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Expression analysis of cannabinoid receptors 1 and 2 in B cells during pregnancy and their role on cytokine production☆

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ABSTRACT

The endocannabinoid system consists in a family of lipids that binds to and activates cannabinoid receptors. There are two receptors so far described, the cannabinoid receptor 1 (CB1) and 2 (CB2). In the context of pregnancy, the endocannabinoid system was shown participates in different key aspects of reproductive events.

B-lymphocytes are pleiotropic cells belonging to the adaptive arm of the immune system. Besides immunoglobulin production, B-lymphocytes were recently shown to be actively involved in antigen presentation as well as cytokine production, thus playing a central role in immunity.

In this study we first aimed to characterize the expression of CB1 and CB2 receptors in B cells during pregnancy and then analyze the impact of their activation in term of cytokine production by B cells from pregnant and non-pregnant mice.

We observed that the expression of CB1 and CB2 receptors in B-lymphocytes is differentially regulated during pregnancy. While CB2 expression is down regulated CB1 is augmented in B-lymphocytes of pregnant mice. Additionally, the treatment of activated B-lymphocytes with specific CB1 and CB2 agonists, showed a different response in term of cytokine production. Particularly, CB1 against boosted the production of the anti-inflammatory cytokine IL-10 by activated B-lymphocytes from pregnant mice.

1. Introduction

The endocannabinoid system (ECS) is composed of endogenous ligands derived from membrane phospholipids that bind to a family of G protein-coupled receptors commonly referred as cannabinoid receptors. So far, two cannabinoid receptors have been described, the endocannabinoid receptor 1 (CB1) and 2 (CB2), being the expression of these receptors broadly distributed in different tissues (Bambang et al., 2012; Sun and Dey, 2012). The ECS has been shown to play a central role in different reproductive processes from embryo implantation to parturition (Karasu et al., 2011). Both, CB1 and CB2, were critically involved in different key events related to pregnancy outcome. In this regard, CB1 knockout mice were shown to be more susceptible to pregnancy loss as compared to control mice (Paria et al., 2001; Wang et al., 2004). In the other hand, signaling through CB2 receptor in immune cells was shown to be associated with the release of anti-inflammatory cytokines such as IL-10, which is known to be fundamental for pregnancy well being (Börner et al., 2006; Correa et al., 2005).

B-lymphocytes are pleiotropic cells belonging to the adaptive arm of the immune system. Due to their unique capacity to produce and release antibodies, B cells were classically referred as effector cells of the immune system (LeBien and Tedder, 2008). However, recent advances in B cell biology have placed the B-lymphocytes as central regulators in immunity (Shlomchik et al., 2001). Besides being very good antigen presenting cells, B-lymphocytes were shown to be able, upon activation, to produce and release a wide range of pro as well as anti-inflammatory cytokines, which in turn helps to shape an immune response (Harris et al., 2000).

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In the context of pregnancy, our laboratory was pioneer in showing how the B cell compartment participates in the process of pregnancy tolerance (Muzzio et al., 2014a,b, 2016) as well as in pregnancy associated pathologies (Jensen et al., 2012; Muzzio et al., 2014b, 2016)

In this study we aimed to characterize the expression of cannabinoid receptors in B cells during murine pregnancy as well as the influence of CB1 and CB2 activation in cytokine production by B-lymphocytes.

2. Material and methods

2.1. Reagents

Lipopolysaccharide (LPS) from Escherichia coli (111:B4) was purchased from Sigma Chemical Co. (St Louis, MI, USA). Selective cannabinoid CB2 receptor agonist SER-601 was purchased from Tocris (United Kingdom). Selective cannabinoid CB1 receptor agonist ACEA, anti CB1 and CB2 antibodies were purchased from ABCAM (United Kingdom). Cytometric Bead Array (CBA array) was obtained from BD Biosciences (Germany). Anti mouse fluorescently labeled anti CD19 (clone 1D3) was purchased from BD Biosciences (Germany). MicroBeads isolation kit was obtained from Miltenyi Biotec (Germany).

