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Ovarian hormones in innate inflammation

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ABSTRACT

Aim: A more vigorous immune system activation is generally seen in women as compared to men. The reasons for these differences are still not understood. By investigating the immune-regulatory role of estrogens, we have previously shown that estradiol (E2) can regulate and ameliorate rheumatoid arthritis models. The aim of this study was to elucidate the role of ovariectomy (ovx) and estradiol (E2) in innate immune responses.

Methods: Female mice were ovx or sham operated. After three weeks, either dorsal air pouches were established by injections of sterile air with subsequent lipopolysaccharide (LPS) injection, or LPS was injected intraperitoneally (i.p). Mice received daily injections with E2 or vehicle for three days before challenge. 6 hours after challenge in the air pouch, blood cells were counted, leukocytes from the pouch were analyzed by flow cytometry, and cytometric bead array or ELISA were used to quantify cytokines collected from the air pouch. Blood cells were counted 1 h after i.p challenge.

Results: Compared to sham, blood leukocyte numbers increased after ovx and ovx + E2 6 h after LPS injections into the air pouch. LPS after ovx induced neutrophil infiltration into the pouch, accompanied by increased levels of MCP-1 and IL-6. Ovx + E2 further enhanced cell infiltration after LPS; however, the cell population diversified by also including more macrophages and monocytes, with reduced MCP-1 and IL-6 levels. Compared to ovx, blood leukocyte numbers increased already 1 h after i.p challenge in ovx + E2 mice.

Conclusion: Our findings suggest that ovarian hormones and estradiol can adjust the acute innate immune reaction by regulating cell recruitment to inflammatory sites, diversify the responding cell population, and at the same time down-regulate production of certain pro-inflammatory cytokines. Our results also suggest a faster responding immune system after E2. Our results bring further information into the intricate relationship between inflammation and sex steroids.

1. Introduction

Sex hormones influence a variety of normal and pathological activities, including regulating the immune system (Straub, 2007). Women often mount a better defense against infection, but are also more prone to autoimmune diseases (Whitacre, 2001; Whitacre et al., 1999). Reduced clinical symptoms of rheumatoid arthritis (RA) often occur during pregnancy, indicating that sex steroids can relieve joint inflammation (Silman and Pearson, 2002; Marder et al., 2016). In contrast, severity of gingivitis can increase during pregnancy, suggesting that sex steroids enhance the immune defense in a more acute, innate inflammation (Wu et al., 2016). In trauma and infection, large studies also suggest that males have increased risk of mortality due to sepsis compared to women (Schoeneberg et al., 2013; Liu et al., 2015).

The regulation of inflammation by sex steroids, and estrogen in particular, has been shown to be a direct effect of estrogen receptor signaling, including regulation of innate cells such as macrophages and monocytes (Straub, 2007). Macrophages are crucial for directing the immune response and recruiting cells during the initiation of an acute inflammatory response. By secreting cytokines and chemokines, macrophages direct the immune system depending on stimulation (Murray and Wynn, 2011). Isolated *in vitro* studies with macrophages and neutrophils (Ghisletti et al., 2005; Pioli et al., 2007; Ito et al., 1995; Campesi et al., 2017) have shown anti-inflammatory effects after E2

Abbreviations: ANOVA, analysis of variance; ANCOVA, analysis of covariance; CBA, cytometric bead array; E2, 17β-estradiol; FMO, fluorochrome-minus-one; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MFI, geometric mean fluorescence intensity; ovx, ovariectomy; RA, rheumatoid arthritis; TNFα, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule-1

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treatment, whereas *in vivo* studies focusing on chronic estradiol (E2) administration and E2's specific effects on macrophages have shown pro-inflammatory effects (Calippe et al., 2010; Calippe et al., 2008). We have previously shown that E2 treatment ameliorates frequency and severity of collagen induced arthritis and regulates both neutrophil and macrophage infiltration into joints (Stubelius et al., 2011). These innate immune cells also participate in acute inflammation. Here, by comparing intact animals to ovariectomized (ovx) mice, and ovx mice treated with E2 (ovx + E2), in two animal models of acute inflammation, we could discern regulatory effects by ovarian hormones in general, and effects specifically dictated by E2 on the innate immune system.

2. Methods

2.1. Animals and experimental procedures

The regional ethical review board in Gothenburg approved all procedures and were carried out according to the ARRIVE guidelines. Mice on C57BL/10.Q wild-type background were a kind gift from professor Rikard Holmdahl at the Karolinska Institute in Stockholm, Sweden (Hultqvist et al., 2004). All mice were housed in a temperature-controlled room with a 06.00–18.00 hours light cycle, consumed a soy-free diet (R70, Lantmännen, Stockholm, Sweden) and tap water *ad libitum*. At 7–10 weeks of age, female mice were anesthetized by Isofluran (Baxter Medical Ab, Kista, Sweden) inhalation and the ovaries were removed after a midline incision of the skin, and flank incisions of the peritoneum. Sham-operations were performed similarly, however, the ovaries were left intact. Carprofen (Orion Pharma AB, Sollentuna, Sweden) was administered subcutaneously as postoperative analgesic. Mice were left to recover for three weeks before experiments were started.

2.1.1. Drug administration

Subcutaneous injections of 17β-Estradiol-3-benzoate (E2; 0.06 mg/kg; Sigma Aldrich, Stockholm, Sweden) or Miglyoil 812 (Omya Peralta, Hamburg Germany; 100 µl/mouse) were administered for three days prior to termination. Mice with air pouches were administered treatment under Isofluran anesthesia.

