implicated in tolerance and regulate the production of autoantibodies and autoantibodies-mediated autoimmune disease [15,16].

Circulating T_{FH} (cT_{FH}) and T_{FR} (cT_{FR}) cells have been identified among the pool of memory $Bcl\text{-}6^-$ $CD4^+$ T cells [17,18]. According to the expression of CXCR3 and CCR6 markers, cT_{FH} cells are classified as $cT_{FH}1$ ($CXCR5^+CXCR3^+CCR6^-$), $cT_{FH}2$ ($CXCR5^+CXCR3^-CCR6^-$), and $cT_{FH}17$ ($CXCR5^+CXCR3^-CCR6^+$), all of them able to efficiently induce antibody response by memory B cells [19]. In addition to IL-21, these different cT_{FH} subsets can also produce, albeit in lower amounts, IL-4 ($cT_{FH}2$), IFN-γ ($cT_{FH}1$), and IL-17 ($cT_{FH}17$) [20,21]. The expression of PD-1 and ICOS (<1% of cT_{FH} cells) identifies a more efficient cT_{FH} cell subset [17,18]. cT_{FR} cells are identified by intracellular expression of FoxP3 [22,23]. Several investigators have reported a positive association between the percentage of different subtypes of cT_{FH} cells and the production of neutralizing IgG against HIV and influenza [18,24–26]. Recently, we have shown that pregnancy favors the expansion of cT_{FH} cells that was directly associated with increased plasma anti-HBsAg IgG titers following HBV immunization in healthy [27] and HIV-1-infected pregnant women [28]. Furthermore, there was a positive correlation with the plasma levels of estrogen (E2) but not with progesterone levels (P4) [27,28].

E2 and P4 are synthesized by the ovarian follicles through the combined functions of the granulosa cells and the theca cells. In physiological concentrations, endogenous E2/ERα signaling is necessary for optimal T cells response both directly, by upregulating the expression of some co-stimulatory molecules (CD40 and CD86) and cytokines (IL-6, IL-12, and IL-23) on DC cells, and directly through amplifying TCR-stimulated T cell activation [29–32]. On the other hand, the effects of E2 and P4 on immune cells during pregnancy are mainly suppressive. During pregnancy, the E2 and P4 levels increase steadily until just prior to delivery. Classically, these hormones are pivotal to support reproductive activity of the female including the development of the ovum, development and maintenance of the corpus luteum, and maintenance of pregnancy, which depends on their effects on maternal immune cells. High levels of both hormones are pivotal to fetal tolerance through inhibition of the potentially embryotoxic maternal $CD4^+$ and $CD8^+$ T cells, as well as Th1 and Th17 subsets [33,34]. This phenomenon depends on the functional expression of intracellular receptors for E2 isoforms (ERα and ERβ) and P4 (PR and glucocorticoid [GR]) on immune cells able to regulate the transcription of different genes [35–39]. In addition to inhibiting the expression of pro-inflammatory cytokines [37–39], E2 and P4 increased the number and functional status of $FoxP3^+$ and $FoxP3^-$ regulatory T cells [37–41], thus helping to prevent fetus rejection. On the other hand, pregnancy appears to favor humoral response [42,43]. Studies have suggested that this event is due to the capacity of E2, via ERα, to promote B cell differentiation and Ig production [44–47]. In the context of normal pregnancy, this biological phenomenon should help to increase fetal protection against different pathogens from maternal IgG placental transfer.[48] We have previously reported [27,28] a positive correlation between plasma E2 levels with cT_{FH} cell subsets in pregnant women. In the present study, we not only demonstrated that pregnancy amplifies the production of anti-HBV IgG following HBsAg immunization, but we also demonstrated, for the first time, the ability of 17β-estradiol (E2) in directly favoring the expansion of T_{FH} cell subsets and IgG production. Furthermore, this hormone amplified in vitro T_{FH} differentiation and IL-21 production from circulating naïve $CD4^+$ T-cells. Finally, both E2 and P4 upregulated the proportions of T_{FR} cells, which could help to prevent autoreactive B cell activation.

Results

Pregnancy-related levels of estradiol correlated with circulating T_{FH} in women immunized against HBV

Our previous study demonstrated that pregnancy favors the expansion of circulating T_{FH} (cT_{FH}) cells [27]. Here, taking into account the gate strategy shown in the Fig. 1A, no difference was observed regarding the percentage of peripheral $CXCR5^+CD4^+$ T cells, expressing or not PD-1 (Fig. 1B) and IL-21 (Fig. 1C), between non-pregnant women (nPW) and pregnant women (PW) in the first 5 weeks of pregnancy, just before receiving HBV vaccination (t0). Nevertheless, 6 months after immunization (t1), the frequency of those cells, mainly the $IL\text{-}21^+PD\text{-}1^+$ subset, was significantly higher in the peripheral blood of PW (Fig. 1B and C). As expected, among women, mean plasma levels of estradiol (nPw [78.50 ± 28 pg/mL] and PW in the third trimester [5.761 ± 1.133 pg/mL]) and progesterone (nPw [4.1 ± 1.8 ng/mL] and PW in the third trimester [57.5 ± 14.9 ng/mL]) were higher in the pregnant group. Moreover, the in vivo estradiol levels were positively correlated with the frequency of $IL\text{-}21^+PD\text{-}1^+CXCR5^+CD4^+$T cells only in PW in the third trimester (Fig. 1E). No relationship was observed between progesterone concentrations and this cell subset in the peripheral blood of nPW (Fig. 1D) and PW (Fig. 1E). With regard to HBV immunization, only one nPW did not reach 10 UI/mL (7 UI/mL) of anti-HBsAg IgG, and, as observed in the Fig. 1F, pregnancy clearly amplified the in vivo production of these neutralizing antibodies following immunization.