2.2. Animals

Eight-weeks-old females C57BL/6 and BALB/c males were purchased from Charles River (France). All mice were maintained in the facilities of the BioTechnikum Greifswald under a 12-h light/12-h dark cycle with free access to water and chow. Animal experiments were carried out according to institutional guidelines after ministerial approval (institutional review board: Landesverwaltungsamt Sachsen-Anhalt [ID: FJ2-1019 to FJ] and Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern [7221.3-1-068/13 to F.J.]). The experiments were conducted in conformity with the European Communities Council Directive 86/609/EEC.

Age-matched virgin C57BL/6 females were mated with BALB/c males. Females were daily inspected for vaginal plugs and presence of a vaginal plug was considered as day 0 of pregnancy. Pregnant females were sacrificed at day 14th of pregnancy.

2.3. Cell isolation

Total B cells were obtained from the spleen of pregnant and non-pregnant females by positive selection using the $CD19^+$ B Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacture's recommendations. In brief, spleens were crushed into a 100 μm cell strainer to obtain a single cell suspension and red blood cells were lysed for 5 min. After washing, cell suspensions were mixed with Anti-CD19 antibodies conjugated to MicroBeads. Next, proceed to magnetically separation. Cell purity was always higher than 95% as analyzed by flow cytometry.

2.4. Cell culture and CBA array

$CD19^+$ B cells from Non-pregnant and Pregnant mice were cultured (1×10^5 cells/well) in RPMI + 10% FBS + 1% antibiotics (streptomycin and penicillin) and divided into four groups: (i) control group; (ii) LPS treated group (10 μg/ml of LPS); (iii) LPS + ACEA treated group (LPS 10 μg/ml and ACEA 100 nM); (iv) LPS + SER treated group (LPS 10 μg/ml and SER 100 nM).

Cells were maintained for 12 h in 5% CO_2 at 37 °C, supernatants were collected and immediately frozen at −70 °C until used.

Concentrations of IL-10 and TNF-α in supernatants were quantified using Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17Cytokine Kit (BD Biosciences) according to the manufacture's recommendations and data were acquired on FACS Canto (BD Biosciences) and subsequently analyzed by FCAP Array software (BD Biosciences).

2.5. Flow cytometry

$CD19^+$ B cells from non-pregnant and pregnant mice were stained for 30 min at 4 °C with anti-CB1(1:50) or anti-CB2 (1:50) antibodies, followed by 1 h incubation with FITC-conjugated anti-rabbit secondary antibody (1:100). Negative control samples were incubated with FITC-conjugated anti-rabbit secondary antibody (1:100).

Data were acquired on FACS Canto (BD Biosciences) and analyzed by using FlowJo software (Tree Star Inc.).

2.6. Statistical analyses

Treatments were assigned completely random to experimental units. Data were analyzed by means of one or two way ANOVA procedures and means were compared by Tukey post hoc tests. Differences between means were considered significant when p value was 0.05 or less. Different letters indicate significant differences between means. Normality and homoscedasticity were tested by Shapiro–Wilk (modified) and Levene test, respectively. Statistical analysis was performed using the software Infostat (Córdoba, Argentina).

3. Results

3.1. Expression levels of cannabinoid receptor 2 (CB2) is strongly reduced while cannabinoid receptor 1 (CB1) is increased in B cells during pregnancy

We began analyzing the expression levels of cannabinoid receptor 1 (CB1) and 2 (CB2) in total $CD19^+$ B cells isolated from the spleen of non-pregnant as well as pregnant mice. As shown in Fig. 1A, pregnant mice showed significantly lower percentages of CB2-expressing $CD19^+$ B cells as compared to non-pregnant age-matched control mice (Fig. 1A). Similarly, when the mean intensify fluorescence (MFI) was analyzed, a clear down-regulation of CB2 expression on $CD19^+$ B cells from pregnant mice was observed (Fig. 1B). Unlike CB2, percentages of $CD19^+$ expressing B cells as well as expression levels of CB1 in $CD19^+$ B cells (MFI) were increased in B cells isolated from pregnant mice as compared to those isolated from non-pregnant control animals (Fig. 1C–D). In summary, during pregnancy, B cells down-regulate CB2 expression while augmenting the expression of CB1.

