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Modulation of NALP3 Inflammasome Genes by Estrogen

Anton P. Chesnokov Mr.

Georgia State University, achesnokov1@student.gsu.edu

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MODULATION OF NALP3 INFLAMMASOME GENES BY ESTROGEN

by

ANTON CHESNOKOV

Under the Direction of Dr. Roberta Attanasio

ABSTRACT

Immunity is known to be sexually dimorphic. This dimorphism may be attributed to the action of different hormones. Aluminum is a component of several vaccines and acts as an adjuvant of immunogenicity. The activation of the Nalp3 inflammasome plays a role in aluminum's adjuvancy. Estrogen affects immune cells by regulating the expression of genes involved in immune-related mechanisms; such as the modulation of cytokine secretion. We hypothesized that estrogen modulates the aluminum-induced secretion of IL-1 β and IL-18. Using an *ex vivo* mouse macrophage model this study examined: (i) the effects of estrogen on Nalp3 inflammasome genes expression and (ii) the estrogen receptor involved in the modulation of these genes. Our results indicate that estrogen up-regulates Nalp3 gene expression via ER α / β heterodimerization, and caspase-1 activity may be indirectly modulated due to the up-regulation of SPI-6 via ER β .

INDEX WORDS: Nalp3 inflammasome, Estrogen, IL-1beta, Aluminum hydroxide

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by

ANTON CHESNOKOV

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

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2011

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ANTON CHESNOKOV

Committee Chair: Dr. Roberta Attanasio

Committee: Dr. Franco Scinicariello

Dr. Andrew Clancy

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iv
LIST OF FIGURES.....	vii
LIST OF ABBREVIATIONS.....	vii
1 INTRODUCTION.....	1
1.1 Purpose of the Study.....	7
2 EXPERIMENTS.....	8
2.1 Animals.....	8
2.2 Ovariectomy.....	8
2.3 Isolation of Bone Marrow Derived Macrophages (BMDM).....	8
2.4 Cell Culture Stimulation	9
2.5 Supernatant and RNA collection.....	9
2.6 ELISA.....	10
2.7 Real-Time PCR.....	10
2.8 Flow Cytometry.....	10
2.9 Statistical Analysis.....	10
3 RESULTS.....	11
3.1 BMDM are F4/80 and CD11(b) Positive	11
3.2 17 β -estradiol Increases Nalp3 Expression	12
3.3 Stimulation of BMDM Decreases the Gene Expression of ASC	14

3.4	Estrogen Receptor Agonists Modulate Caspase-1	15
3.5	<i>Serpinb9</i> is Up-regulated in Stimulated Macrophages via ER β	16
3.6	BMDM Constitutively Express IL-1 β mRNA, but at Much Lower Levels than when Stimulated.....	17
3.7	17 β -Estradiol does not Modulate IL-1 β Secretion in BMDM Stimulated with Aluminum and LPS.....	18
4	DISCUSSION.....	19
5	CONCLUSIONS.....	24
6	REFERENCES.....	25

LIST OF FIGURES

Figure 1.1 Murine BMDM were F4/80 Positive	11
Figure 1.2 Murine BMDM were CD11(b) Positive.....	11
Figure 2.1 17 β -estradiol Increased Endogenous Nalp3 Gene Expression.	13
Figure 2.2 17 β -estradiol Increased Nalp3 Gene Expression in Stimulated Macrophages.....	13
Figure 3.1 Stimulation of BMDM with Aluminum and LPS Down-regulated ASC Gene Expression	14
Figure 4.1 Estrogen Receptor Beta Increased Caspase-1 Gene Expression.....	15
Figure 5.1 Estrogen Receptor Beta Increased <i>Serpinb9</i> Expression in Stimulated Macrophages	16
Figure 6.1 BMDM Increased Production of IL-1 β mRNA after Stimulated	17
Figure 7.1 Estrogen Receptor Agonists Did Not Modulate IL-1 β Secretion in BMDM Stimulated with Aluminum and LPS	18

LIST OF ABBREVIATIONS

Al.....	Aluminum Hydroxide
LPS.....	Lipopolysaccharide
M ϕ	Macrophage
BMDM.....	Bone Marrow-Derived Macrophage
E2.....	17 β -estradiol
PPT.....	Propylpyrazoletriphenol
DPN.....	Diarylpropionitrile
Nalp3.....	Nucleotide-binding site and leucine-rich repeats with N-terminal pyrin domain containing 3
ASC.....	Apoptosis-associated speck-like protein containing a caspase recruitment domain
IL-.....	Interleukin
SPI-6.....	Serine protease inhibitor-6

1 INTRODUCTION

Since Edward Jenner developed a smallpox vaccine at the turn of the 19th century, vaccinations have been an efficient and common means of protecting the population from infectious diseases. Today, a large number of vaccines are administered for many different kinds of diseases. An adjuvant of a vaccine allows for a more pronounced stimulation of the immune system, i.e. resulting in higher antibody titers. Aluminum has been administered in vaccines as an adjuvant since the 1930's. Rhoads (1930) found an aluminum precipitated antigen to provide immunity against polio in Rhesus macaques. In 1932, Park and Schroder reported a higher percentage of children obtained immunity when vaccinated with an aluminum precipitated diphtheria toxin. Eventually a large study by Volk and Bunney (1939) included 1,800 children and found that the best response was induced in patients who took the aluminum precipitated toxoid. The aluminum adjuvant is known to increase the effectiveness of vaccines by increasing antibody titers (Volk and Bunney 1941; Gupta 1998). The initial findings suggested that absorption of the toxoid/antigen onto the aluminum resulted in the precipitation of the toxoid/antigen from it, resulting in a steady release of antigen (Carlinfanti 1949; Gupta 1998). In theory, this steady release would maintain stimulation of the immune response longer and is a result of the adjuvant's action.

It has been recently established that a protein complex known as the 'Nalp3 inflammasome,' found within, but not limited to, monocytes, macrophages, and dendritic cells (Eisenbarth, Colegio et al. 2008), allows for aluminum's adjuvancy. The complex is composed of several proteins: Nalp3, ASC, pro-Caspase-1, and possibly CARDINAL. The protein nucleotide-

binding site (a.k.a. nucleotide-binding oligomerization domain) and leucine-rich repeats with N-terminal pyrin domain containing 3 (Nalp3), seems to be the first cellular proteins to be involved. This protein is constitutively expressed, but without appropriate signals remains in an inactive conformation with interactions with HSP90 and SGT1. (Mayor, Martinon et al. 2007). Upon stimulation with danger associated molecular patterns, such as ATP or pathogen associated molecular pattern, Nalp3 is presumed to detach from HSP90 and SGT1 and exposes the pyrin domain (Duncan, Bergstralh et al. 2007; Mayor, Martinon et al. 2007). The activation of Nalp3 is followed by the binding of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) to Nalp3's pyrin domain (Mariathasan, Newton et al. 2004; Franchi and Gabriel 2008). This model suggests that ASC (as well as CARDINAL, in humans), once bound to Nalp3, allows for the binding of pro-caspase-1, resulting in caspase-1 activation (Agostini, Martinon et al. 2004; Mariathasan, Newton et al. 2004; Petrilli, Dostert et al. 2007; Church, Cook et al. 2008). The activated caspase-1 can now cleave pro-cytokines IL-1 β and IL-18, turning them into active cytokines (Thornberry 1992; Gu, Kuida et al. 1997; Eisenbarth, Colegio et al. 2008; Franchi and Gabriel 2008). Aluminum salts activate the Nalp3 protein, either directly or indirectly and aluminum's adjuvancy manifests in the secretion of the IL-1 β and IL-18 cytokines (Li, Nookala et al. 2007; Eisenbarth, Colegio et al. 2008; Gregorio, Tritto et al. 2008; Li, Willingham et al. 2008).