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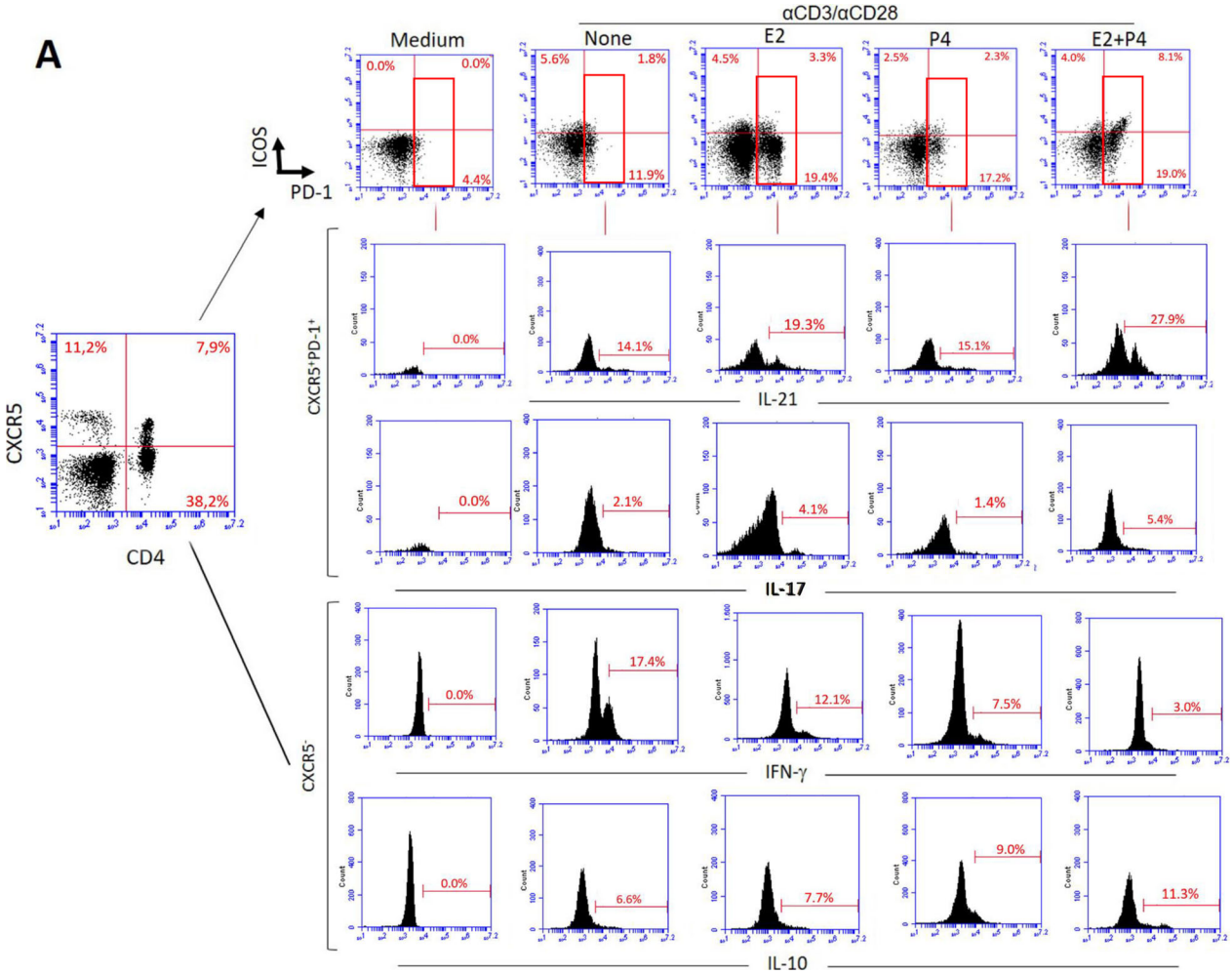


Figure 1. The impact of pregnancy on the frequency of cT_{FH} cells and the anti-HBsAg IgG in women immunized against HBV. PBMC cultures (1×10^6/mL) obtained from nPW (n = 10) and PW (n = 10) just before (t0) and after (t1) anti-HBV vaccine were maintained for 4 h in the presence of PMA (20 ng/mL) plus Ionomycin (600 ng/mL). In (A), representative flow cytometry dot-plots and histograms of cytokine-producing $CXCR5^+CD4^+$ T cells positives for PD-1 and IL-21. In (B) and (C), the mean (± SD) percentage of $CXCR5^+CD4^+$T cells, expressing or not PD-1, and $IL\text{-}21^+PD\text{-}1^+CXCR5^+CD4^+$T cells, respectively. The data are shown as mean ± SD from five independent experiments with four samples for experiment. Significance was calculated by comparing nPW ($n = 10$) versus PW ($n = 10$), and the p-values, obtained by using unpaired Student's t-test, are presented in the figures. In (D) and (E), the correlation of Pearson was applied to evaluate the relationship between the proportion of $IL\text{-}21^+PD\text{-}1^+CXCR5^+CD4^+$T cells and the plasma levels of estradiol and progesterone, respectively. In (F), the plasma titers of anti-HBsAg IgG in nPW and PW just before and after immunization against HBV. The mean ± SD of 10 nPW and 10 PW plasma samples were compared by using unpaired Student's test and the significant p-values shown in the figure.

17-β-Estradiol and progesterone modulate differently non-cT_{FH} and cT_{FH} cell subsets

The co-expression of CXCR5 and PD-1 is used to identify cT_{FH} cells [1–4]. Following the gate strategies shown in Fig. 2A, we observed that 17-β-estradiol (E2, 5 ng/mL), but not progesterone (P4, 50 ng/mL), increased the percentage of T_{FH} cells among $CD4^+$ T cells in PBMC cultures activated via TCR/CD28 (Fig. 2B). In addition, E2 increased the frequency of ICOS+ cells (Fig. 2C). A similar but less impressive effect was observed in cultures treated with 1 ng/mL of E2 (data not shown).

With regard to the cytokine profile, E2 but not P4, increased the proportion of cT_{FH} cells positive for IL-21 and to a lesser extent IL-17, with no change in frequency of $IL\text{-}4^+$ or $IFN\text{-}\gamma^+$ cell subsets (Fig. 2D). Concerning the role of both hormones on cytokine-producing non-cT_{FH} cells, pregnancy-related doses of P4 but not E2, significantly reduced the percentage of $CXCR5^-CD4^+$ T-cells capable of producing IL-4 and IL-17 (Fig. 2E). The frequency of $IL\text{-}21^+$ non-cT_{FH} cells was low and was not affected by either hormone. Both E2 and P4 reduced the proportion of the $IFN\text{-}\gamma^+$ subset (Fig. 2E). On the other hand, P4 elevated the frequency of IL-10-producing non-T_{FH} cells mainly in the presence of E2 (Fig. 2E).

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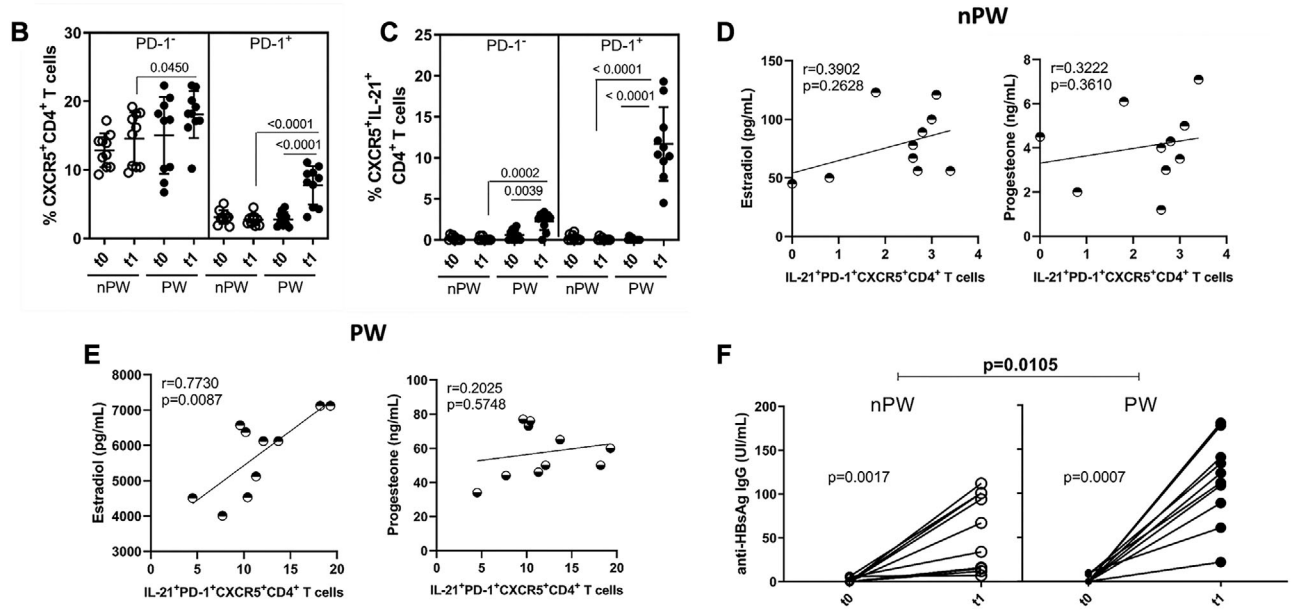


Figure 1. *Continued*

Neither E2 nor P4 altered the proportion of T_{FR} cells ($CXCR5^+FoxP3^+CD4^+$ T-cells) (Fig. 3A; Supporting Information Fig. S1). However, E2 and mainly P4, significantly upregulated the percentage of IL-10^+ T_{FR} cells (Fig. 3B; Supporting Information Fig. S1). E2 also significantly increased the frequency of IL-10-secreting $FoxP3^-CXCR5^+T_{FH}$ cells (Fig. 3C; Supporting Information Fig. S1); this effect was potentiated by the addition of P4. No changes were observed in cell cultures maintained in the presence E2 and/or P4 alone (data not shown).