3.2. Effect of CB1 and CB2 agonist treatment in the production of the anti-inflammatory cytokine IL-10 by B cells from pregnant and non-pregnant mice

Having observed that the expression pattern of cannabinoid receptors is differently modulated in B cells during pregnancy, we next wondered in which extend this may affect cytokine production by B cells. To test this, we isolated $CD19^+$ B cells from the spleen of non-pregnant as well as pregnant mice and further stimulated them with LPS in the presence or absence of the specific CB2 (SER-601) or CB1 (ACEA) agonists. Levels of pro as well as anti-inflammatory cytokines were assayed in supernatants. As expected, LPS induced the production of IL-10 by B cells isolated from non-pregnant and pregnant mice (Fig. 2A–B). However, when $CD19^+$ B cells from non-pregnant or pregnant mice were stimulated with

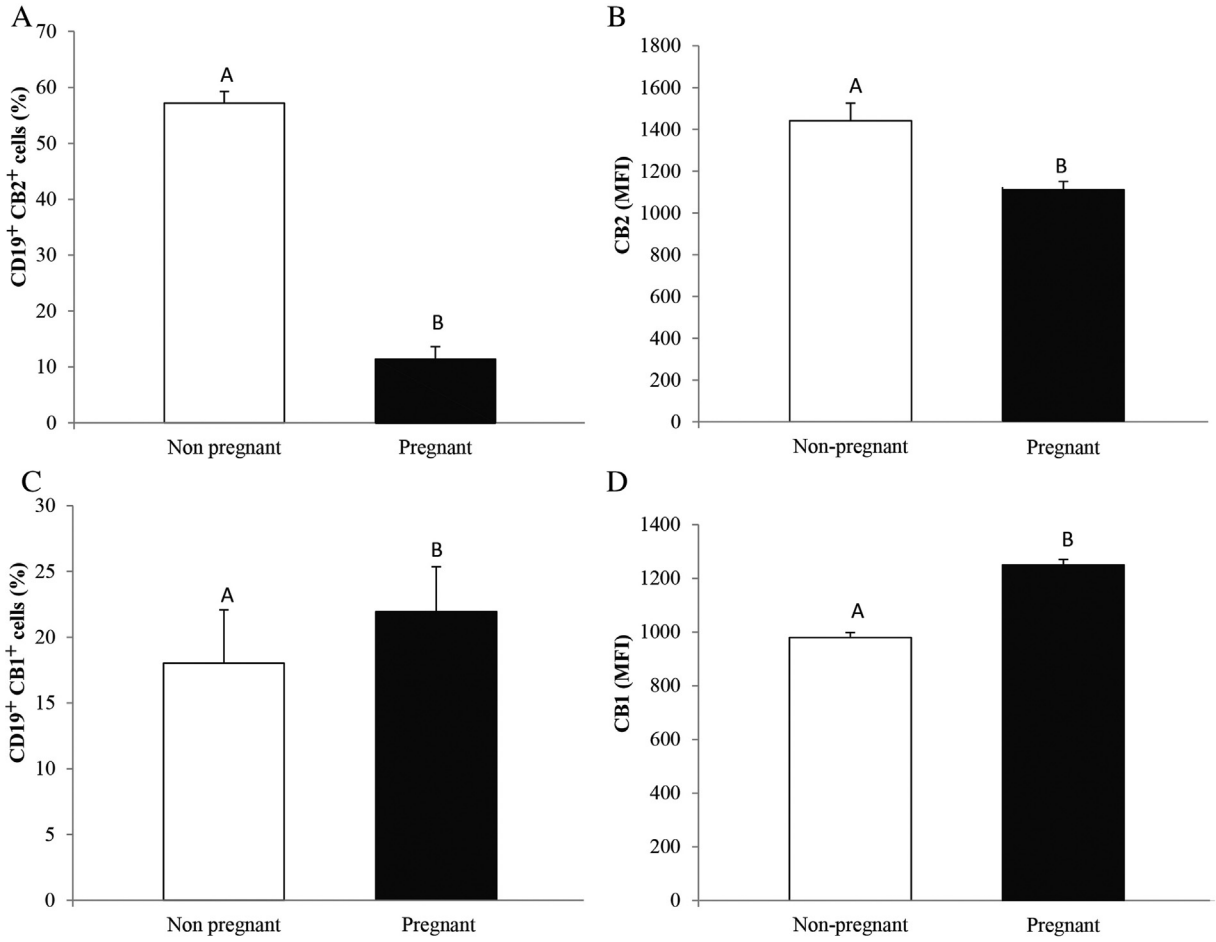

Fig. 1. Expression levels of cannabinoid receptor 2 (CB2) and cannabinoid receptor 1 (CB1) in B cells during pregnancy. Bar graphs A and C show percentages of $CD19^+$ $CB2^+$ and $CD19^+CB1^+$ B cells respectively. Bar graphs B and D display median fluorescence intensity (MFI) of CB2 and CB1 in $CD19^+$ B cells respectively. White bars: non-pregnant mice. Black bars: pregnant mice (day 14th of pregnancy). Data are expressed as means ± SEM. Statistics: A ≠ B, $p < 0.05$ (n = 5–6 animals per group).

LPS in the presence of CB2 agonist a strong suppression of IL-10 production was observed (Fig. 2A). Similarly, CB1 agonist induced a reduction of IL-10 production by LPS-stimulated B cells from non-pregnant mice (Fig. 2B). In contrast, when B cells from pregnant mice were stimulated with LPS in the presence CB1 agonist a significantly higher production of IL-10 was observed (Fig. 2B). In summary, CB2 agonist treatment reduced the production of IL-10 by LPS-activated B cells from both, pregnant and non-pregnant mice while CB1 agonist treatment reduced the production of IL-10 by LPS-activated B cells from non-pregnant mice but strongly induced it in LPS-stimulated B cells isolated from pregnant mice.

3.3. Impact of CB1 and CB2 agonist treatment in the production of the pro-inflammatory cytokine TNF-α by B cells from pregnant and non-pregnant mice

Knowing that the production of IL-10 was differentially modulated by CB2 and CB1 agonists in LPS-activated B cells from non-pregnant and pregnant mice, we next wanted to analyze whether the production of pro-inflammatory cytokine, TNF-α was also affected. As shown in Fig. 2C and D, neither CB2 nor CB1 selective agonists could significantly alter the production of pro-inflammatory cytokine TNF-α by LPS activated $CD19^+$ B cells isolated from non-pregnant or pregnant mice. Nevertheless, it is important to note that LPS activated B cells from pregnant mice produced lower levels of TNF-α as compared to LPS-activated B cells from non-pregnant control mice (Fig. 2C and D). In summary, TNF-α production by B cells was not affected by CB2 or CB1 agonist treatment.

