Accepted Manuscript

Title: ☆Progesterone and estradiol exert an inhibitory effect on the production of anti-inflammatory cytokine IL-10 by activated MZ B cells

Author: I. Bommer D.O. Muzzio M. Zygmunt F. Jensen

PII: S0165-0378(16)30055-9
DOI: http://dx.doi.org/doi:10.1016/j.jri.2016.05.008
Reference: JRI 2411

To appear in: *Journal of Reproductive Immunology*

Received date: 14-3-2016
Revised date: 20-5-2016
Accepted date: 27-5-2016

Please cite this article as: Bommer, I., Muzzio, D.O., Zygmunt, M., Jensen, F., ☆Progesterone and estradiol exert an inhibitory effect on the production of anti-inflammatory cytokine IL-10 by activated MZ B cells.Journal of Reproductive Immunology http://dx.doi.org/10.1016/j.jri.2016.05.008

Progesterone and estradiol exert an inhibitory effect on the production of anti-inflammatory cytokine IL-10 by activated MZ B cells

Bommer I[1#], Muzzio DO[1#], Zygmunt M[1], Jensen F[1, 2, 3]

1: Research Laboratory, Department of Obstetrics and Gynecology, University of Greifswald, Greifswald, Germany.

2: Laboratory for Immunology of Pregnancy, Center for Pharmacological and Botanical Studies (CEFYBO-CONICET-UBA), Buenos Aires, Argentina.

3: Institute of Health Sciences, National University Arturo Jauretche, Buenos Aires, Argentina.

Correspondence should be addressed to: Prof. Dr. Federico Jensen. Laboratory for Immunology of Pregnancy, Center for Pharmacological and Botanical Studies (CEFYBO-CONICET-UBA).

Address: Paraguay 2155 floor 17th, Buenos Aires C1121ABG, Argentina.

E-mail: fjensen@unaj.edu.ar or federico.jensen@outlook.com

Phone: +5411-45083680 (120)

Fax: +5411-45083680 (101)

# Both authors contributed equally to this work.

This study was supported by grants from the Fritz Thyssen Foundation to FJ (Az. 10.12.2.155) and intramural founding from Medical Faculty, Greifswald University.

Abstract

The main message of this work is the fact that female sex hormones, progesterone and estradiol, whose levels significantly rise during pregnancy, inhibit the production of anti-inflammatory cytokine IL-10 with no apparent effect on pro-inflammatory cytokine TNF-α by activated MZ B cells. This is an important piece of information and helps to better understand how the maternal immune system controls the balance between immune tolerance and immune activation during pregnancy leading to the simultaneously acceptance of the semi-allogeneic fetus and the proper defense of the mother against pathogens during this critical period of time.

Introduction

Proper establishment, progress and success of mammalian pregnancy rely on the capability of the maternal immune system to undergo a series of adaptations tending to tolerate the presence of the semi-allogeneic fetus (Robertson and Moldenhauer, 2014; Seavey and Mosmann, 2008). Among others well described immune-mechanisms; our laboratory has demonstrated that pregnancy induces strong modifications in B cell development (Muzzio et al., 2014). These modifications include a strong reduction on B cell lymphopoiesis from the bone marrow, which in turn induces B cell lymphopenia in the spleen and a re-organization of the main B cell population in this tissue, the marginal zone (MZ) and follicular (FO) B cells (Muzzio et al., 2014). These two B cells populations have different but complementary functions (Cerutti et al., 2013). Due to their pre-activated phenotype depicted by high expression levels of TLRs and strategic localization in the spleen, the MZ B cells can

rapidly react against pathogens giving rise to plasmablasts that produce large amount of IgM but also IgG and IgA (Cerutti et al., 2013). Hence they represent the first barrier against infections (Martin et al., 2001). In the context of pregnancy, we showed that MZ B cells numbers are increased in the spleen (Muzzio et al., 2014) and seems to be crucial for pregnancy wellbeing (Muzzio et al., 2016).

