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Poly (I:C) and LPS induce distinct immune responses by ovarian stromal fibroblasts☆

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ABSTRACT

Despite its anatomical location, the ovary is a site of pathogen exposure in the human female reproductive tract (FRT). However, the role of ovarian stromal fibroblasts in immune protection is unclear. We generated a population of ovarian stromal fibroblasts derived from normal human ovaries that expressed the pattern recognition receptors TLR3, TLR4, RIG-I, & MDA5. Poly (I:C) and LPS, respective mimics of viral and bacterial infections, selectively upregulated antiviral gene expression and secretion of chemokines and antimicrobials. Poly (I:C) exclusively stimulated the expression of interferon (IFN) β, IFNλ1, and the IFN-stimulated gene OAS2. Poly (I:C) also significantly increased secretion of elafin, CCL20, and RANTES, but had no effect on SDF-1α. In contrast, LPS had no effect on IFN or ISG expression but significantly increased secretion of RANTES and SDF-1α. Secretions from poly (I:C)-treated fibroblasts had both greater anti-HIV activity and induced higher levels of CD4 + T cell chemotaxis than those from LPS-treated cells. Our studies demonstrate a potential key role for ovarian fibroblasts in innate immune protection against incoming pathogens in the normal ovary.

1. Introduction

The female reproductive tract (FRT) is a unique mucosal site whose immune system has evolved to optimize conditions for successful reproduction and immune protection (Wira et al., 2015). The ovary represents a target site for sexually-transmitted infections (STIs). Movement from the vagina to the upper FRT (uterus, Fallopian tubes, and ovaries) occurs rapidly, potentially exposing a large mucosal surface to foreign pathogens (Zervomanolakis et al., 2007; Kunz et al., 1996). In recent studies Simian Immunodeficiency Virus (SIV) deposited in the vagina reached the ovaries and infected immune cells, thus demonstrating that viral pathogens can breach the surface epithelium to infect intra-ovarian CD4 + T cells (Barouch et al., 2016; Stieh et al., 2016). In studies with the human ovary, we found that CD4 + T cells and macrophages are present and readily infectible by HIV *in vitro* (Shen et al., 2017). The ovary is also a target for bacterial STIs such as *Neisseria gonorrhea* and *Chlamydia trachomatis*, which can lead to Pelvic Inflammatory Disease (PID) (Brunham et al., 2015). PID has been proposed as a risk-factor for ovarian cancer (Lin et al., 2011), although other studies have shown no association (Rasmussen et al., 2013). Therefore, ovarian infections can potentially cause considerable pathology leading to increased morbidity and mortality in women.

The innate immune system is the first line of defense against incoming pathogens and is essential for reducing the possibility of a successful transmission event. Pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and Retinoic Acid Inducible Gene I (RIG)-like receptors (RLRs) recognize conserved moieties present on multiple classes of viral, bacterial, and fungal pathogens (Brubaker et al., 2015). TLR3, RIG-I, and melanoma differentiation-associated protein 5 (MDA5) are three well-characterized PRRs that recognize dsRNA (poly (I:C)), a common viral marker (Kato et al., 2008). In contrast, TLR4 recognizes lipopolysaccharide (LPS) present on the surface of Gram-negative bacteria (Chow et al., 1999). Recognition by PRRs leads to the rapid induction of downstream signaling cascades that culminate in the expression of interferons (IFN), interferon-stimulated genes (ISG), antimicrobials, cytokines, and chemokines that increase immune protection, inhibit pathogen survival, and recruit immune cells to the site of exposure. For example, antimicrobials such as human beta-defensin 2 (HBD2), elafin, CCL20, RANTES, stromal-derived factor 1α (SDF-1α) can inhibit HIV infection (Sun et al., 2005; Ghosh et al., 2010; Cocchi et al., 1995a; Ghosh et al., 2009; Oberlin et al., 1996), while functioning as chemokines for immune cells (Jin et al., 2008).

While the innate immune system has been extensively studied in the human FRT, most research has focused on epithelial cells and immune cells in the vagina, cervix, and endometrium. In contrast, the ovary has

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Table 1
Patient characteristics.

Patient	Age	Menopausal Status	Reason for Surgery
1	34	Pre-menopausal	Adenomyosis
2	41	Pre-menopausal	Adenomyosis
3	46	Post-menopausal	Fibroids
4	48	Post-menopausal	Prolapse
5	50	Post-menopausal	Prolapse

not been adequately investigated, and little is known about the role of normal ovarian fibroblasts in the innate immune response. In this study, we isolated and cultured purified populations of ovarian fibroblasts derived from normal human ovaries. The fibroblasts were exposed to poly (I:C), a viral ligand, and LPS, a bacterial ligand. We measured the expression of a panel of antiviral genes and secreted anti-HIV proteins in response to these treatments, as well as their effect of CD4+ chemotaxis and infection by HIV.

2. Methods and materials

2.1. Source of ovarian tissue

Ovarian tissue was obtained from five women (average age 43.8yrs; range 34-50yrs) (Table 1) undergoing hysterectomy surgery at Dartmouth-Hitchcock Medical Center (Lebanon, NH). All tissues used were distal to the sites of pathology and determined to be unaffected by disease upon inspection by a pathologist. All investigations involving human subjects were conducted according to the principles expressed in the Declaration of Helsinki and carried out with the approval from the Committee for the Protection of Human Subjects (CPHS), Dartmouth Hitchcock Medical Center, and with written informed consent obtained from the patients before surgery.