The cytokines IL-1 β and IL-18 are included in the IL-1 gene family, but produce different effects on the cells they target. IL-1 β is an endogenous pyrogen, or inflammatory inducer, and acts on many different types of tissues; it induces fever, dilation of vascular endothelium, and increasing expression of cell adhesion molecules (Parham 2009). IL-1 β can also activate B-cells

and T-cells. IL-18 contributes to inflammation and can be produced by different cell types, including macrophages (Stoll, Jonuleit et al. 1998; Murakami, Otsuki et al. 2005; Dinarello 2006), and, together with IL-2 or IL-12, co-stimulates the production of interferon- γ and other cytokines from T-cells and macrophages, among others (Dinarello 1996, 2006, 2009). This co-stimulation aids in the development of type 1 T helper cells, via interferon- γ (Dao, Ohashi et al. 1996; Stoll, Jonuleit et al. 1998), which function to activate macrophages (Parham 2009).

Studies have shown that after having been stimulated with appropriate factors, mice deficient in Nalp3, ASC, or Caspase-1 are unable to secrete cytokines (Mariathasan, Newton et al. 2004; Franchi and Gabriel 2008; Kool, Petrilli et al. 2008; Li, Willingham et al. 2008). Aluminum does not stimulate the production of cytokines, but rather only activates the Nalp3 protein; the synthesis of cytokines involves a separate pathway, including Toll-like-receptor ligands such as lipopolysaccharides (LPS) (Li, Nookala et al. 2007; Li, Willingham et al. 2008). In addition to the Nalp3 protein's importance in the secretion of these cytokines, higher antibody titers have also been attributed to Nalp3 gene expression; titers against a specific antigen are decreased in Nalp3 knock-out mice (Eisenbarth, Colegio et al. 2008; Li, Willingham et al. 2008).

Varying effectiveness of vaccines between the sexes has been reported (Giron-Gonzalez, Moral et al. 2000; Card, Carey et al. 2006; Cook 2008). All the factors for this sexual dimorphism are not known, but it has been shown that estrogen plays a role in immunity and/or on immune cells (Hu, Mitcho et al. 1988; Deshpande, Khalili et al. 1997; Kramer and Wray 2002; Masseoud 2008; Nguyen, Masseoud et al. 2011). Estrogen has been found to affect IL-1 production of monocytes/macrophages, seemingly increasing IL-1 production (Yoshida, Nakamura et al. 1996), with negative feedback at higher concentrations (Hu, Mitcho et al. 1988; Polan, Da-

niele et al. 1988; Polan, Loukides et al. 1989). However, a more recent study found that human peripheral blood monocytes increase IL-1 β secretion at high levels of estradiol (100 nM) (Pioli, Weaver et al. 2006). In correlation with this, 17 β -estradiol (E2) up-regulates IL-1 β mRNA expression (Harris, Feldberg et al. 2000; Pioli, Weaver et al. 2006). Estrogen-estrogen receptor complex is known to enhance the IL1- β promoter (Ruh, Bi et al. 1998). Also, an estrogen responsive B box protein has been identified to interact with proIL-1 β , enhancing secretion (Munding, Keller et al. 2006). Further, the IL-1 β transcription factor, NF- κ B, was shown to interact with estrogen receptor α (Ghisletti, Meda et al. 2005; Gionet, Jansson et al. 2009), but the effects are not clear.

However, a contradictory report suggests a down regulation of IL-1 secretion monocytes/macrophages in response to E2 (Deshpande, Khalili et al. 1997; Morishita, Miyagi, and Iwamoto 1999). Another study showed that sera from male mice express higher responses to LPS stimulation with respect to cytokine secretion (Marriott, Bost et al. 2006). This same study showed that male macrophages secrete more IL-1 β *in vitro*, than females. Further, a trend of lower IL-1 β levels was observed from cells of postmenopausal women undergoing estrogen replacement therapy than those not undergoing the therapy (Rogers and Eastell 1998). Similarly, postmenopausal women were found to have increased IL-18 levels than fertile women (Cioffi, Esposito et al. 2002). In addition to contrary data, human monocyte-derived macrophages were shown not to alter IL-1 β gene expression levels in the presence of E2 at low or high concentrations (Corcoran, Meydani et al. 2010). Also, a study by Lewis et al. (2008) shows no effect on IL-1 β secretion in microglia when treated with 17 β -estradiol, but a reduction when treated

with DPN, an ER β agonist. Estrogen has also been shown to impact IL-18 mRNA production, which was inhibited in the mouse uteri (Murakami, Otsuki et al. 2005).

In addition to the direct modulation of IL-1 β and IL-18 gene expression, estrogen receptors can up-regulate the expression of a caspase-1 inhibitor, serine protease inhibitor 6 (SPI-6), which also inhibits granzyme B, a cytotoxic enzyme secreted by cytotoxic T-cells and natural killer cells. SPI-6 gene expression is induced in response to both LPS and IL-1 β in some immunoprivileged cells, suggesting a negative feedback mechanism for controlling inflammation and/or protection from granzyme B degradation (Kannan-Thulasiraman and Shapiro 2002; Bots, de Bruin et al. 2007; Andrew, Simkins et al. 2008). The SPI-6 gene, *Serpinb9*, contains an estrogen responsive unit downstream of the transcription site and is responsible for estrogen induced expression (Krieg, Krieg, and Shapiro 2001; Krieg, Krieg et al. 2004). Regardless of the influence that estrogen may have on the expression of SPI-6, Stout-Delgado et al. (2007) found no difference in expression between genders in hepatocytes from mice challenged with a viral infection. The expression of SPI-6 is well established in the dendritic cell model, an antigen presenting cell of the myeloid lineage (Andrew, Simkins et al. 2008); immunohistological data of multiple tissues showed negative staining for SPI-6 in macrophages (Bladergroen, Strik et al. 2001). However, preliminary data from our lab suggests SPI-6 expression in the peritoneal macrophage cell line PMJ2, but estrogenic modulation was not present (unpublished). The expression in macrophages and the potential estrogenic modulation of this gene may imply a mechanism for estrogenic control of the Nalp3 inflammasome activity, namely the ability of caspase-1 to cleave pro-cytokines IL-1 β and IL-18.

The clinical implications of estrogenic modulation of the inflammasome genes can be applied to macrophagic myofasciitis and several chronic inflammatory diseases, collectively known as cryopyrin-associated periodic syndrome (CAPS). CAPS is attributed to mutations within the Nalp3 gene (Hoffman, Mueller et al. 2001; Verma, Eriksson et al. 2010; Hoffman and Brydges 2011). Agostini et al. (2004) showed that monocytes from individuals with Muckle-Wells syndrome, a type of CAPS, constitutively secrete IL-1 β , and these levels were only slightly lower than stimulated macrophages from normal donors. Murine bone marrow derived macrophages containing a Nalp3 mutation that is common among sick individuals produce constitutive IL-1 β and the addition of pathogen associated molecular patterns induced further secretion without the aid of a Nalp3 activator, such as ATP (Meng, Zhang et al. 2009). Therapy for CAPS often addresses the suppression of IL-1 β , via antagonists, namely, Anakinra. Macrophage myofasciitis is a newly described chronic inflammatory disease which is attributed to the inability of the body to remove the aluminum that is injected with a vaccine and thus causing chronic stimulation of macrophages at the site; the presence of aluminum within macrophages at the injection site of vaccines have repeatedly been reported (Lacson, D'Cruz et al. 2002; Lach and Cupler 2008). If estrogen has the ability to regulate the inflammatory response, via Nalp3 inflammasomal modulation, then an increase in systemic estrogen has the potential to influence the degree and duration of chronic inflammation in patients with macrophage myofasciitis or CAPS.