17-β-Estradiol and progesterone elevated the in vitro frequency of differentiated T_{FH} cells

In order to determine the ability of both hormones in modulating the in vitro differentiation of T_{FH} cells, naïve $CD4^+$ T-cells were activated for 5 days in the presence of conditioning cultures containing a combination of recombinant human cytokines IL-12, IL-23, and TGF-β [49] with or without E2 and/or P4 [50]. As shown in Fig. 4A and B, the addition of E2 and P4 elevated the proportion of cT_{FH} cells. By contrast, only E2 amplified the release of IL-21 (Fig. 4C). Neither hormone had any significant effect on the percentages of differentiated $T_{FH}1$ ($CXCR3^+CCR6^-$), $T_{FH}2$ ($CXCR3^-CCR6^-$), and $T_{FH}17$ ($CXCR3^-CCR6^+$; Supporting Information Fig. S2).

The effects of hormones on B cell subsets and in vitro IL-21 and IgG production

It is known that E2 and P4 can modulate B cell activation [51,52] and plasma cell generation [53]. In the present study, following the strategy shown in Fig. 5A, the addition of E2 plus P4 led to a reduction in the percentage of naïve B cells ($CD19^+IgD^+CD27^-$) in PBMC cultures activated with SEB (Fig. 5B). The memory B cell subset ($CD19^+IgD^-CD27^+$) was upregulated by E2, but not P4 (Fig. 5C). P4 significantly reduced the proportion of plasmablasts (Fig. 5D), and by contrast, increased the frequency of plasma cells in SEB-activated PBMC cultures (Fig. 5E).

As T_{FH} cells help B cells to generate antibody-producing plasma cells [1–4], our objective was to evaluate the ability of those hormones in modulating the in vitro production of IgG in T_{FH}/B cell co-cultures activated with SEB. E2, in the presence of P4, increased the production of total IgG (Fig. 6A). Furthermore, E2 also increased the production of IL-21 (Fig. 6B).

Discussion

Both E2 and P4 alter the function of many body systems including the immune system. While P4 exerts essentially immunosuppressive actions, immune effects of E2 are concentration- and context-dependent [54–57]. In physiological concentrations, E2, via ERα, amplified many immune events, such as TCR-stimulated T-cell activation and expression of MHCII [29,30], expression of CD40 and CD86 [31] on human myeloid DCs, as well as DC-derived IL-6, IL-23, and IL-12 production in response to TLR ligands [32]. Nonetheless, like P4, pregnancy-related doses of E2 downregulate some effector T cell-dependent inflammatory processes [58,59]. Pregnancy favors humoral responses [40–43], and the findings obtained in the present study suggest that this phenomenon is mainly related to the property of E2 to modulate the proportion of different circulating T_{FH} (cT_{FH}) cell subsets.

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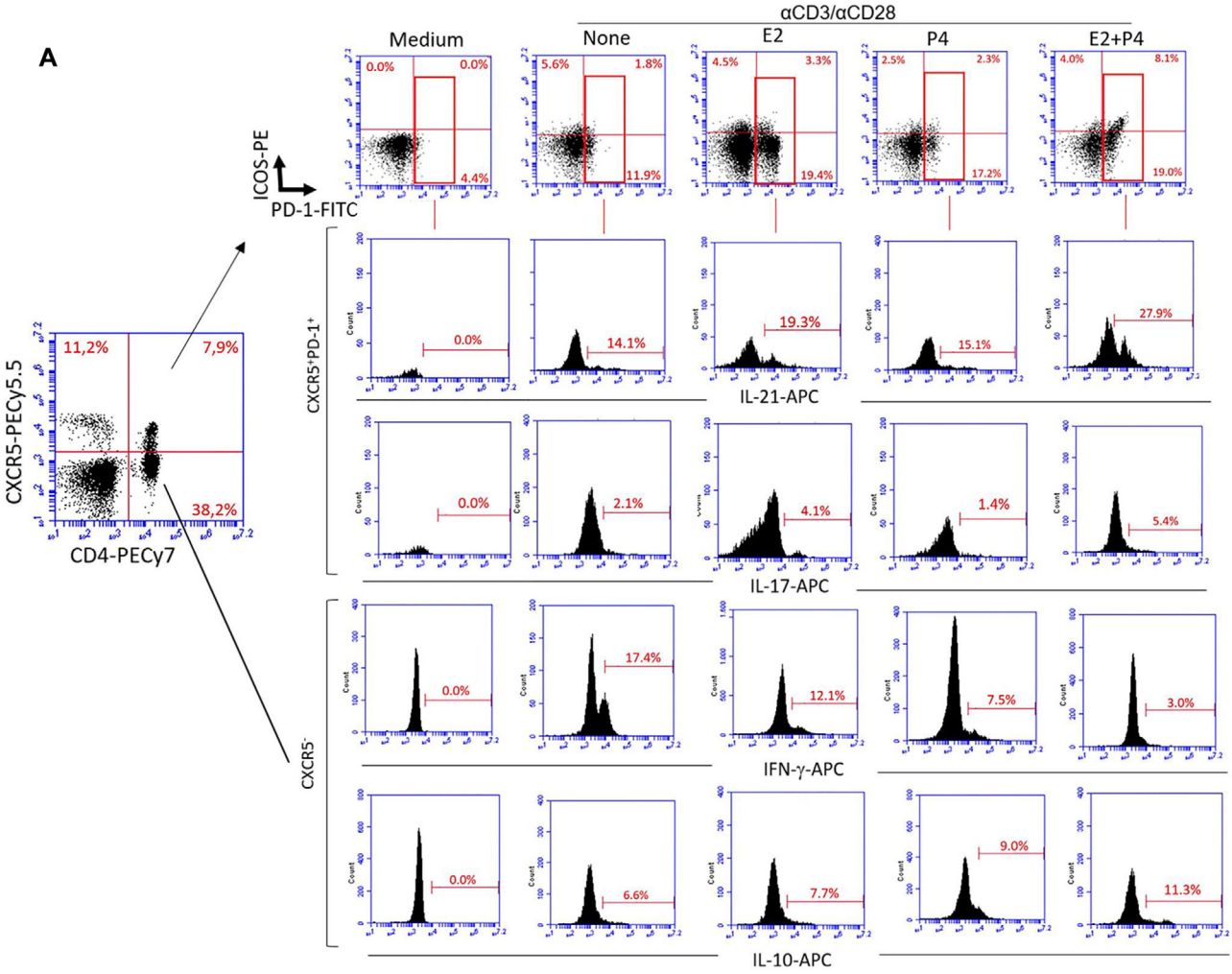