In addition to the production of antibodies, in the recent years it has been extensively demonstrated that, once activated, B cells produce large amounts of pro- as well as anti-inflammatory cytokines that are fundamental in orchestrating an immune response (Shen and Fillatreau, 2015).

Based on this, we aimed in this work to investigate the effect of pregnancy-associated hormones, progesterone and estradiol in the production of cytokines by activated MZ B cells.

Material and methods

Animals

Eight-weeks-old female BALB/c (H2d) mice were provided by the BioTechnikum Greifswald. All mice were maintained in the facilities of the BioTechnikum Greifswald under a 12-hour light/12-hour dark cycle and were given *ad libitum* access to food and water. Animal experiments were carried out according to institutional guidelines after ministerial approval (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. After the animals were euthanized, spleens were taken for MZ B cell isolation.

Cell isolation

Splenocytes were isolated using standard methods (Muzzio et al., 2014). Briefly, spleens were crushed in a 100 µm cell strainer to obtain a single cell suspension and red blood cells were lysed for 5 minutes. Lymphocytes were isolated using Lympholyte density gradient (Cedarlane, Ontario, Canada). After washing, cell suspensions were counted using a Neubauer Chamber. For the isolation of splenic B cells, CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used as stated on the provided protocol. Isolation of MZ B cells was performed using MZ and FO B Cell Isolation Kit, mouse (Miltenyi Biotec, Bergisch Gladbach, Germany) as described by the manufacturer. We typically obtained ~90% purity as checked by Flow Cytometry.

Flow cytometry

The presence of PR on freshly isolated $CD19^+$ B cells was performed using standard methods (Muzzio et al., 2014). Cells were first fixed with paraformaldehyde 4% solution and then permeabilized using 0,1% saponin solution. Rabbit anti PR (Santa Cruz Biotechnology, Heidelberg, Germany) was used as primary antibody, which was later detected by a goat anti-rabbit FITC antibody (BD Biosciences, Heidelberg, Germany). The unstained control was treated likewise but lacked the primary antibody.

Immunofluorescence

Magnetic isolated $CD19^+$ B cells were stained as mentioned for flow cytometry and observed under 100x amplification. DAPI was used to stain cell nuclei.

Western blot

$CD19^+$ isolated B cells were treated with RIPA Lysis and Extraction Buffer (Thermo Thermo Fisher Scientific, Bonn, Germany) and homogenates were centrifuged at 14,000 × g for 20 min at 4°C and the supernatant containing the proteins was transferred to a fresh tube. Protein concentration was assessed using the Pierce BCA Protein Assay (Thermo Thermo Fisher Scientific, Bonn, Germany) as indicated by the manufacturer.

30 µg of protein were transferred into an 8 polyacrylamide gel and a SDS-PAGE was performed at 100 V. After the electrophoresis proteins were transferred into PVDF membranes in transfer buffer containing 20% methanol (v/v), 0,19 M glycine and 0.025 M Tris-base pH 8,3. For protein detection, membranes were incubated with rabbit anti-progesterone receptor for 2 h. After three washing steps with TBST (TBS with 0.5% Tween) for 5 min each, the membranes were then incubated with an anti rabbit HRP-conjugated (Thermo Thermo Fisher Scientific, Bonn, Germany) for 1 h at RT and then with EMD Millipore Immobilon Western Chemiluminescent HRP Substrate (ECL) (Fischer-Scientific, Pittsburgh, USA)

Cell culture

$2x10^5$ magnetic isolated MZ B cells were cultured on 96-well flat-bottom plates in 200 µL culture media (RPMI 1640 supplemented with fetal-bovine serum and

antibiotics) for 24 hs at 37°C and 5% CO_2. Some wells were additionally stimulated with 10 µg/mL LPS 0111:B4 (Sigma-Aldrich, Munich, Germany) and 250 pg/mL anti-CD40 antibody (BD Biosciences, Heidelberg, Germany). Some of the stimulated wells were also treated with 50 ng/mL progesterone (Sigma-Aldrich, Munich, Germany), 50 pg/mL estradiol (Sigma-Aldrich, Munich, Germany) or the combination of both. Both concentrations reflect physiological hormone levels between days 10-20 of gestation in mice (Chung et al., 2012). For the last 5 hs of stimulation, 50 pg/mL PMA (Merck Millipore, Darmstadt, Germany), 500 pg/mL Ionomycin (Sigma-Aldrich, Munich, Germany) were added in the culture media. After stimulation, supernatant was separated from cells by centrifugation and frozen at -80°C for later analysis.