2.1.2. The air pouch model of inflammation

Dorsal pouches were raised by subcutaneous injection of 5 ml sterile-filtered air on day 1, and were maintained by re-injecting 3 ml of sterile-filtered air on day 4. Before the injections, mice were briefly anesthetized with Isofluran. On day 7, inflammation was induced by injecting 10 µg of LPS (Escherichia Coli O55:H5, Sigma Aldrich) dissolved in 1 ml sterile PBS (PAA Laboratories, Pasching, Austria) into the air pouch. Six hours after LPS injection, mice were anesthetized with ketamine (Pfizer AB, Täby, Sweden) and medetomidin (OrionPharma, Espoo, Finland) for blood withdrawal, and then euthanized by cardiac puncture. The uteri were weighed and the exudates of the pouches were collected by washing 3 times with 1 ml ice-cold RPMI 1640 without phenol red (PAA Laboratories). The studies were repeated three times with similar results in cell infiltration and subsequently pooled when possible.

2.1.3. Intraperitoneal LPS injections

Ovx mice were injected with either 10 µg of LPS or PBS as control. One hour later, mice were euthanized, blood was collected and the peritoneal cavity was lavaged with 3 ml PBS. The total number of leukocytes was counted using an automated cell counter (Sysmex Europe GmbH, Nordenstedt, Germany).

2.2. Single cell preparation and immune-phenotyping by flow cytometry

The collected cells from the pouch was centrifuged and the supernatants were stored at –20 °C until further analyzed. Cells were further washed with PBS, re-suspended in FACS-buffer containing PBS, 10% fetal calf serum (Sigma Aldrich) and 1% sodium azide (Sigma Aldrich). A skin section was cut from the pouch's surrounding tissue and weighed to ensure equal representation. The skin was incubated for 1 h at 37 °C with 2 µg/ml Collagenase IV (Sigma Aldrich), mashed through a 70 µm filter, washed with PBS, re-suspended in FACS-buffer, and then analyzed by FACS. Expression of cell surface markers was detected using fluorochrome-conjugated antibodies following Fc-blocking (anti-mouse CD16/CD32; clone 2.4G2); anti-CD11b V450 (clone M1/70; BD Biosciences, San Jose, CA, USA). From Biolegend, San Diego, CA USA: anti-F4/80 FITC (clone BM8), anti-Gr1 PerCp (Clone RB6-8C5), anti-CD200R PE (clone OX-110), anti-CD31 FITC (clone 390), and anti-CD106 VCAM Pe/Cy7 (clone 429 MVCAM.A). Neutrophils were defined as being: $CD11b^+Gr1^+F4/80^-$, monocytes being $CD11b^+Gr1^+F4/80^+$, and macrophages defined as being $CD11b^+Gr1^-F4/80^+$ and analyzed using Boolean gates to diminish overlap of cells into multiple populations. Fluorescence-minus-one (FMO) was used as control. Cells were acquired by a FACSCanto II (BD Biosciences) equipped with Diva Software (BD Biosciences), and data were further processed using FlowJo software (Three Star, Inc., Ashland, VA, USA). Data are presented as percentage positive and viable singlet leukocytes. Geometric mean fluorescence intensity (MFI) was calculated for CD200R expression on live cells.

2.3. Cytokine analysis

Cytometric bead array (CBA; BD Biosciences) was used to determine levels of monocyte chemotactic protein-1 (MCP-1), IL-6, tumor necrosis factor alpha (TNFα) and IL-10 from the air pouch exudate according to the manufacturer's instructions. Samples were run on a FACSCanto II. Detection limits were 5, 17.5, 52.7, and 7.3 pg/ml for IL-6, IL-10, MCP-1, and TNF-α respectively. IL-1β was determined using ELISA IL-1β ELISA MAX set (Biolegend) using manufacturer's instructions, detection limit 16 pg/ml.

2.4. Statistical analysis

Statistical analysis was performed using SPSS software 20.0.0 (IBM Corp) and GraphPad Prism (GraphPad Software) version 6.0c. Logarithmic transformation of data was used when appropriate to ensure normal distribution. T-test was used when comparing two independent groups. One-way analysis of variance (ANOVA) was used for comparison of three independent groups; all groups were statistically compared followed by Tukey's *post hoc* multiple comparisons test. Whenever Levene's test revealed unequal variances between the groups, Dunnett's T_3 *post hoc* test was used instead. An analysis of covariance (ANCOVA) followed by a Tukey's *post hoc* test was used when adjustments for covariates were needed, *i.e.* when two experiments were pooled and for day-to-day variation at the termination of experiments. P values < 0.05 were considered statistically significant.

3. Results

3.1. Blood leukocytes increase after LPS challenge in ovx mice with the air pouch model

Mice were either sham or ovx operated, treated with either vehicle or E2 and then the wet uteri weight was recorded. Treatment with E2 (0.06 mg/kg) for three days resulted in mean uterine weight of 64 mg (± 9.6 mg, SD), which was comparable to the mean uterine sham level of 71 mg (± 27 mg, SD, n.s). Ovx was confirmed to have a reduced mean uterine weight of 16 mg (± 4.9 mg, SD), with a statistical difference of $p < 0.0001$ compared to both sham and E2 levels.

Mobilizing and recruiting cells to the site of inflammation is an important step for initiation and perpetuation of the inflammatory reaction. 6 hours after LPS challenge we counted cells in the blood. We