Figure 2. The effect of E2 and P4 on non-T_{FH} and T_{FH} cells. PBMC cells (1×10^6/mL) from healthy women ($n = 20$) were stimulated with anti-CD3/anti-CD28 beads (10 μL/mL) in the presence or absence of 17β-estradiol (E2, 5 ng/mL) and/or progesterone (P4, 50 ng/mL) every 3 days. In (A), representative flow cytometry dot-plots and histograms of cytokine-producing $CXCR5^-CD4^+$ T (non-T_{FH}) and different cT_{FH} (total $CXCR5^+PD\text{-}1^+$ and $ICOS^+CXCR5^+PD\text{-}1^+$ subset) cells. In (B) and (C), the mean percentage of the total cT_{FH} and $ICOS^+$ cT_{FH} cell subset, respectively. In (D), the mean percentage of different cT_{FH} cells, and (E) non-T_{FH} cells able, to produce IL-21, IL-4, IL-17, and IFN-γ after the addition of PMA and Ionomycin 4 h before finishing culture time. Data are shown as mean ± SD of five independent experiments with four samples per experiment. Significance was calculated by comparing different cell cultures conditions (medium, E2, P4, and E2 plus P4; two-away ANOVA). The *p*-values are indicated in the figure.

Taking into account the co-expression of CXCR5, PD-1 markers, we observed a direct correlation between the plasma estradiol levels, but not progesterone, and the frequency of IL-21-producing cT_{FH} cells in PW in the third trimester. Further, after completing the hepatitis B immunization schedule, the plasma titers of anti-HBsAg IgG were also higher in PW as compared with nPW. Although we have not analyzed the frequency of cells in the second semester of pregnancy, these findings, along with other studies [27,28,40–43], suggest that pregnancy-related levels of estradiol should improve the cT_{FH} cell function. Indeed, here, E2, but not P4, directly increased the frequency of both cT_{FH} ($PD1^+CXCR5^+$) and $ICOS^+T_{FH}$ cell subsets in PBMC cultures containing polyclonally activated T cells. The expression of ICOS on activated T cells, and signaling via ICOS–ICOSL interactions are critical for T_{FH} cell differentiation and GC formation [60]. The study by Rider et al. [61] revealed the ability of E2 to increase CD40L (CD154) expression on $CD4^+$ T cells. It is possible that in addition to upregulating ICOS, E2 also increases CD40 ligand (CD154) expression on T_{FH}. In addition to ICOS and CD154, some cytokines, particularly IL-21 released by T_{FH} cells, have also been implicated in B cell activation and antibody class switching [62,63]. Along with IL-21, small amounts of IL-4, IL-17, and IFN-γ can also be produced by $cT_{FH}2$, $cT_{FH}17$, and $cT_{FH}1$ cell subsets, respectively [64]. In agreement with published literature [5,65] regarding cytokine profiles, most T_{FH} cells from healthy women were positive for IL-21, and E2 but not P4, increased the proportion of IL-21 and IL-17 producing cells without any changes in the percentage of $cT_{FH}1$ or $cT_{FH}2$ cells.

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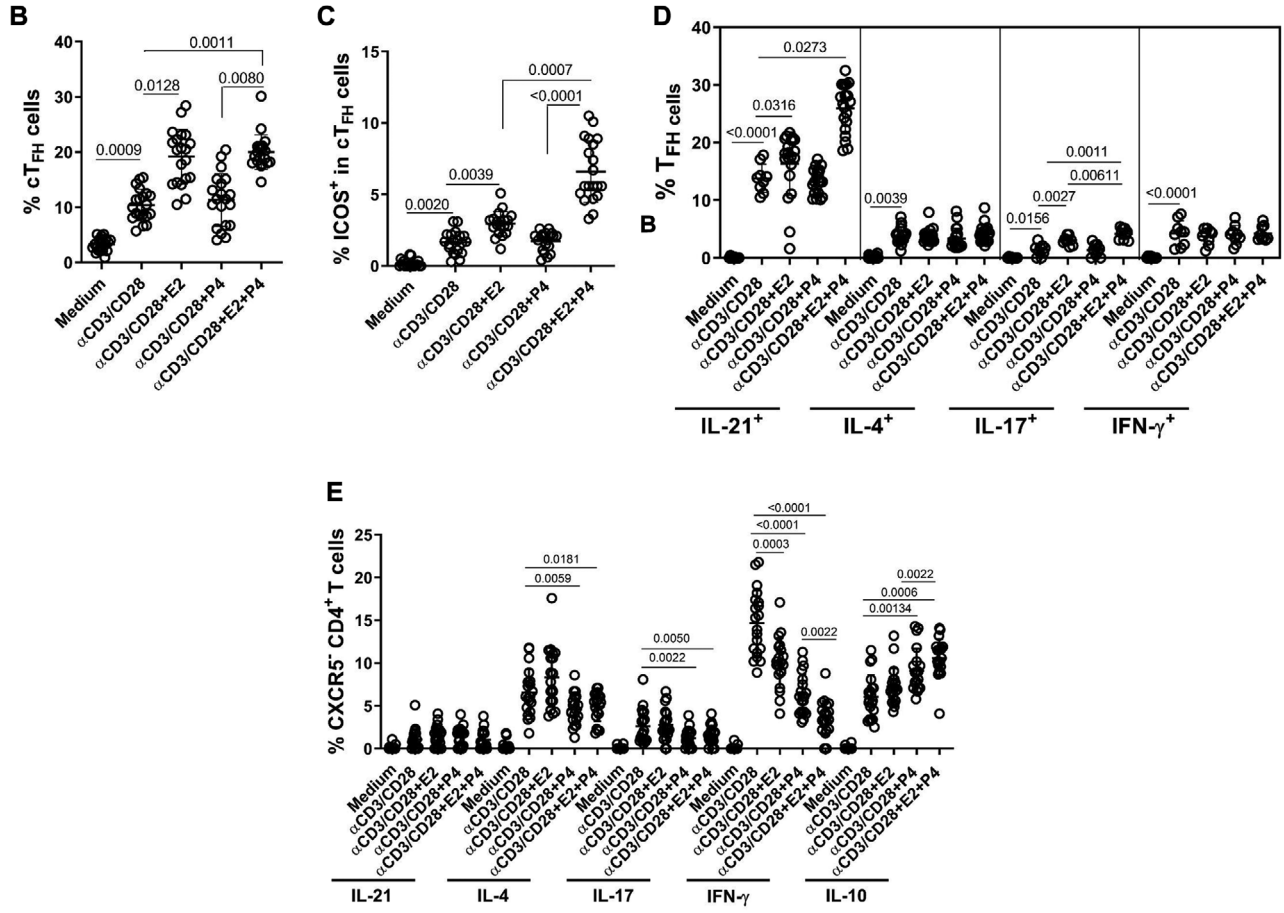


Figure 2. *Continued*

In comparison with cT$_{FH}$1 cells, cT$_{FH}$2 and cT$_{FH}$17 phenotypes are known to provide efficient help to naïve B cells [19]. It is known that IL-4$^+$IL-21$^+$CXCR5$^+$CD4$^+$ T-cells positively correlated with total IgE in the blood of allergic patients [66,67], and the absence of E2 effect on this phenotype in the present study is related to exclusion criteria applied to our cohort, whereby none of the women recruited for the study suffered from any allergic disease. However, the inability of E2 to modulate the in vitro cT$_{FH}$1 cell subset may also be associated with lower IFN-γ production during pregnancy. Indeed, high E2 and P4 levels reduced the frequency of CXCR5$^-$ Th1-like cells. Moreover, P4 but not E2 decreased the proportion of non-T$_{FH}$ cells positive for IL-21, IL-4, and IL-17. These results suggested that E2 and P4 have different immunomodulatory effects in non-T$_{FH}$ and T$_{FH}$ cells. Lower IFN-γ production by Th1-like cells should be pivotal to preventing fetus rejection by embryotoxic maternal effector CD4$^+$ T and CD8$^+$ T cells [29,30], but this phenomenon should contribute to known maternal susceptibility to infection by intracellular pathogens [68–71]. Finally, the ability of pregnancy-related doses of E2 and P4 in controlling IFN-γ production can also explain why pregnancy is typically a stabilizing period in the clinical course of MS [72,73], a demyelinating autoimmune disease of the CNS. During the third trimester of pregnancy, the MS relapse rate can be 70% lower when compared with the period prior to pregnancy [74].