Analysis of Cytokine Production on supernatants

Culture media was analyzed using Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 (BD Biosciences, Heidelberg, Germany) following manufacturer's instructions. The measurement was performed in a FACSCanto A (BD Biosciences, Heidelberg, Germany) and data was analyzed using the Kit-provided Software (FCAP Array v3.0.1).

Statistical analysis

Data were analyzed with PRISM software (version 5.01, GraphPad). ANOVA followed by Tukey multiple post t test or Kruskal–Wallis test as appropriated were applied to evaluate differences of means of multiple groups. Significant differences between groups were indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results and Discussion

Like other immune cell subsets, B cells express progesterone as well as estrogen receptors (Cunningham and Gilkeson, 2011; Zhang et al., 2014). The presence of progesterone receptors on $CD19^+$ B cells was confirmed by flow cytometry and immunofluorescence (Fig. 2A-B) while the corresponding western blot analysis further specified the predominance of the progesterone receptor subtype A (PRA, Fig. 2C).

However, B cells in uterine tissue are represented in very low frequencies; the fact that they are found increased in recurrent abortion suggests that they may have a pro-inflammatory phenotype (Lachapelle et al., 1996). On the other hand, regulatory B cells are found in high frequencies in spleen, including non-circulating MZ B cells and MZ precursors.

To dissect the effect of female sex hormones, estradiol and progesterone in MZ B cells, pure isolated MZ B cells were stimulated *in vitro* with LPS and CD40L with or without the addition of estradiol, progesterone or both hormones and cytokines levels were assayed in the supernatant. As expected, LPS/CD40 stimulated-MZ B cells produced pro (TNF-α) as well as anti-inflammatory (IL-10) cytokines (Figure 1 A-B). The addition of estradiol caused a slight, not significant, reduction of IL-10 production and no appreciable effect of TNF-α production by LPS/CD40 activated MZ B cells (Fig. 1A-B). Similarly, progesterone added alone, induced a reduction of IL-10 production that reached significance and a modest, not significant, reduction of TNF-α production by LPS/CD40 activated MZ B cells (Fig. 1A-B). When both hormones were added together the inhibition of IL-10 production by activated

MZ B cells was significantly boosted while no changes on TNF-α production was observed (Fig. 1B). Next to IL-10 and TNF-α, other cytokines related to Th1/Th2/Th13 responses were measured on the supernatants. While IL-17A, IFN-γ, IL-4 and IL-2 were not detectable, IL-6 levels showed no significant variation under stimulation (data not shown). In summary we demonstrated in this study that female sex hormones, particularly progesterone, whose levels significantly raise during pregnancy, have a negative effect on the production of the potent anti-inflammatory cytokine IL-10 by activated MZ B cells with no appreciable effect on TNF-α production.

Previously, we have shown that pre-activated MZ B cells numbers were increased in the spleen during pregnancy and proposed that this may represent an evolutionary acquired mechanism tending to overcome the strong reduction of B cell lymphopoiesis occurring during this critical period of time, thus ensuring a proper defense (Medina et al., 1993; Muzzio et al., 2014). Indeed, using a mouse model of pregnancy failures (Bobé et al., 1986) we could later confirm the crucial role of MZ B cells in pregnancy wellbeing by showing that pregnant mice developing normal pregnancies but not those suffering from pregnancy failures display an expansion of the MZ B cell compartment (Muzzio et al., 2016).