Preliminary data from our lab show that in the presence of 17 β -estradiol Nalp3 gene expression is up-regulated when the peritoneal macrophage PMJ2 is stimulated with ATP; however, this modulation is not present when stimulated with aluminum hydroxide (unpublished). If

estrogen modulates the expression of Nalp3 inflammasome genes the diseases associated with it can be understood on a gender basis. Also, controlling hormonal levels in these patients may be viewed as a possible therapeutic approach.

There are many factors involved in the processing of both steroids and cytokines, making it difficult to obtain a clear understanding of estrogen's impact on IL-1 β . Currently, there are no studies that have observed estrogen's effects on aluminum adjuvancy. This study seeks to understand estrogen effects on Nalp3 associated gene expression. Determining the impact that estrogen may have on aluminum induced cytokine secretion and on Nalp3 inflammasome gene expression may help understand how estrogens impact aluminum adjuvancy. It is hypothesized that 17 β -estradiol will: (i) decrease aluminum induced IL-1 β secretion from murine bone marrow-derived macrophage, and (ii) up-regulate SPI-6 gene expression via estrogen receptor β . In support of this hypothesis, cells treated with E2 and diarylpropionitrile (DPN), an ER β agonist, are expected to show lower levels of IL-1 β in the supernatant, and this will correlate with an increase in gene expression of SPI-6.

1.1 Purpose of the Study

No study has addressed the influence of estradiol on aluminum adjuvancy. This study investigates how estradiol modulates the genes of the Nalp3 inflammasome, which is a key factor in aluminum adjuvancy. The genes of direct interest to the inflammasome are: Nalp3, ASC, and Caspase-1. Other genes that influence the function or outcome of the inflammasome were also investigated: namely, IL-1 β and SPI-9. This study also attempts to determine the estrogen receptor responsible for the modulation of Nalp3 inflammasome genes by using specified estrogen receptor agonists.

2 EXPERIMENTS

2.1 Animals

Female BALB/c mice (Charles River Laboratories, Inc., Wilmington, MA) were housed together in an individually ventilated cage with a 12hr day and night cycle (lights from 7am – 7pm). Cages and water were replaced once a week. Chow was isoflavone and soy-free (Stewart's Feed, #5K96). All animal procedures were conducted in agreement with the institutions policies and by the guidelines of the IACUC approved protocol.

2.2 Ovariectomy

At the age of 10.5 weeks, mice were anesthetized with 5% isoflurane gas in 100% medical grade oxygen. Mice were administered subcutaneously buprenorphine (0.1 mg/kg). While on a heating pad the mice's ovaries were removed by dorsal entry. The incisions were stapled and allowed to fall off naturally.

2.3 Isolation of Bone Marrow Derived Macrophages (BMDM)

At 6 months of age, mice were anesthetized with 5% isoflurane gas in 100% medical grade oxygen and euthanized by axillary cut-down. Femur and tibia bones were removed and tissue was removed. The bone epiphyses were removed in a biosafety cabinet and bone marrow was flushed out with RPMI1640 (Gibco) until the bone was white. The marrow plugs were mechanically disrupted with a 19-G needle and filtered through a 70 μ m cell strainer. Each mouse's bone marrow was cultured in two non-culture treated petri dishes with 20 ml of complete media (RPMI1640 supplemented with 10% FBS, 5 mM glutamine, 1 mM HEPES, and 1% Pen-Strep (Gibco), plus 10 ng/ml M-CSF (Leinco). On day 3, 5 ml of media was removed and an

additional 10 ml of complete media was added; the complete media was replaced on day 6. On day 7 cells were identified by flow cytometry with staining for F4/80 (Invitrogen) and CD11(b) (BD Pharmingen).

2.4 Cell Culture Stimulation

On day 8 of culture, cells were counted and plated in 12-well plates at 5×10^5 cells per well in 2 ml. After 24 hrs the media was replaced with 1 ml of hormone-free media (complete media without phenol red and containing charcoal-stripped FBS) supplemented with either ethanol dissolved 17β -estradiol (E2) at 1 or 10 nM, propylpyrazoletriophenol (PPT) at 5 nM, or diarylpropionitril (DPN) at 10 nM, or vehicle (ethanol at 0.001%) for 12 hrs. PPT served as an ER α agonist (Stauffer, Coletta et al. 2000), whereas DPN served as an ER β agonist (Meyers, Sun et al. 2001). Cells were then primed with lipopolysaccharide (LPS) at 100 ng/ml for 1 hr with their respective hormone. This was replaced with media containing their respective hormone, LPS, and aluminum hydroxide (Al) at 125 μ g/ml for 6 hrs. Positive controls were primed/stimulated with LPS without hormones or aluminum for 7 hrs and ATP at 5 mM for the last 20 min. All reagents were obtained from Sigma-Aldrich.

2.5 Supernatant and RNA collection

Supernatants were removed and centrifuged at 10,000 x g for 10 min at 4°C, then frozen at -80°C. Aluminum containing groups were washed 3X with PBS before RNA extraction. RNA was obtained using the RNeasy kit (Qiagen) according to manufactures protocol, and stored at -20°C.

2.6 ELISA

ELISA was conducted using a mouse IL-1 β ELISA Set (BD OptEIA). Coating buffer was a 1 M phosphoric acid solution. Assay diluent was a 5% FBS in PBS solution. The stop buffer was a 1 M phosphoric acid solution. Samples were diluted 1/50. None stimulated groups (no aluminum or LPS) were conducted in either duplicates or triplicates. Aluminum containing samples were assayed in triplicates. Multiple assays were performed.

2.7 Real-Time PCR

Total RNA (2 μ l) was reverse transcribed with AMV reverse-transcriptase (Roche) in the presence of RNase inhibitor (Roche) using random primers (Roche). Diluted (1/20) cDNA underwent real-time PCR using Taqman[®] Gene Expression Assay (Applied Biosystems) in a 7500 Fast-Realtime machine (Applied Biosystems). Relative quantification was derived by $\Delta\Delta$ Ct, with β -actin as the endogenous control.

2.8 Flow Cytometry

Cells (3×10^5) were stained for either anti-mouse F4/80 PE conjugate (Invitrogen) or anti-mouse CD11(b) FITC conjugate (BD Pharmingen). F4/80-PE antibody was compared to an isotype control (Biolegend), whereas the CD11(b)-FITC antibody was compared to an unstained cell sample.

2.9 Statistical Analysis

One-way ANOVA analysis was conducted with post-analysis using Tukey's multiple comparison test to determine significance. A p-value of <0.01 was considered statistically significant.

3 RESULTS

3.1 BMDM were Positive for F4/80 and CD11(b)

After 7 days of culture, bone marrow derived macrophages (BMDM) were stained for F4/80 expression to determine the purity of the derived macrophages. Over 98% of the cells expressed F4/80, deeming them murine macrophages (Figure 1.1). These cells were also assayed for a common monocyte/macrophage receptor, CD11(b), which was expressed at >90% of the cells (Figure 1.2).