In addition to reducing pro-inflammatory cytokines, the highest frequency of IL-10$^+$non-T$_{FH}$ cells was seen in cell cultures stimulated in the presence of E2 and P4. Although we did not analyze FoxP3 expression, these observations are in agreement with other studies that demonstrate the ability of pregnancy-related doses of E2 and P4 to favor the expansion of IL-10$^+$CD25$^+$FoxP3$^+$ CD4$^+$ Tregs [34–37]. E2 and P4 modulated the proportion of different subtypes of IL-10-secreting T$_{FH}$ cells. While E2 and P4 increased the proportion of the IL-10$^+$T$_{FR}$ phenotype, identified by additional expression of FoxP3, only E2 increased the percentage of IL-10$^+$FoxP3$^-$T$_{FH}$ cells. It is known that T$_{FR}$ cells are particularly important in the suppression of autoreactive B cell responses [75]. However, FoxP3$^-$IL-10$^+$ T$_{FH}$ cell subset can promote GC response [19,76,77]. Therefore, during normal pregnancy it is possible that cT$_{FR}$ expansion controls production of autoantibodies rather than reducing maternal Ig production against different pathogens. In fact, a direct correlation between plasma estrogen levels with both frequencies of IL-21-secreting T$_{FH}$ cells and in vivo anti-HBsAg IgG production following HBV immunization was observed in both healthy and HIV-1-infected pregnant women [27,28]. In the present study, E2, mainly in the presence of P4, directly increased

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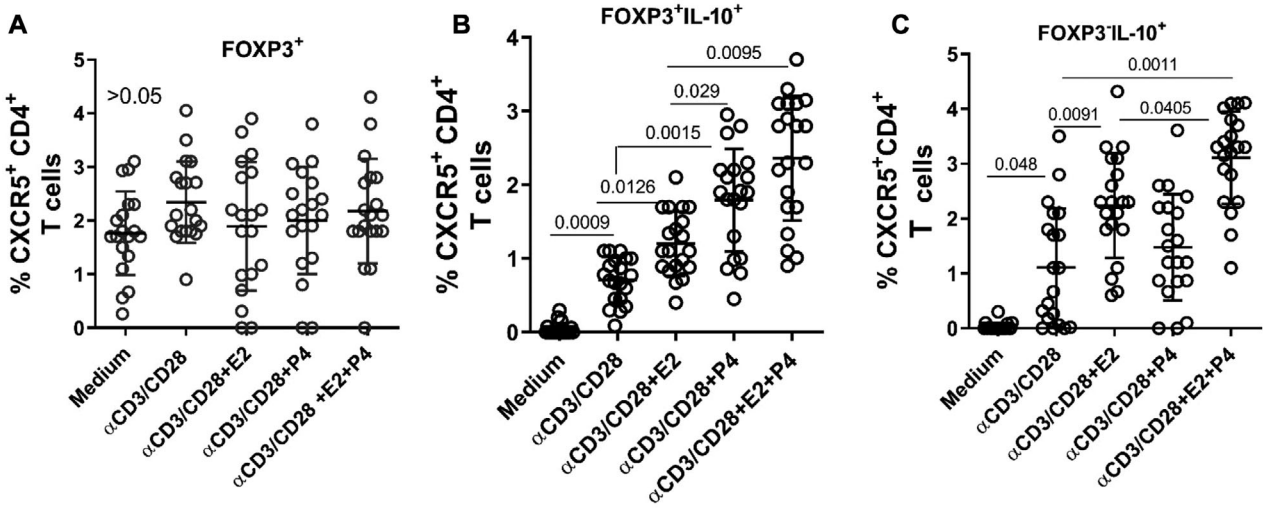


Figure 3. The effect of E2 and P4 on T_{FR} cell frequency. PBMC cells (1×10^6/mL) from healthy women ($n = 20$) were stimulated with αCD3/αCD28 beads (10 μL/mL) in the presence or absence of 17β-estradiol (E2, 5 ng/mL) and/or progesterone (P4, 50 ng/mL) for 3 days. The mean percentage of $FOXP3^+CXCR5^+$ $CD4^+$ T (A), $FOXP3^+CXCR5^+IL\text{-}10^+CD4^+$ T (B), and $FOXP3^-CXCR5^+IL\text{-}10^+CD4^+$ T (C) cells were determined after incubation of PBMC cultures with PMA and inomycin for 4 hours before finishing culture time and measured by flow cytometry. Data are shown as mean ± SD of five independent experiments with 4 samples per experiments. Significance was calculated by comparing different cell cultures conditions (medium, E2, P4, and E2 plus P4; two-way ANOVA). The *p*-values are indicated in the figure.