4. Discussion

We demonstrated in this study that the expression of CB1 and CB2 receptors in B-lymphocytes was differentially regulated during pregnancy in mice. While CB1 expression was increased, the expression of CB2 was down regulated in B cells from pregnant mice. Cannabinoid receptors are expressed on immune cells including B cells, T cells, neutrophils and natural killer (NK) cells, with CB2 levels known to be higher than those of CB1 (Galiègue et al., 1995). Among these immune cells, B-lymphocytes express the highest levels of CB2 (Munro et al., 1993). Furthermore, CB2 has been shown to actively participate in crucial events related to B cell development and differentiation. Indeed, CB2 deficient mice (CB2 KO mice) display a strong reduction in the total numbers of marginal zone B cells (MZ B cells) (Basu et al., 2011), which are one of the most prominent B cell subsets in the spleen. In addition, CB2 has been shown to control the retention of MZ B cell in the marginal sinus area of the spleen (Muppidi et al., 2011). In the other hand, our laboratory has recently demonstrated that MZ B cell numbers are increased in the spleen during pregnancy (Muzzio et al., 2014a). Furthermore, we showed that this augmentation seems to be crucial for pregnancy wellbeing as pregnant mice suffering from pregnancy failures display lower numbers of MZ B cells compared to normal pregnant mice (Muzzio et al., 2016). Based on this, we would have expected