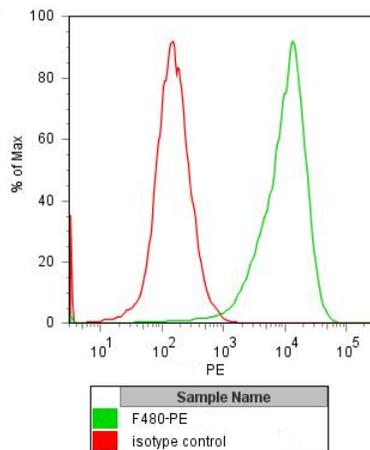


Figure 1.1 BMDM were F4/80 Positive. Bone Marrow Derived Macrophages (BMDM) (3×10^5 cells) were stained with F4/80 antibody or an isotype control (PE conjugates) and analyzed by flow cytometry. Percentage is of 20,000 recorded events.

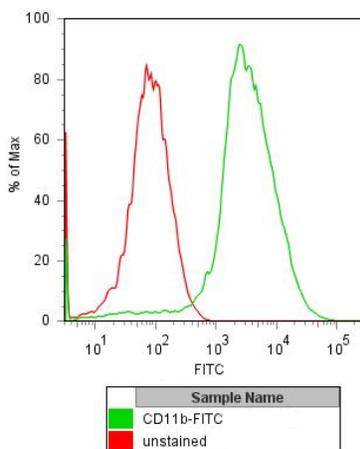


Figure 1.2 BMDM were CD11(b) Positive. Bone Marrow Derived Macrophages (BMDM) (3×10^5 cells) were stained with CD11(b) antibody (FITC conjugate) or unstained and analyzed by flow cytometry. Percentage is of 20,000 recorded events.

3.2 17 β -estradiol Increased Nalp3 Expression

To determine how Nalp3 gene expression responds to particular estrogen receptor agonists, BMDM were stimulated with aluminum hydroxide (Al) and LPS and cDNA was assayed by real-time PCR (Figure 2.1-2). Estradiol (E2) up-regulated Nalp3 gene expression in a dose dependant manner; in unstimulated cells the gene expression increased 2 and 2.5-fold with E2 at 1 and 10 nM, respectively. This similar pattern was observed in the stimulated groups, however it was not statistically significant ($p>0.05$). The Nalp3 gene expression in the no hormone group increased ~56-fold after stimulation; whereas in the presence of 1 nM E2 the increase was ~62-fold, and at 10 nM E2 it was some ~68-fold. This increase – after treatment with 10 nM E2 – in stimulated macrophages was 20% more than the no hormone group. After stimulation and in the presence of diarylpropionitrile (DPN), an estrogen receptor (ER) beta agonist, Nalp3 gene expression increased to a similar degree as macrophages stimulated in the presence of E2 at 1 nM; propylpyrazoletriphenol (PPT), the ER α agonist, did not express this similar effect. Unstimulated macrophages treated with either PPT or DPN did not up-regulate Nalp3 expression.

The increase in endogenous expression of Nalp3 expression in response to E2 and not either of the ER agonists suggests that this modulation is attributed to the heterodimerization of ER α and β , both of which bind E2. The up-regulation in the presence of E2 may deem these cells more readily responsive to Nalp3 activating molecules.

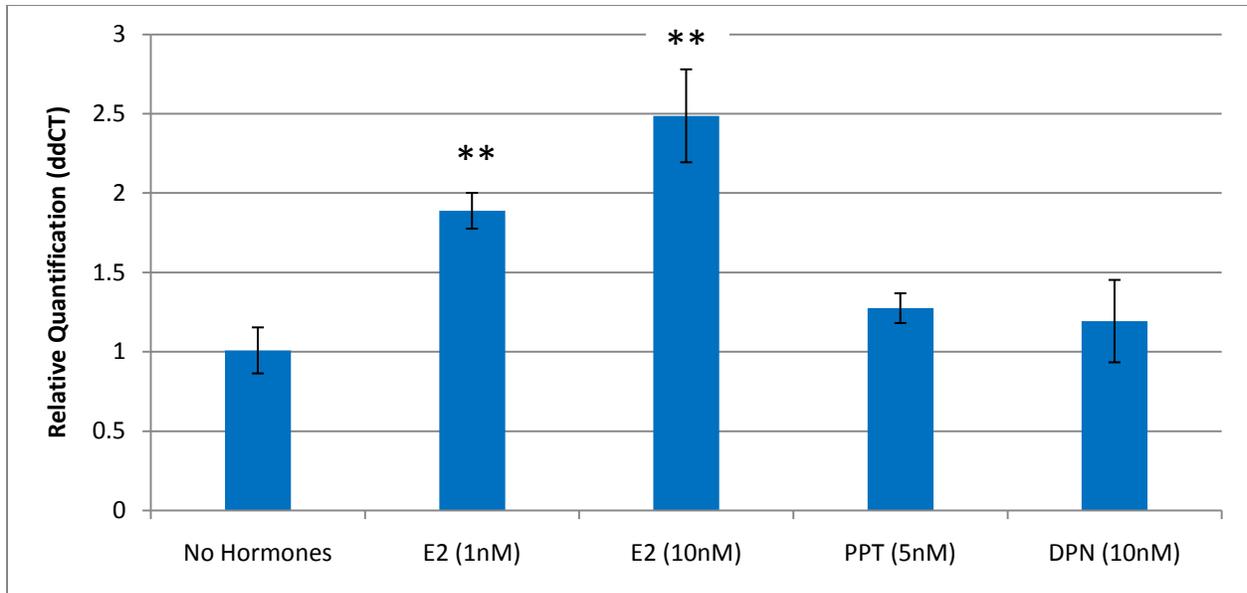


Figure 2.1 17 β -estradiol Increased Endogenous Nalp3 Gene Expression. BMDM were cultured in the presence or absence of estrogen receptor agonists for 17 hrs. “No Hormones” group contains no estrogen receptor agonists, and is the reference point of expression. Hormone containing groups are: E2, estradiol; PPT, ER α agonist; DPN, ER β agonist. Error bars represent the standard deviation of triplicate samples assayed in triplicates. ** ($p < 0.001$) derived using Tukey’s multiple comparison test.