In recent years, the misleading concept of B cells being just antibody producing cells has been extensively revised. In this regard, it has been shown that B cells can produce and release a large range of pro as well as anti-inflammatory cytokines that in turn regulates an immune response (Mauri and Menon, 2015; Shen and Fillatreau, 2015). While pro-inflammatory cytokines produced by B cells such as IL-6, INF-γ and TNF-α induce the differentiation of effector $CD4^+$ T

cells, production of the potent anti-inflammatory cytokine IL-10 strongly influence inflammatory immune responses (Shen and Fillatreau, 2015).

Here we confirmed the capacity of MZ B cells to produce both, pro and anti-inflammatory cytokines upon activation. Notably, if MZ B cells were activated in the presence of pregnancy-associated hormones, E2 and P4, a strong reduction of IL-10 ~~release~~ production was observed. This effect was not observed for the pro-inflammatory cytokine TNF-α.

Taking into account that these two cytokines work balancing an immune response, the fact that pregnancy-associated hormones inhibited the production of anti-inflammatory but not pro-inflammatory cytokines by activated MZ B cells might be interpreted as mechanisms triggered during pregnancy in order to ensure an activation of the maternal immune system and proper defense of the mother against infections.

Previous studies have shown an association of progesterone to Th2 mediated suppression of Th1 immune responses in autoimmune diseases (Østensen et al., 2012). However the increase of IL-10 production observed in our experiments demonstrates an opposite effect and emphasizes the versatile impacts of progesterone on different cell subsets within the immune system. The above given results substantiate the role of MZ B cells as a fast compensatory branch of the immune system intercepting the general B cell reduction during pregnancy and thus providing an early immune response to blood-borne pathogens (Cerutti et al., 2013; Muzzio et al., 2014).

Our results bring a new and not yet described mechanism of how the maternal immune system control the fine balance between immune tolerance and

activation during pregnancy in order to support the presence of the fetus without losing the capability to fight pathogens.

Acknowledgements

We would like to thank Mr. Jens Ehrhardt for his supportive expertise and major assistance and Dr. Jens van den Brandt from the ZSF (Zentrale Service- und Forschungseinrichtung für Versuchstiere) Greifswald for the generous supply of BALB/c (Hd2) mice.

References

Bobé, P., Chaouat, G., Stanislawski, M., Kiger, N., 1986. Immunogenetic studies of spontaneous abortion in mice. II. Antiabortive effects are independent of systemic regulatory mechanisms. Cell. Immunol. 98, 477–85. doi:10.1016/0008-8749(86)90306-0

Cerutti, A., Cols, M., Puga, I., 2013. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. Nat. Rev. Immunol. 13, 118–32. doi:10.1038/nri3383

Chung, E., Yeung, F., Leinwand, L.A., 2012. Akt and MAPK signaling mediate pregnancy-induced cardiac adaptation. J. Appl. Physiol. 112, 1564–1575.

Cunningham, M., Gilkeson, G., 2011. Estrogen receptors in immunity and autoimmunity. Clin. Rev. Allergy Immunol. 40, 66–73. doi:10.1007/s12016-010-8203-5

Martin, F., Oliver, A.M., Kearney, J.F., 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity 14, 617–29. doi:10.1016/S1074-7613(01)00129-7

Mauri, C., Menon, M., 2015. The expanding family of regulatory B cells. Int. Immunol. 27, 479–86. doi:10.1093/intimm/dxv038

Medina, K.L., Smithson, G., Kincade, P.W., 1993. Suppression of B lymphopoiesis during normal pregnancy. J. Exp. Med. 178, 1507–15. doi:10.1084/jem.178.5.1507

Muzzio, D.O., Soldati, R., Ehrhardt, J., Utpatel, K., Evert, M., Zenclussen, A.C., Zygmunt, M., Jensen, F., 2014. B Cell Development Undergoes Profound Modifications and Adaptations During Pregnancy in Mice. Biol. Reprod. 91, 115–115. doi:10.1095/biolreprod.114.122366

Muzzio, D.O., Ziegler, K.B., Ehrhardt, J., Zygmunt, M., Jensen, F., 2016. Marginal zone B cells emerge as a critical component of pregnancy well-being. Reproduction 151, 29–37. doi:10.1530/REP-15-0274