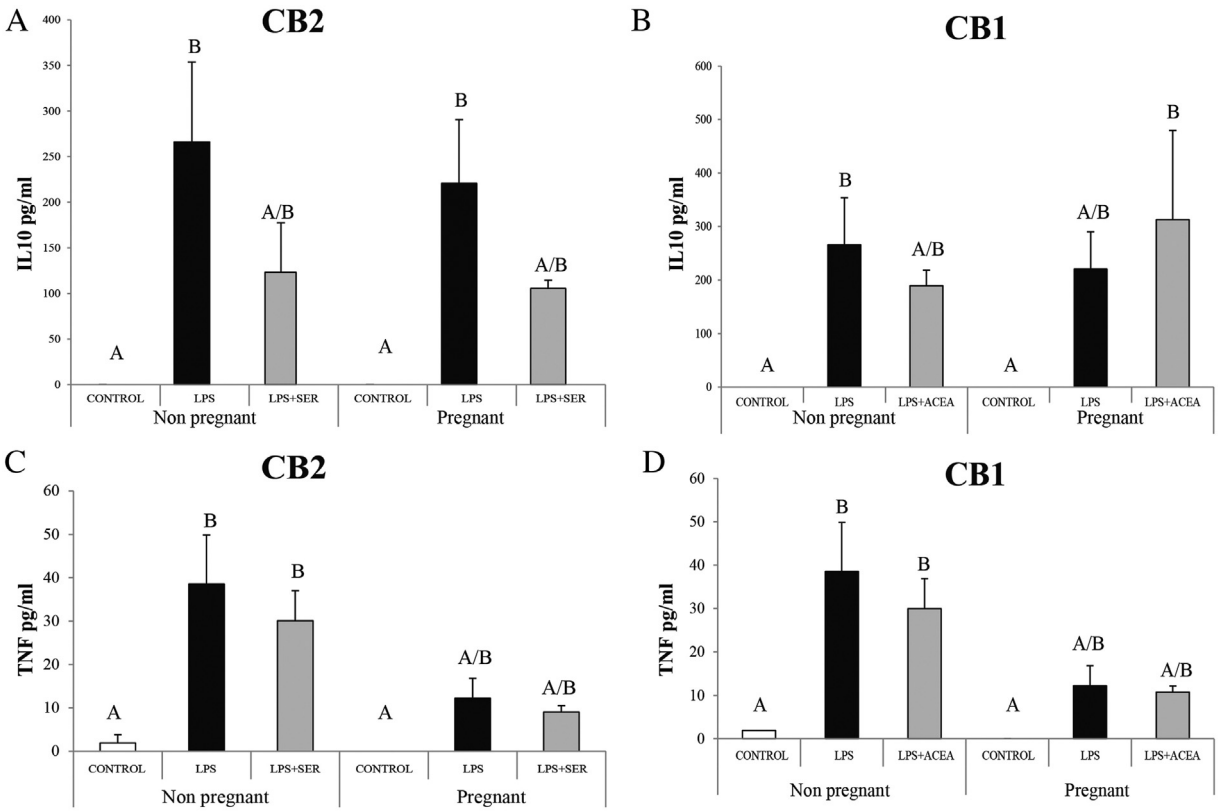

Fig. 2. Effect of CB1 and CB2 agonist treatment on IL-10 and TNF-α production by CD19⁺ B cells isolated from non-pregnant and pregnant mice. CD19⁺ B cells were isolated from the spleen of non-pregnant and pregnant mice (day 14th of pregnancy) and further activated *in vitro* with LPS in the presence/absence of specific CB2 or CB1 agonist. Levels of cytokines were assayed in the supernatants.
CB2 agonist induced a strong suppression of IL-10 production by LPS activated B cells from non-pregnant or pregnant mice (n = 3–5) (A). CB1 agonist treatment induced a reduction of IL-10 production by LPS-stimulated B cells isolated from non-pregnant mice but induced an increase of IL-10 production by LPS-activated B cells from pregnant mice (n = 3–5) (B). Neither CB2 (n = 3–5) (C) nor CB1 (n = 3–5) (D) selective agonists could significantly alter the production of pro-inflammatory cytokine, TNF-α, by LPS activated CD19⁺ B cells isolated from non-pregnant or pregnant mice. Data are expressed as means ± SEM. Statistics: A ≠ B, $p < 0.05$.

an increased expression of CB2 in B cells during pregnancy, instead of the observed decrease, that may account for the increased number of MZ B cells described during this period. However, it is worth to note that we analyzed the expression of CB2 in total B cells but not in pure isolated MZ B cells, which makes difficult to clearly dissect its participation in the establishment of MZ B cell compartment during pregnancy. In addition, unlike CB2, we observed an increase in CB1 expression on B cells during pregnancy, which would indicate a role of this receptor in B cells behavior during this critical period of time.

Having found that CB1 and CB2 expression was differentially regulated in B cells during gestation we next wanted to analyze in which extend this affect or impact the B cell functionality, particularly in term of cytokine production. We treated LPS activated B cells from pregnant and non-pregnant mice with CB1 and CB2 specific agonists and then analyzed the production of