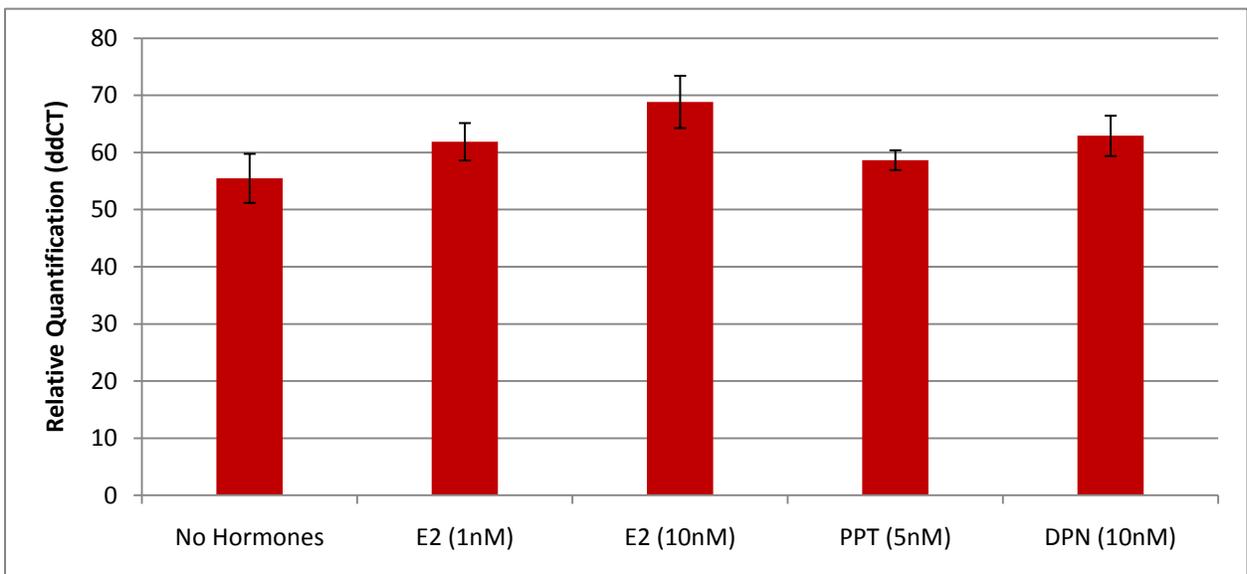


Figure 2.2 17 β -estradiol Increased Nalp3 Gene Expression in Stimulated Macrophages. BMDM were cultured in the presence or absence of estrogen receptor agonists for 12 hrs prior to stimulation with LPS (100 ng/ml) for 1 hr and another 6 hrs with aluminum hydroxide (125 μ g/ml). Quantification is normalized to a non-stimulated and no hormone containing group (not shown). Hormone containing groups are: E2, estradiol; PPT, ER α agonist; DPN, ER β agonist. Error bars represent the standard deviation of triplicate samples assayed in triplicates.

3.3 Stimulation of BMDM Decreased the Gene Expression of ASC

For Nalp3 to active caspase-1, via proteolytic cleavage, the protein ASC is required to link the two proteins. Figure 3.1 shows how estrogen receptor agonists affect ASC gene expression before and after stimulation of BMDM with Al and LPS. Estradiol did not affect the endogenous expression of ASC. The estrogen receptor α and β agonists, PPT and DPN, respectively, down-regulated the expression of this gene in unstimulated cells; however, the reduction was minor. After stimulation, the macrophages expressed about half as much ASC. This decrease was not altered in the presence of any of the hormones tested.

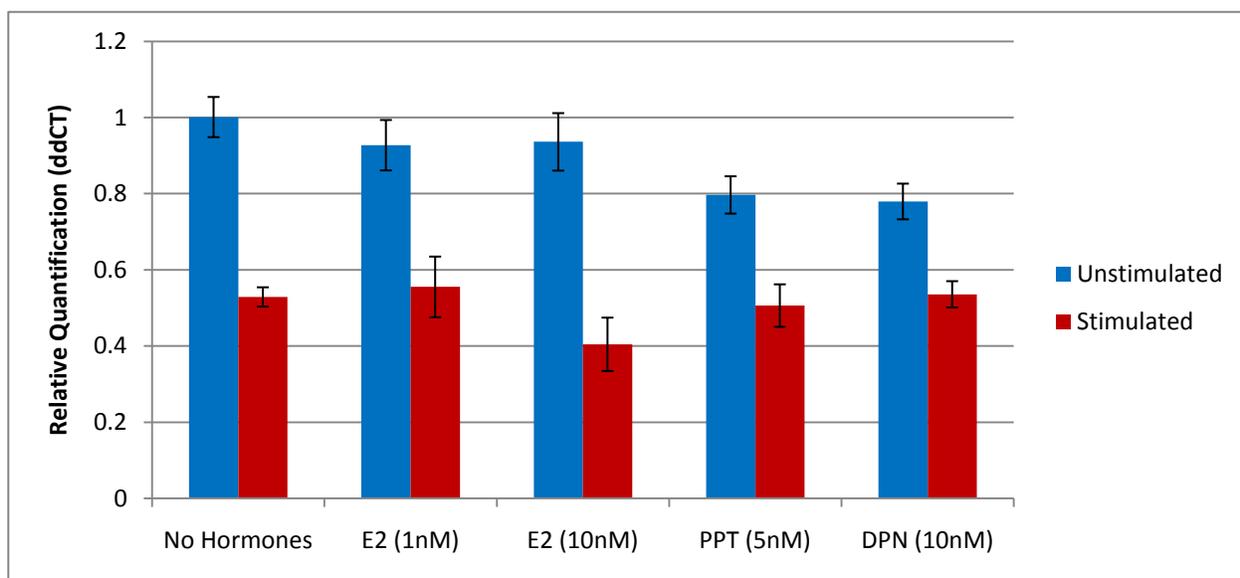


Figure 3.1 Stimulation of BMDM with Aluminum and LPS Down-regulated ASC Gene Expression. BMDM were cultured in the presence or absence of estrogen receptor agonists for 12 hrs prior to stimulation with LPS (100 ng/ml) for 1 hr and another 6 hrs with aluminum hydroxide. Quantification is normalized to the unstimulated and no hormone containing group. Hormone containing groups are: E2, estradiol; PPT, ER α agonist; DPN, ER β agonist. Error bars represent the standard deviation of triplicate samples assayed in triplicates.

3.4 Estrogen Receptor Agonists Modulated Caspase-1

The expression of caspase-1 is vital to the adjuvancy effects of aluminum on macrophages, so it was of interests to determine whether estrogen modulates the expression of this gene (Figure 4.1). DPN, an ER β agonist, up-regulated caspase-1 in stimulated macrophages; DPN resulted in over a 3-fold increase, whereas no hormones yielded a 2.5-fold increase. The increase was modest when cells were treated with estradiol, for both 1 nM and 10 nM concentrations. PPT, an ER α agonist, increased the gene expression 3-fold, slightly higher than estradiol. This would suggest that the gene may be modulated in stimulated macrophages by ER α , and to a greater extent, ER β . Endogenous levels of the gene were not altered in the presence of any of the tested hormones.

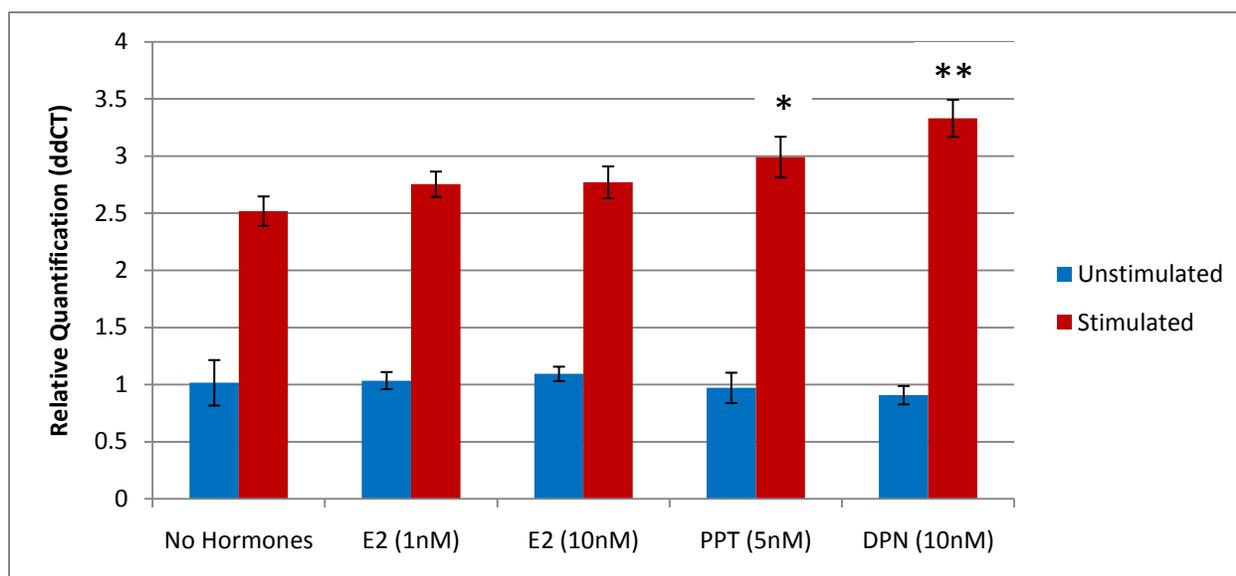


Figure 4.1 Estrogen Receptor Beta Increased Caspase-1 Gene Expression. BMDM were cultured in the presence or absence of estrogen receptor agonists for 12 hrs prior to stimulation with LPS (100 ng/ml) for 1 hr and another 6 hrs with aluminum hydroxide. Quantification is normalized to a non-stimulated and no hormone containing group. Hormone containing groups are: E2, estradiol; PPT, ER α agonist; DPN, ER β agonist. Error bars represent the standard deviation of triplicate samples assayed in duplicates (Unstimulated) or triplicates (Stimulated). *($p < 0.01$), ** ($p > 0.001$), derived using Tukey's multiple comparison test as compared with stimulated No Hormone group.