Østensen, M., Villiger, P.M., Förger, F., 2012. Interaction of pregnancy and autoimmune rheumatic disease. Autoimmun. Rev. 11, A437–46. doi:10.1016/j.autrev.2011.11.013

Robertson, S.A., Moldenhauer, L.M., 2014. Immunological determinants of implantation success. Int. J. Dev. Biol. 58, 205–17. doi:10.1387/ijdb.140096sr

Seavey, M.M., Mosmann, T.R., 2008. Immunoregulation of fetal and anti-paternal immune responses. Immunol. Res. 40, 97–113. doi:10.1007/s12026-007-8005-x

Shen, P., Fillatreau, S., 2015. Antibody-independent functions of B cells: a focus on cytokines. Nat. Rev. Immunol. 15, 441–451. doi:10.1038/nri3857

Zhang, L., Chang, K.-K., Li, M.-Q., Li, D.-J., Yao, X.-Y., 2014. Mouse endometrial stromal cells and progesterone inhibit the activation and regulate the differentiation and antibody secretion of mouse B cells. Int. J. Clin. Exp. Pathol. 7, 123–33.

Figure Legend

Figure 1. Female sex hormones, estradiol (E2) and progesterone (P4), inhibit the production of anti-inflammatory cytokine IL-10 by activated Marginal Zone B cells. MZ B cells were isolated from the spleen of virgin, fertile BALB/c females (n=6) and activated *in vitro* with LPS and CD40L in the presence or not of E2, P4 or both hormones. Levels of IL-10 and TNF-α were assayed in the supernatants. Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 as analyzed by the one-way ANOVA, followed by a Tukey multiple comparison test.

Figure 1

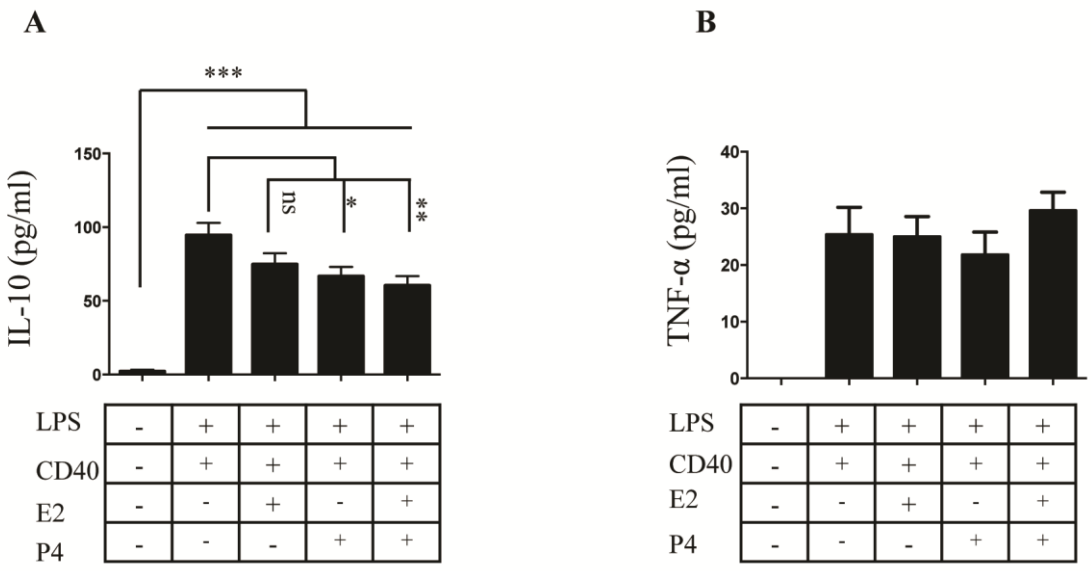

Figure 2. Splenic B cell predominantly expresses PR-A. *CD19*$^{+}$ B cells were isolated from the spleen and analyzed for PR expression by flow cytometry (a), immunofluorescence (b) and western blot (c).