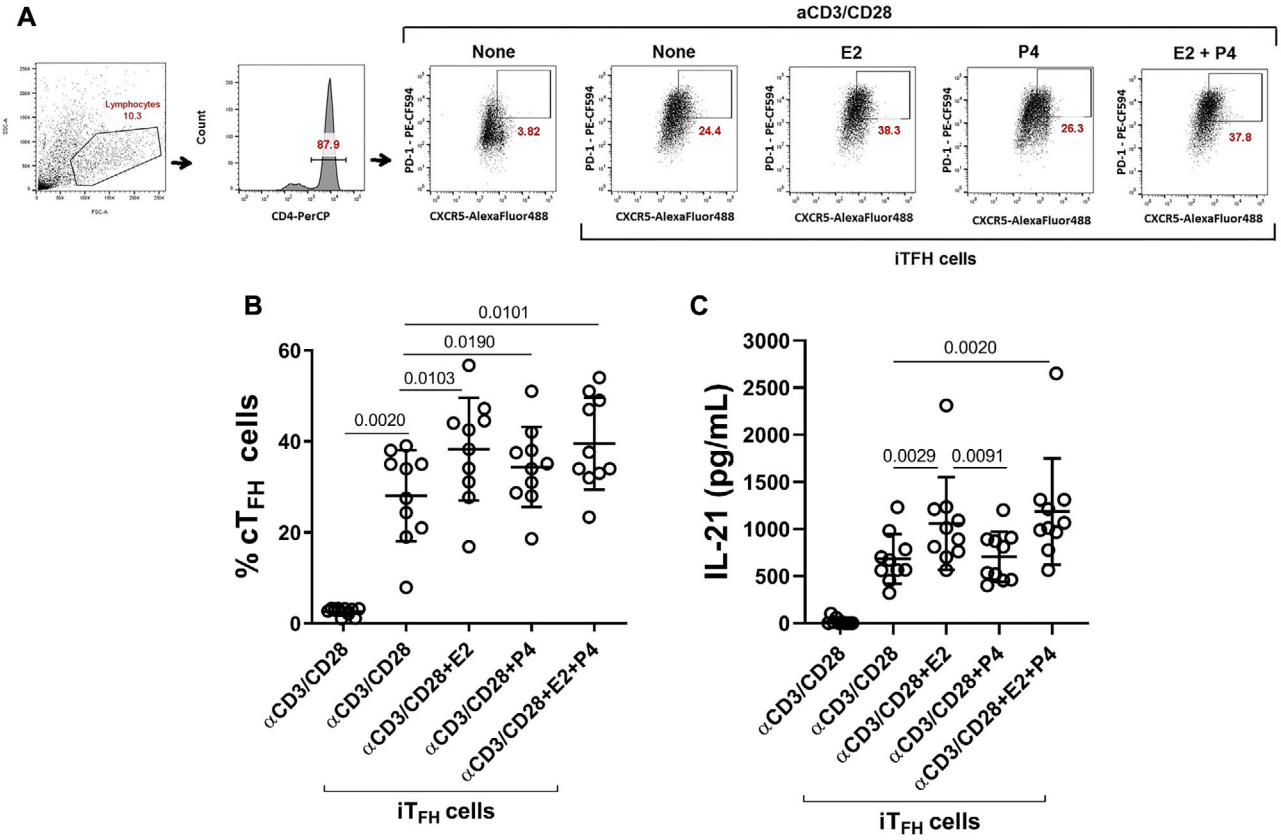


Figure 4. The impact of E2 and P4 on in vitro cT_{FH} cell differentiation. Naïve $CD4^+$ T cells (0.8×10^6/2 mL) from healthy women ($n = 10$) were stimulated with α-CD3/CD28 beads (10 μL/mL) in the presence of hrIL-12 (0.5 ng/mL), hrIL-23 (25 ng/mL), and hrTGF-β (5 ng/mL). In some wells, 17β-estradiol (E2, 5ng/mL) and/or progesterone (P4, 50 ng/mL) were added at the start of the cell culture period. After 5 days, the differentiated frequency of T_{FH} (iT_{FH}) cells was determined by cytometry. In (A) representative dot-plots of T_{FH} cells ($PD\text{-}1^+CXCR5^+CD4^+$T cells). The mean percentage of cT_{FH} cells ($CXCR5^+PD\text{-}1^+CD4^+$ T cells) (B) and IL-21 secretion (C) were analyzed by flow cytometry and ELISA, respectively. Data are shown as mean ± SD of five independent experiments with four samples per experiment. Significance was calculated by comparing different cell cultures conditions (medium, E2, P4, and E2 plus P4; two-way ANOVA). The *p*-values are indicated in the figure.

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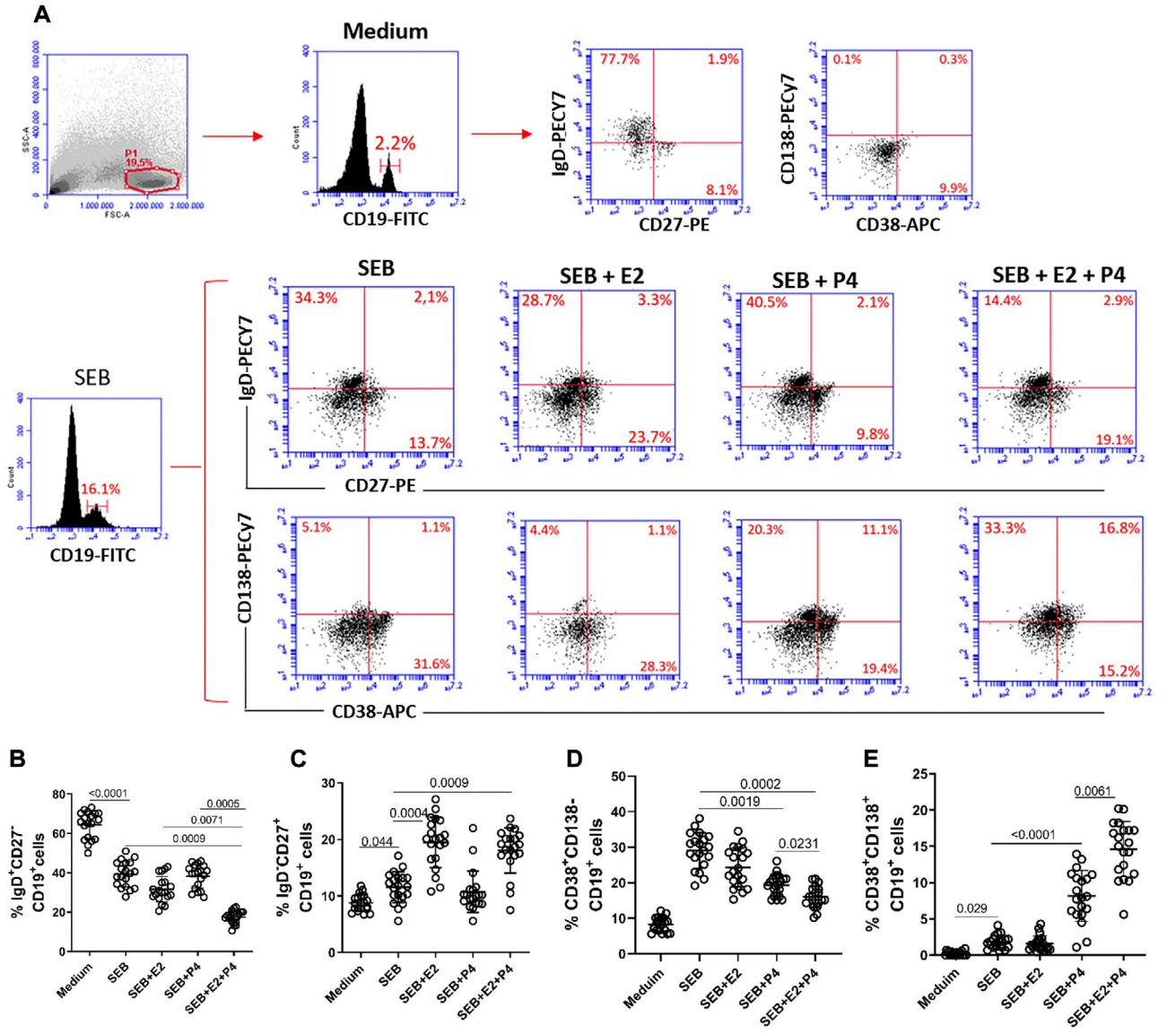


Figure 5. The effect of E2 and P4 on the frequency of B cell subsets. PBMC cultures (1×10^6/mL) from healthy women ($n = 20$) were stimulated with SEB (1 μg/mL) for 6 days and, following the strategies in (A), we determined the frequency of (B) naïve ($CD3^-$ $CD19^+$ $CD27^-$ IgD^+), (C) memory ($CD3^-$ $CD19^+$ $CD27^+$ IgD^-), (D) plasmablasts ($CD3^-$ $CD19^+$ $CD38^+$ $CD138^-$), and (E) plasma cells ($CD3^-$ $CD19^+$ $CD38^+$ $CD138^+$) by flow cytometry. Data are shown as mean ± SD of five independent experiments with four samples from women per experiment. Significance was calculated by comparing different cell cultures conditions (medium, E2, P4, and E2 plus P4; two-way ANOVA). The *p*-values are indicated in the figure.