3.5 *Serpinb9* was Up-regulated in Stimulated Macrophages via ER β

To determine a possible mechanism by which estrogen may inhibit IL-1 β secretion, the expression of the gene *Serpinb9*, which codes for a natural inhibitor of caspase-1, was determined (Figure 5.1). In the BMDM cell model, activation with LPS and aluminum increased the expression of *Serpinb9*; this suggests that *Serpinb9* is an inflammatory response gene. After activation, macrophages in the presence of DPN, an ER β agonist, increased *Serpinb9* expression 5-fold, as compared to the unstimulated and no hormone containing group; whereas a stimulated cells without hormones only increased gene expression by roughly 2-fold. This deems *Serpinb9* an ER β responsive gene, but up-regulation only occurs after cell stimulation/activation; DPN, and PPT, down-regulated the endogenous gene expression levels of *Serpinb9* to half the level of both no hormone and estradiol containing groups.

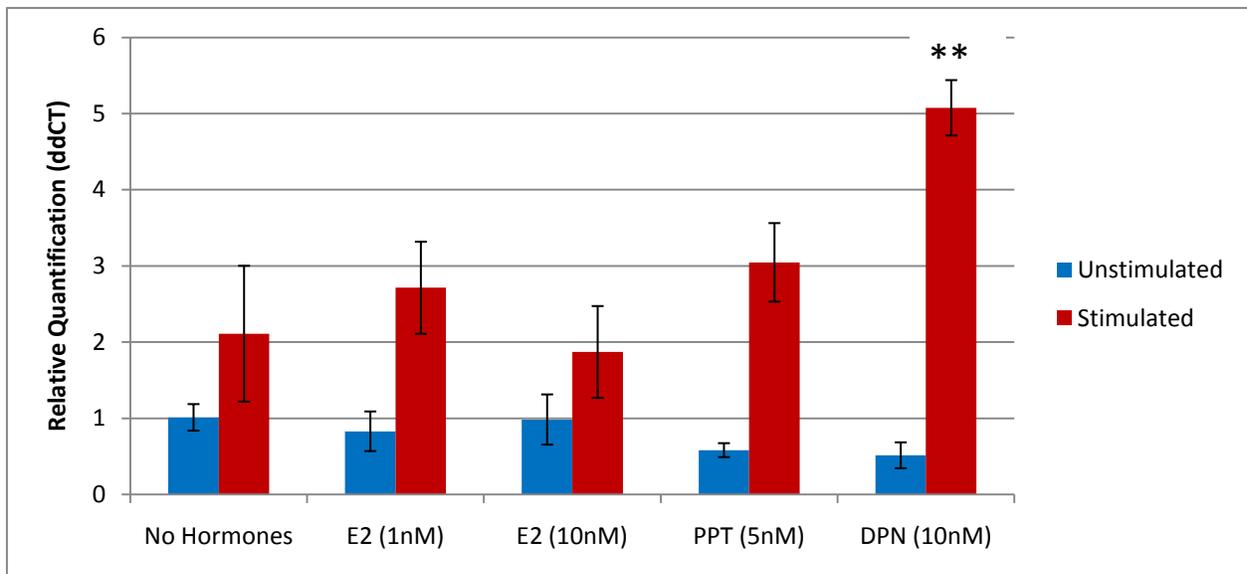
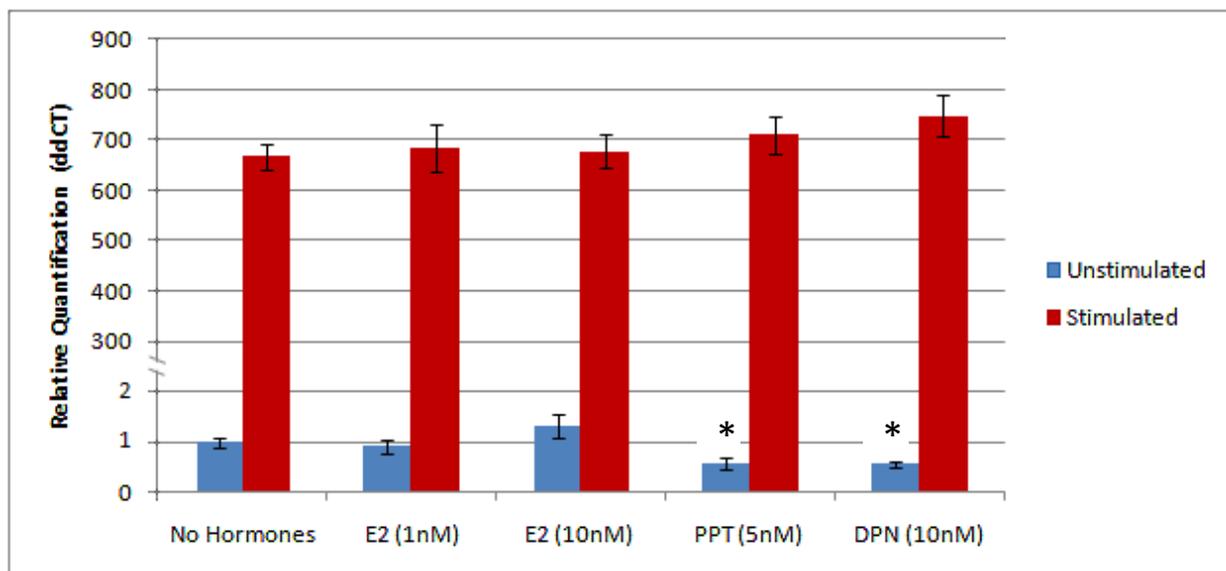


Figure 5.1 Estrogen Receptor Beta Increased *Serpinb9* Expression in Stimulated Macrophages. BMDM were cultured in the presence or absence of estrogen receptor agonists for 12 hrs prior to stimulation with LPS (100 ng/ml) for 1 hr and another 6 hrs with aluminum hydroxide. Quantification is normalized to a non-stimulated and no hormone containing group. Hormone containing groups are: E2, estradiol; PPT, ER α agonist; DPN, ER β agonist. Error bars represent the standard deviation of triplicate samples assayed in triplicates. ** ($p > 0.001$), derived using Tukey's multiple comparison test as compared with stimulated No Hormone group.

3.6 BMDM Constitutively Expressed IL-1 β mRNA, but at Much Lower Levels than when Stimulated

IL-1 β secretion is central to the Nalp3 inflammasome's function, so determining how estradiol might influence its gene expression can help in determining possible modulation of aluminum adjuvancy by estradiol. BMDM constitutively express IL-1 β mRNA, and PPT and DPN down-regulated these levels (Figure 6.1). However, these levels are minute when compared to stimulated cells. After stimulated with LPS and Al, IL-1 β gene expression in BMDM increased about 650-fold. Unlike the endogenous levels, in this model none of the estrogen receptor agonists influenced the levels of IL-1 β gene expression in activated cells (Figure 6.1).