pro as well as anti-inflammatory cytokines. Activation of CB2 receptor was shown to enhance the production of the potent anti-inflammatory cytokine IL-10 in LPS-activated murine macrophages (Correa et al., 2005). Unlike the observed effect in macrophages, CB2 activation by specific agonist in B cells from non-pregnant and pregnant mice induced a reduction of IL-10 production, clearly indicating that activation of CB2 receptor in different immune cells has completely different effects. In keeping with this, CB1 activation in B cells from non-pregnant mice has also induced a mild decrease of IL-10 production. Nonetheless, when B cells from pregnant mice were treated with specific CB1 agonist, an increase on IL-10 production was observed.

Pregnancy has been classically associated with a shift from a pro-inflammatory Th1 profile dominating the phase of embryo implantation into a Th2, anti-inflammatory profile until parturition when Th1 cytokines rise again (Mor and Cardenas, 2010; Sykes et al., 2012). Indeed, alteration of the fine balance between Th1 and Th2 cytokines during pregnancy induced by bacterial infections or their products (LPS) provoke pregnancy failures (Mor and Cardenas, 2010). The fact that CB1 activation induced the production of IL-10 by LPS-activated B cells from pregnant mice, but not from non-pregnant animals could be interpreted as a mechanism triggered in order to control the over activation of B cells, under the presence of pathogens or their components, and thus compromising the continuity of pregnancy. Further doses of LPS as well as the mechanisms involved in this effect should be tested in the future.

Overall, we demonstrated in this study that the expression of cannabinoid receptors is differentially modulated during pregnancy more likely indicating a physiological role of these proteins in this critical period of life. The results involving cytokines production by activated B cells treated with specific cannabinoid receptor agonists at least partially confirm this.

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