IgG production by B cells co-cultured with purified cT_{FH} cells. Further, in the supernatants of these cultures, higher IL-21 levels were quantified after the addition of E2. Interestingly, in SEB-activated PBMC cultures, while E2 elevated the percentage of memory B cells, P4 reduced the proportion of plasmablasts but enhanced the percentage of plasma cells. In line with our study, another study published by Parr and Parr [50] in ovariectomized mice demonstrated the ability of P4 to elevate the number of plasma cells in the uterus at the outset of pregnancy. Here, the capacity of P4 to elevate in vitro plasma cells, coupled with its inability to increase IgG production in the absence of E2, suggests that both hormones are necessary for optimal humoral immune response.

The effects of hormones depend not only on their concentrations, but also on the cell maturation stage. In the present study, although P4 did not change the percentage of $CXCR5^+PD\text{-}1^+$ $CD4^+$ T cells in activated PBMC cultures, it did amplify just like E2, the differentiation of circulating naïve $CD4^+$ T cells into T_{FH} cells; no changes were observed in the proportion of $T_{FH}1$, $T_{FH}2$, and $T_{FH}17$ subsets. E2 also increased the levels of IL-21. These results suggested that during pregnancy these hormones can boost maternal neutralizing antibody production following maternal primary immunization. The differential effects of P4 on naïve $CD4^+$ T cells could be related to the differential expression of the receptor for this hormone. Regardless of the

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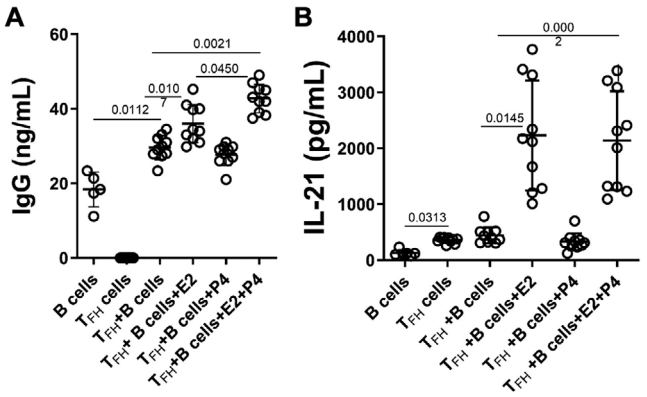


Figure 6. The effect of E2 and P4 on IgG and IL-21 production by SEB-activated T_{FH}/B cell co-cultures. Purified T_{FH} cells ($CD4^+CD45RA^-CXCR5^+$) and B cells from healthy women ($n = 10$) were co-cultured in the presence of medium (negative control) or stimulated with SEB (1 μg/mL) for 6 days. Both IgG production (A) and IL-21 release (B) were dosed in the supernatants through ELISA. Data are shown as mean ± SD of four independent experiments with two to three samples per experiment. Significance was calculated by comparing different cell cultures conditions (medium, E2, P4, and E2 plus P4; two-way ANOVA). The *p*-values are indicated in the figure.

existence of membrane isoforms, the biological effects of E2 and P4 effects are mainly mediated by intracellular ER (α and β) isoforms and PGR, respectively [78,79]. In humans, ERα signaling increases the production of B cell activating factor (BAFF), a vital cytokine for survival and maturation of B cells [48], as well as increasing Ig production [38,80]. Interestingly, estrogen response elements (ERE) region for ERα was identified within the heavy chain switch (S) regions, indicating that this hormone can directly regulate class switch recombinations [42]. In a very preliminary analysis, the quantification of ERα and PGR via real-time PCR in one woman from our cohort demonstrated a higher expression of ERα on $CD45RA^-CXCR5^+CD4^+$ T cells when compared with $CD45RA^-CXCR5^-$ ones. Of note, this finding will be investigated by us in a larger number of women before a definitive statement can be made for this issue.

During normal pregnancy, a delicate balance between T_{FH} and T_{FR} phenotypes is probably important to avoid immune disorders mediated by excessive antibody production. Indeed, mice deficient in T_{FR} cells developed late-onset spontaneous autoimmune diseases mediated by antibodies.[81] Sex differences in T_{FH} cells help B cells contribute to sexual dimorphism in severity of experimental model for rheumatoid arthritis.[82] Moreover, uncontrolled IgG production in animals lacking functional ERα on $CD4^+$ T-cells [83] should also be related to a loss of functional T_{FH} cells. Furthermore, an imbalance of T_{FR} and T_{FH} cells could result in abnormal GC response and contribute to progression of pathogenesis of autoimmune diseases, as observed in lupus [84]. In the murine model of lupus and patients, E2 treatment led to increased serum anti-dsDNA IgG, and peripheral lymphoid expansion of high-affinity antibody-positive B cells [85,86]. In humans, production of high levels of pro-inflammatory cytokines and placental transfer of these anti-dsDNA IgG can complicate pregnancy with an increased risk of miscarriage, premature delivery, and preeclampsia, as well as heart problems in the newborn.[87]

In summary, our findings suggested that a combined and balanced effect of high E2 and P4 levels on the non-T_{FH} and T_{FH}/T_{FR} axis is important not only to prevent fetal rejection but, also, to increase fetal protection against different pathogens from immunized maternal IgG placental transfer by booster functional cT_{FH} cell subsets.

Materials and methods

Subjects

For our study, peripheral blood was collected from 30 healthy female volunteers (20–35 years old) after completing a self-administered questionnaire regarding their menstrual status, reproductive history, and use of oral contraceptives. Women with regular menstrual cycles (28 ± 2 days) who had not taken hormone-containing medication in the last 3 months were recruited for the study. In order to attenuate interaction of endogenous E2 and P4 with the in vitro immune assays, blood samples were taken at the beginning of the follicular phase of the menstrual cycle (days 6–7). As we aimed to evaluate the impact of pregnancy on the immunization against hepatitis B virus (HBV), pregnant (n = 10, 22–31 years old) or not (n = 10 from 30 healthy female volunteers, 23–35 years old) women, who were not previously immunized to this virus (anti-HBsAg IgG <10 UI/m), received a three-dose hepatitis B vaccine on a 0, 1, and 6-month schedule, beginning in the first trimester among PW. In those women, the evaluation of anti-HBsAg IgG and T_{FH} cells was performed just before the first dose (t0) and approximately 15 days (t1) after the application of the last dose of the vaccination schedule (6 after the first dose). In the present study, subjects with autoimmune and allergic diseases or cancer, immunocompromised individuals, smokers, illicit substance users, and those with a clinical or serological indication of acute or chronic disease, such as influenza, HCV, HBV, and HIV-1/2 were excluded. Additionally, with regard to CMV, 83% of non-PW ($n = 25/30$) and 80% of PW (8/10) were IgG positives for the virus, but none was seropositive for anti-CMV IgM, which excluded active infection.

The subjects were recruited from the Federal University of the State of Rio de Janeiro, Fernando Figueiras Institute /IOC, and the Institute for Immunology/University of California (UC/Irvine), with written consent being obtained from all subjects.