6.1 BMDM Increased Production of IL-1 β mRNA after Stimulated. BMDM were cultured in the presence or absence of estrogen receptor agonists for 12 hrs prior to stimulation with LPS (100 ng/ml) for 1 hr and another 6 hrs with aluminum hydroxide. Quantification is normalized to a non-stimulated and no hormone containing group. Hormone containing groups are: E2, estradiol; PPT, ER α agonist; DPN, ER β agonist. Error bars represent the standard deviation of triplicate samples assayed in triplicates. *($p < 0.05$), derived using Tukey's multiple comparison test as compared with non-stimulated No Hormone group.

3.7 17 β -estradiol did not Modulate IL-1 β Secretion in BMDM Stimulated with Aluminum and LPS

To determine if estrogen receptor agonists modulate the amount of IL-1 β secreted from BMDM in response to Al and LPS, ELISA assays were conducted on the supernatants from the cell cultures stimulated with these reagents. No significant modulation was observed in any of the stimulation groups (Figure 2.1). No secretion was detected in groups that were not stimulated with Al and LPS (data not shown).

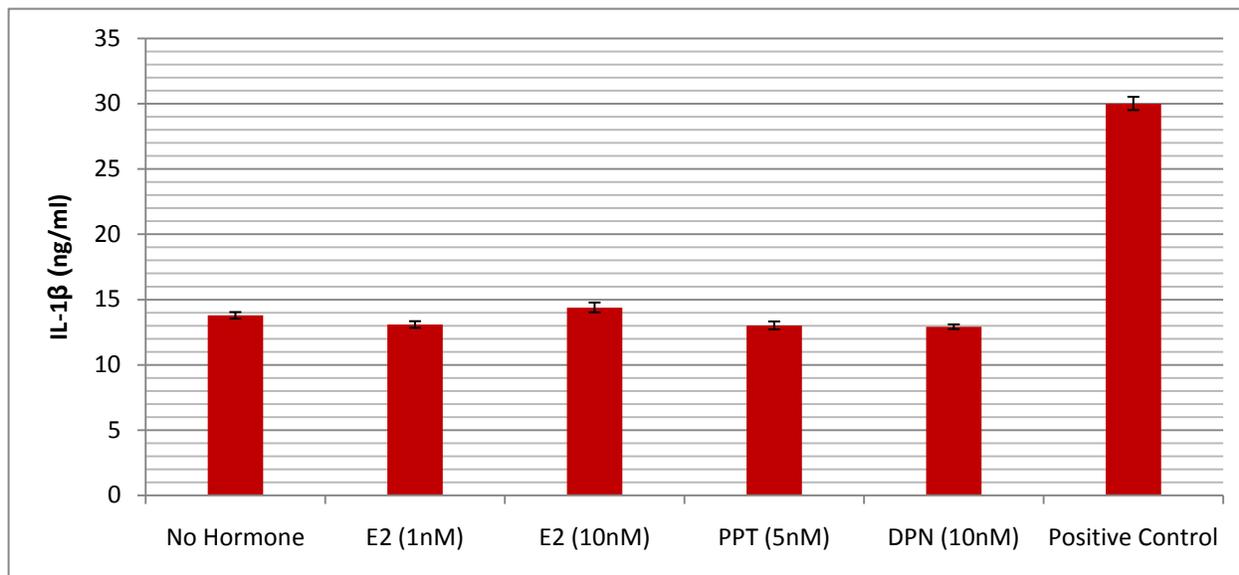


Figure 7.1 Estrogen Receptor Agonists Did Not Modulate IL-1 β Secretion in BMDM Stimulated with Aluminum and LPS. All groups were cultured in hexlicates, except E2 (10 nM) which was conducted in triplicates. Cells were stimulated with LPS (100ng/ml) for 1 hr, followed by aluminum hydroxide (125 μ g/ml) for 6 hr, except the Positive Control, which was stimulated with LPS for 7 hr and ATP (5 mM) for the last 20 min. Hormone containing groups are: E2, estradiol; PPT, ER α agonist; DPN, ER β agonist. Error bars represent standard error of the mean from six different samples, except E2 (10 nM) which is of three culture samples, assayed three different times in triplicates each time.

4 DISCUSSION

Estrogen is known to modulate several parameters of the immune system (reviewed in Bouman, Heineman, and Faas 2005). Classical estrogen receptors, ER α and ER β , are well established transcription factors that function by dimerization in either a homo- or a hetero- fashion (Kumar and Chambon 1988; Ogawa, Inoue et al. 1998). Here we investigated how 17 β -estradiol (E2) might modulate the gene expression of the Nalp3 inflammasome genes - Nalp3, ASC, and Caspase-1 – with and without lipopolysaccharide (LPS) and aluminum hydroxide (Al) activation of macrophages. In addition to E2, two estrogen receptor (ER) agonists were used: propylpyrazoletriophenol (PPT), an ER α agonist, and diarylpropionitrile (DPN), an ER β agonist. Bone marrow derived macrophages (BMDM), which express the characteristic macrophage markers F4/80 and CD11(b) (Fig. 1.1 & 1.2, respectively), are a common *in vitro* model in the study of the Nalp3 inflammasome. We also investigated the influence E2 and the ER agonists might have on IL-1 β gene expression and secretion in these cells.

If E2 has a role in modulating Nalp3 expression, then the implications may be extensive. Several chronic inflammatory diseases, collectively called CAPS, result from a constitutively active Nalp3 protein; expression of Nalp3 leads to uncontrolled secretion of IL-1 β , an endogenous pyrogenic cytokine. Estrogen has been implicated in rheumatoid arthritis (reviewed in Islander, Jochems et al. 2011), and Nalp3 induced IL-1 β has been implicated in gout (Joosten, Netea et al. 2010). To date, no reported study has determined whether E2 regulates the Nalp3 gene. Our results show that E2 up-regulates the endogenous levels of Nalp3 mRNA, and that this up-regulation is likely a result of heterodimerization of ER α and β ; PPT and DPN, ER α and ER β agonists, respectively, did not show any up-regulation in this gene. Further, the subsequent

increase in Nalp3 expression after increasing the E2 concentration to 10 nM suggests that this up-regulation is dose-dependent. This up-regulation by E2 was not apparent in the stimulated macrophages. Instead, stimulated cultures that were treated with E2 at 10 nM show a slight increase in Nalp3 gene expression, but the significance was not evident. The clear up-regulation of the Nalp3 gene in macrophages that have not been stimulated with LPS and AI may enable the cells to respond more strongly or quickly to a Nalp3 activator and hence would express the phenotypic manifestation of caspase-1 activation more readily. This would be a kinetically based response and whose effects have peaked within the six hours of stimulation that the current model used, and whose residual effects are seen by the slight variation in the stimulated cells. To verify this hypothesis, a kinetic study with multiply time points would have to be conducted. Even though E2 did not significantly influence Nalp3 gene expression in stimulated cells, the statistically significant endogenous up-regulation of Nalp3 by E2 may be implicated in the cryopyrin-associated periodic syndrome, wherein increased estrogen levels may lead to an immune flare-up.