2.2. Isolation of ovarian fibroblasts

Ovarian tissues were minced into 1–2 mm fragments and subjected to digestion using an enzyme mixture containing final concentrations of 0.05% collagenase type IV (Sigma-Aldrich, St. Louis, MO) and 0.01% DNAse (Worthington Biochemical, Lakewood, NJ). After enzymatic digestion for 1 hr at 37 °C, cells were dispersed through a 250-μm mesh screen (Small Parts, Miami Lakes, FL), washed, and suspended in Hank's Balanced Salt Solution (Thermo Fisher, Logan, UT) followed by further filtration through a 20-μm nylon mesh filter (Small Parts). Epithelial sheets and large debris were retained on the 20-μm filter, while the stromal fraction containing fibroblasts and immune cells passed through and were collected as part of the filtrate.

2.3. Ovarian fibroblast cell culture and treatment

To establish a purified *in vitro* cell culture system of human ovarian fibroblasts, the stromal filtrate was centrifuged (500 x *g*, 10 min), the pellet resuspended in complete media, and placed in a 75cm^2 cell culture flask (Thermo Fisher). Medium was changed every 2 days. Complete medium consisted of DMEM/F12 supplemented with 20 mM HEPES (Invitrogen), 2 mM L-glutamine (Invitrogen), 50 mg/ml Primocin (Invivogen) and 10% heat-inactivated defined Fetal Bovine Serum (FBS) (Thermo Fisher). After reaching confluence, the cells were trypsinized and 1x10^6 cells added to a fresh 75cm^2 flask. This was repeated at least once more before the cells were recovered and plated (1×10^6 cells/ml) in 24-well cell culture dishes (CytoOne, USA Scientific, Ocala, FL) in 500 μl of complete medium with 10% charcoal-dextran stripped FBS (Gemini, West Sacramento, CA) for at least 48 h prior to treatment. Cells were treated with HMW-poly (I:C) (Invivogen) at 2.5–25 μg/ml or Ultra-pure LPS-EK (derived from the *Escherichia coli* K12 strain) (Invivogen) at 0.1–1 μg/ml for up to 48 h.

2.4. TaqMan real-time RT-PCR

Total mRNA was isolated and purified using an RNeasy mini kit (Qiagen, Valencia, CA) with on-column DNase digestion using the RNase-Free DNase set (Qiagen) according to the manufacturer's recommendations. 400 ng of total RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's recommendations. Relative mRNA expression levels of genes of interest were measured using the 5′ fluorogenic nuclease assay in real-time quantitative PCR using TaqMan chemistry on the ABI 7300 Prism real-time PCR instrument (Applied Biosystems, Carlsbad, CA). PCR was conducted using the following cycle parameters: 95 °C, 12 min for 1 cycle (95 °C, 20 s; 60 °C, 1 min), for 40 cycles. Analysis was conducted using the sequence detection software supplied with the ABI 7300. Relative expression levels were expressed as a fold-increase in mRNA expression and calculated using the formula $2_t^{-\Delta\Delta C}$.

2.5. Flow cytometry

Prior to treatment with poly (I:C) or LPS, cells were analyzed for surface expression of CD45-VioletFluor 450 (Tonbo Biosciences, San Diego, CA, USA), CD54-PE (Biolegend, San Diego, CA), CD73-PE (R&D Systems, Minneapolis, MN), CD90-APC (Thermo Fisher), CCR6-PE-Cy5.5 (Biolegend), PD-L1-PE-Cy7 (BD Biosciences, San Jose, CA), and EpCam-FITC (BD Bioscicences). Each marker was analyzed independently. Flow cytometry was performed using a MACSQuant flow cytometer (Miltenyi Biotec) using MASCQuantify software. Analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR).

2.6. Cytokine secretion

Elafin, Human Beta Defensin 2 (HBD2), CCL20, Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), and Stromal-Derived Factor (SDF-1α) in cell culture supernatants were measured using a custom microsphere multiplex assay developed by our group (Boesch et al., 2013).

2.7. CD4 + T cell chemotaxis

Chemotaxis assays were performed using Incucyte Clearview 96-well cell migration plates (Essen Biosciences, Ann Arbor, MI) and analyzed using an Incucyte Zoom (Essen Biosciences) as per the manufacturer's instructions. Briefly, the apical chamber of the cell migration plates was coated with Protein G (Thermo Fisher) and ICAM-I (Thermo Fisher) prior to addition of activated CD4 + T cells (see below). Undiluted conditioned media from ovarian fibroblast cultures was placed in the basolateral chamber and chemotaxis of activated CD4 + T cells measured every 30 min for the following 24 h using the Incucyte Zoom. Analysis of chemotaxis was performed using the Incucyte Zoom software module.

2.8. Virus

HIV-GFP-BaL: Replication-competent GFP-encoding infectious molecular clone (IMC) (Dr. Christina Ochsenbauer, University of Alabama at Birmingham), pNLENG1i-BaL.ecto (Ochiel et al., 2010), was derived from pNLENG1-ires (Gelderblom et al., 2008) to express heterologous BaL *env* gene sequences in an isogenic backbone as described (Ochsenbauer et al., 2012; Edmonds et al., 2010). Such reporter viruses, collectively referred to as Env-IMC-GFP, express GFP upon infection of HIV-1 susceptible cells (Ochiel et al., 2010; Rodriguez-Garcia et al., 2013).

2.9. HIV infection of CD4 + T cells

CD4 + T cells were isolated by negative bead selection (Miltenyi