PBMC cultures

For our study, PBMCs (1×10^6 /mL), obtained by centrifugation on the Ficoll–Hypaque density gradient, were cultured in 24-well flat-bottomed microplates with 2 mL of AIM-V serum-free medium without phenol red (ThermoFisher Scientific Inc.). These cells were stimulated for 3 days with anti-CD3/anti-CD28

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Dynabeads™ (10 μL/mL) or with 1μg/mL of Staphylococcal enterotoxin B (SEB) from *Staphylococcus aureus* (Sigma–Aldrich Co) for 6 days. The effects of hormones on cT_{FH} cells were evaluated after addition of pregnancy-related doses [87] of E2 (5 ng/mL) and/or P4 (50 ng/mL; Sigma–Aldrich Inc.) at the beginning of cell cultures. In order to optimize the detection of intracellular cytokines, these cell cultures were stimulated with phorbolmyristate acetate (PMA, 20 ng/mL; Sigma–Aldrich) plus Ionomycin (600 ng/mL; Sigma–Aldrich) for 4 h in the presence of brefeldin A (10 μg/mL; BD Biosciences, San Diego, CA, USA). The cell cultures were maintained in a humidified incubator (5% CO_2) at 37°C.

Flow cytometry

To determine the percentage of different cT_{FH} and B-cell subtypes, we used mouse anti-human monoclonal antibodies (mAbs) directed against surface (CD3-APC/FITC, CD4-FITC/PECy7, CD19-FITC/APC, CXCR5-PECy7/PerCPCy5.5/AlexaFluor488/FITC, CCR6-PE, CXCR3-PECy7/BV421, PD1-APC/PECF594/FITC/PE, CD45RA/PECy7, ICOS-BB515/PE/BV786, CD3-APC/PE, CD38-PE, CD138-PECy7, IgD-PECy7, and CD27-FITC/PE) and intracellular (IL-21-PE/APC, IFN-γ-APC, IL-10-APC, IL-17-PECy7/APC, IL-4-APC, IL-6-PE, FoxP3-PE) markers. Antibodies and isotype controls were purchased from BioLegend (San Diego, CA, USA). Briefly, the cells were incubated with various combinations of mAbs for 30 min at room temperature in the dark, according to manufacturer's instructions. The cells, washed with PBS containing 1% BSA, were permeabilized by incubating them with Cytofix/Cytoperm solution (BD Pharmigen, San Diego, CA) at 4°C for 20 min. The mAbs for intracellular staining were incubated for 30 min at 4°C. The cells were acquired on Attune NxT Flow Cytometer (ThermoFisher Scientific Inc.), Accuri C6 (Accuri™ Ann Arbor, MI, USA) or FACS LRS II Fortessa (BD Biosciences, San Diego, CA, USA) and analyzed by using FlowJo© or Cflow Software. Isotype control antibodies and single-stained samples were used to periodically check the settings and gates on the flow cytometer. After acquisition of 100 000–200 000 events, lymphocytes were gated based on forward and side scatter properties after the exclusion of dead cells and doublets. All analyzes were in accordance with "*Guidelines for the use of flow cytometry and cell sorting in immunological studies*." [88]

In vitro differentiation of T_{FH} cells

Naïve $CD4^+$ T-cells were obtained from PBMCs by negative selection using magnetic columns according to manufacturer's instructions (EasySep™, StemCell Technology, Canada). The purity of $CD4^+$ T-cell was >98%, as measured by flow cytometry (data not shown). To induce T_{FH} cell differentiation, 0.8×10^6 of naïve $CD4^+$ T-cells were resuspended in 2 mL of AIM-V serum-free medium without phenol red (ThermoFisher Scientific Inc.) and stimulated in 24-well flat-bottom tissue culture plates with anti-CD3/anti-CD28 Dynabeads™ (10 μL/mL) plus the recombinant human IL-12 (0.5 ng/mL), IL-23 (25 ng/mL), and TGF-β (5 ng/mL) [88]. In some wells, E2 (5 ng/mL) and/or P4 (50 ng/mL) (Sigma–Aldrich Inc.) were added at the beginning of cell cultures. The cell cultures were maintained for 5 days at 37°C in a humidified 5% CO_2 incubator.

T_{FH}/B cell co-culture assays

For cT_{FH} and B cell cocultures, T_{FH} cells were sorted as $CD4^+CXCR5^+CD45RA^-$ after staining PBMC with CD4/PerCP, CXCR5/Alexa Fluor 488, and CD45RA/APC. The sorting was conducted using BD FACS ARIA II (BD Biosciences, San Diego, CA, USA). To acquire B cells, PBMCs were submitted to negative selection using magnetic bead-based kits according to manufacturer's instructions (EasySep™, StemCell Technology, Canada). The cells were resuspended in AIM-V serum-free medium without phenol red (ThermoFisher Scientific Inc.). Using a round-bottom 96-well plate, cT_{FH} (1×10^4/100 μL) and B (2×10^4/100 μL) cells were stimulated with 1 μg/mL of Staphylococcal enterotoxin B (SEB) from *Staphylococcus aureus* (Sigma–Aldrich Co) with or without hormones (E2 [5 ng/mL] and P4 [50 ng/mL]; Sigma–Aldrich Inc.) The cells were maintained at 37°C in a humidified 5% CO_2 incubator for 6 days. Just prior to analyzing the cells by flow cytometry, the supernatants were collected for quantification of IL-21 and total IgG via ELISA.

Enzyme-linked immunosorbent assay

The quantification of IL-21 and total IgG antibodies in supernatants of SEB-activated T_{FH}/B cell co-cultures were performed using ELISA kits (Invitrogen, ThermoFischer), according to manufacturer's instructions. Recombinant human IgG, at concentrations ranging from 1.6 to 100 ng/mL, were used to construct the standard curve. Results concerning IL-21 were interpolated from a standard curve plotted using recombinant human IL-21 ranging from 78 to 5000 pg/mL. Peripheral progesterone and estradiol levels were also measured using Abcam's ELISA kit (Cambridge, USA), according to manufacturers' instructions. Reagents supplied by the manufacturer were used to construct the standard curve from 0 to 1000 pg/mL for estrogen and 0 to 40 ng/mL for progesterone. The anti-hepatitis B (HBsAg) titers were determined through ELISA kits [Bioclin (Belo Horizonte, BRA), according to manufacturers' instructions. Reagents supplied by the manufacturer were used to construct the standard curve from 0 to 500 mUI/mL to anti-HBsAg IgG.

Statistical analysis

Statistical analysis was performed using Prism 5.0 software (GraphPad Software, San Diego, CA). To compare more than two

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groups (control, E2, P4, E2 + P4), we used two-way ANOVA. The nonparametric Mann–Whitney *U*-test and the Student's *t*-test were applied to determine whether the two groups were statistically different for nonparametric and parametric variables, respectively. We used Spearman and Pearson´s correlation coefficient test to determine the relation of different hormones and cT_{FH} cells, as appropriate. Significance in all experiments was defined as $p < 0.05$.

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Conflict of Interest: All authors declare that there are no commercial and financial conflicts of interest.

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Abbreviations: **Bcl-6**: transcription factor B cell lymphoma-6 · **CMV**: cytomegalovirus · **cT_{FH}**: circulating T follicular helper · **cT_{FR}**: circulating follicular regulatory T cells · **E2**: 17β-estradiol · **ER**: estrogen receptor · **HbsAg**: hepatitis B superficial antigen · **HBV**: hepatitis B virus · **ICOS**: inducible T-cell co-stimulator · **mAbs**: monoclonal antibodies · **nPW**: non-pregnant women · **P4**: progesterone · **PD-1**: programmed cell death receptor-1 · **PMA**: phorbol 12-myristate 13-acetate · **PW**: pregnant women · **Th**: T helper

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