ASC is an adaptor protein, linking caspase-1 to Nalp3, resulting in caspase-1 activation. The ASC gene was shown to decrease 2-fold in response to the LPS and AI stimuli. Indeed, Taxman et al. (2006) observed a sharp decrease in ASC mRNA expression in the human monocytic cell line THP-1, after 6 hrs of infection with *Porphyromonas gingivalis*, a gram-negative bacterium. The down-regulation of ASC in response to 6 hrs of stimulation may suggest a negative feedback to the inflammatory response, preventing the cell from inducing inflammation for too long of a period. The hormonal effects on the expression of ASC were not seen in either the unstimulated or stimulated cultures, suggesting that this gene is not modulated by estrogen.

The presence of caspase-1 is vital for an effective Nalp3 response. Caspase-1 is constitutively expressed and is up-regulated in response to LPS and AI (Figure 4.1). The endogenous level of caspase-1 gene expression is not affected by estrogen receptor agonists in cells that are not stimulated. However, DPN was found to significantly increase the levels of caspase-1 mRNA in stimulated macrophages. This increase suggests that ER β is an activator of this gene; interestingly though, neither of the E2 concentrations led to this increase. An increase in caspase-1 gene expression was observed in response to E2 in pigs' endometrium (Ashworth, Ross et al. 2010); however, these animals were not challenged with any type of PAMP and thus do not correlate with our data. Our data suggest that ER β up-regulates caspase-1 gene expression only in the presence of other stimuli, likely LPS, which is known to recruit a multitude of transcription factors resulting in an interaction. This suggests that ER β requires the presence of other transcription factors to function as an activator of transcription. Indeed, Quaedackers et al. (2007) showed ER α to directly interact NF- κ B proteins p50 and p65, which are up-regulated in LPS stimulated cells (Zandi, Rothwarf et al. 1997). Also, E2 was shown to increase NF- κ B activity in splenic macrophages (Suzuki, Yu et al. 2008). Thus, it is possible to imagine how NF- κ B transcription factors may interact with estrogen receptors. Because 17 β -estradiol binds both ER α and β , the lack of caspase-1 gene expression increase after stimulation of macrophages in the presence of E2 may be due to antagonism by ER α , whereby ER α acts as a decoy for E2, preventing enough ER β ligation for the activation of caspase-1.

Several studies have suggested that the protein SPI-6, gene name *Serpinb9*, can inhibit caspase-1 activity (Annand, Dahlen et al. 1999; Kannan-Thulasiraman and Shapiro 2002). Furthermore, *Serpinb9* has been shown to be up-regulated in response to estrogens (Krieg, Krieg,

and Shapiro 2001; Krieg, Krieg et al. 2004). As mentioned above, caspase-1 is crucial to the secretion of IL-1 β and its inhibition would result in lower secretion levels of IL-1 β ; thus, if estrogen modulates IL-1 β secretion it would be of interest to determine if *Serpinb9* up-regulation would correlate. Our data show that endogenous levels of *Serpinb9* were not affected by either of the E2 concentrations, but that the ER α and β agonists, PPT and DPN, respectively, decreased the expression levels roughly 2-fold (Figure 5.1); however this was not statistically significant. LPS is known to increase the expression of *Serpinb9* (Kannan-Thulasiraman and Shapiro 2002; Andrew, Simkins et al. 2008), which would explain the up-regulation of the gene among all the stimulated groups. More interestingly though, was the large and significant up-regulation of *Serpinb9* in response to DPN after stimulating the macrophages. This is contrary to Dr. David J. Shapiro's team at the University of Illinois, which consistently attributes ER α to the up-regulation of *Serpinb9* (Krieg, Krieg et al. 2004; Jiang, Ellison et al. 2007). Our data does suggest an increase similar to that of E2, at 1 nM, in response to the ER α agonist. Taking this into account, it is worth noting the lack in increase in E2 at 10 nM, which suggest an E2 threshold in acting as an activator of transcription. However, these data points contain lots of variation and are not statistically significant.

Several studies have attributed ER β to decreased immune responses in microglia (Baker, Brautigam and Watters 2004; Lewis, Johnson et al. 2008), the macrophages of the central nervous system. The 5-fold increase in *Serpinb9* in response to DPN, as compared to the roughly 2-fold increase in other stimulated hormonal groups, would suggest that ER β has a potential to inhibit IL-1 β secretion via SPI-6. However, our data did not support such a hypothesis; the IL-1 β secretion levels remained similar. One possibility for a lack of reduction in IL-1 β secretion can

be due to *Serpina9* not being translated and thus no phenotypic modulation is seen. Indeed, macrophages were negative for PI-9, the human homolog, in lymphoid tissue samples; however, these samples were derived from normal human tissues (Bladergroen, Strik et al. 2001).

The secretion of IL-1 β from macrophages stimulated with LPS and AI for six hours did not differ in the presence of any of the ER agonists tested (Figure 7.1). These data correlate well with the lack of modulation of IL-1 β gene expression (Figure 6.2), which is in agreement with human monocyte-derived macrophages (Corcoran, Meydani et al. 2010). The inability of the DPN up-regulated SPI-6, whose increased protein levels are assumed due to the increased mRNA levels, to reduce the amount of IL-1 β secretion may be offset by a corresponding up-regulation of caspase-1. Another explanation is that *Serpina9* might not have been up-regulated before the six hour mark. Without kinetic gene expression data we cannot determine if the observed up-regulation of *Serpina9* by DPN only just occurred at the six hour mark, and that the manifestation of caspase-1 inhibition – reduced IL-1 β secretion – can only be observed after this time point. Lewis et al. (2008) showed that DPN decreased IL-1 β secretion from LPS stimulated microglia, after 24 hours of stimulation. Our study chose six hours because LPS stimulated IL-1 mRNA peaks at this time point (Dinarello, Muegge, and Durum 2000).

Other factors that may have led to the lack of observable differences in IL-1 β secretion is the presence of M-CSF, a growth factor essential to BMDM, or the presence of LPS concentrations that are too high. Cells respond upon reaching a threshold level of stimulation; further, the response is not expressed until intracellular biochemical responses reach a particular threshold. This phenomenon explains the varying cellular responses to different concentrations of stimuli. M-CSF is known to influence IL-1 β secretion and activation of macrophages in general

(Evans, Kamdar et al. 1995; Popova, Kzhyshkowska et al. 2011). Its presence was perhaps not essential, but is common in the macrophage milieu. The M-CSF concentrations used in this study (10 ng/ml) could have masked the effects that E2 might have had on the secretion of IL-1 β by exacerbating the macrophage's response to the LPS and Al stimuli. The LPS concentration, 100 ng/ml, may also have been too high. A response curve was not conducted on these cells, so similarly to the M-CSF, LPS might have masked the potential effects of estrogen on IL-1 β secretion.

5 CONCLUSIONS

Estrogen receptor agonists estradiol, PPT, and DPN modulate gene expression of particular Nalp3 inflammasome genes. Nalp3 gene expression was increased by estradiol at 1 nM and to a higher degree with 10 nM. Nalp3 modulation by estradiol may be attributed to heterodimerization of estrogen receptor (ER) α and β ; PPT and DPN, ER α and ER β agonist, respectively, did not show any increase in gene expression. Caspase-1 was up-regulated by all three agonists; caspase-1 gene expression is modulated by both ER α and ER β , but ER β seems to have a higher degree of influence. IL-1 β gene expression was not modulated by estradiol, PPT, or DPN in this model. SPI-6 gene expression in macrophages was up-regulated in response to stimulation with Al and LPS, and this up-regulation was greatly exacerbated in the presence of DPN. However, the up-regulation of SPI-6 gene expression did not result in decreased IL-1 β secretion. In conclusion, estrogen receptor agonists did not modulate IL-1 β secretion in murine bone marrow-derived macrophages stimulated with aluminum and LPS, while in the presence of M-CSF.

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