**Functional Regulation of Dendritic Cells by Estrogen**

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**Layman’s Abstract**

Systemic Lupus Erythematosus is an autoimmune disease that arises from multiple genes. Pro-inflammatory responses and misrecognition of healthy tissue characterize the disease. It was expected that in vitro common estrogen, and a steroidal compound, as well as chemical substances from the immune system will influence the differentiation of dendritic cells—the primary entity of an immune response. qPCR was used to measure gene expression of Interleukin-6, a pro-inflammatory chemical substance, from activated dendritic cells. It was found that estrogen exposure yielded higher levels of interleukin-6, a pro-inflammatory response cytokine, only in dendritic cells cultured from the femurs of C57BL6 mice.

**Abstract**

Systemic Lupus Erythematosus is an autoimmune disease in which pro-inflammatory episodes occur from a combination of hormones and cytokines. The cells that primarily begin an immune response are dendritic cells. There is a correlation between expression of high levels of interleukin-6 and pro-inflammatory responses. Bone marrow cultures from Balb/c and C57BL6 mice 6-8 weeks of age, allowed for generation of dendritic cell cultures in RPMI 1640 media (1% L-Glutamine, 1% Pen/strep, and without phenol red) with either Granulocyte/Macrophage-Colony-stimulating factor (GM-CSF) or FMS-related tyrosine kinase 3 Ligand (FLT3L). Estradiol (E2) and ICI 182, 780 were added to separate dendritic cell cultures. After one week, dendritic cell cultures were stimulated with lipopolysaccharide to begin an in-vitro immune response. The next day RNA samples were collected for cDNA synthesis. Real-time PCR was run to obtain interleukin-6 expression levels. Flow cytometry was also run to decipher which dendritic cells were plasmacytoid or conventional, as well as CD11c expression. It was found that estradiol increased the expression of interleukin 6, thereby a rise in pro-inflammatory responses, in the C57BL6 mice but not Balb/c. E2 and ICI treatments did not affect dendritic cell differentiation either, but GM-CSF was able to culture dendritic cells from bone marrow more efficiently providing more mature sized dendritic cells.

**Introduction**

Difficulty of the immune system to recognize healthy tissue(s) from infected tissue(s) is the basis of autoimmunity. Constant stimuli are being misinterpreted: chemical, antigenic, and/or infectious agents (Wallace, Hahn, Butts, and Sternberg, 143-167).Communication between the central nervous system (CNS) and the immune system is directed into two paths instead of one, causing over production of cytokines (chemical substance(s) secreted by the immune system that effects the cells’ growth or cell-cell communication). When disruptions between signals going from the immune system to the CNS and vice versa are interrupted, inflammatory responses like inflamed joints, and autoimmunity to self-tissue(s) are prone to occur—conditions that provide the basis to study Systemic Lupus Erythematosus (Wallace, Hahn, Butts, and Sternberg, 143-167).Hormones and cytokines are the means of communication throughout the immune system, so when autoimmunity is involved the brain’s activity increases and becomes overstimulated. The brain sends its signals via two pathways: hypothalamic-pituitary-adrenal axis (HPA), and hypothalamic-pituitary-gonadal axis (HPG) Miscommunication between the HPA and HPG is the primary step of autoimmunity (Wallace, Hahn, Butts, and Sternberg, 143-167).

While no cure or well understood etiology for Systemic Lupus Erythematosus (SLE) has been determined, treatments have been postulated. Systemic Lupus Erythematosus (SLE) is a polygenetic, autoimmune disease commonly characterized by a facial malar rash. Systemic Lupus Erythematosus is diagnosed with criteria created by the American College of Rheumatology: malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, kidney disorder, neurological disorder(s), blood disorder(s), immunologic disorder, and abnormal antinuclear antibodies (American College of Rheumatology).

 The rash spreads throughout the face covering the nose, cheeks, and forehead—deemed the butterfly rash. Cranial overstimulation occurs, which is related to disease flares and hormone release, specifically estrogen. For example, a hormone such as estrogen from the HPG and glucocorticoid production from the HPA will be sent into the body at insufficient or surplus levels causing an increase in disease activity. Some cases of Systemic Lupus Erythematosus are treated immediately with glucocorticoid treatment in an effort to decrease disease flares. With increased signals being produced throughout the body, the immune system becomes over productive as well. It has been empirically found that estrogen levels are related to how active the immune system is; thus, women are more prone to being diagnosed with SLE.

Although T-cells and B-cells orchestrate immune responses, dendritic cells (DCs)—first discovered by Ralph Steinman and Zanvil A. Cohn in 1973, at Rockefeller University—tell these leukocytes what needs to be done in the immune system, presenting the dendritic cells’ antigens to T-cells and B-cells (The Rockefeller University, 2014). Rapid initiation of the immune response can’t occur without dendritic cells. (Brasel *et al*, 2000). These cells are easily influenced, being a hematopoietic cell; influenced mainly by cytokines causing DCs to differentiate into either mature, activated DCs or macrophages.

Dendritic cells are a relative minority amongst the cells of the immune system, and it has been discovered [empirically] that cytokines Granulocyte/Macrophage Colony-stimulating Factor (GM-CSF) and FMS-related tyrosine kinase 3 Ligand (FLT3L) can generate DCs from a bone marrow culture (Brasel *et al*, 2000 and Inaba *et al*, 1992**).** Alongside DCs being generated, however, macrophages can appear in the cultures as well. The optimum time to culture DCs in the cytokines is 6-8 days (Brasel *et al*, 2000 and Inaba *et al*, 1992). Fully matured DCs should express major histocompatibility complex (MHC) class II products based on the antigens expressed on the cells—the key to a T-cell and B-cell immune reaction (Inaba *et al*, 200). Estradiol (E2) promotes differentiation of the DC antigens CD11c+ CD11bintermediate DCs and increases MHC class II expression (Paharkova-Vatchkova *et al*, 2003). MHC class II expression is crucial for development of T-cells and ultimately the immune system**.** An important antigen of dendritic cells and other immune cells are siglec antigens. These antigens are responsible for binding ligands to the cell and cellular signaling. DCs cultured in GM-CSF and FLT3L are potentially affected by E2 that’s added to the media, in the case that different subsets of DCs form. These subsets of DCs are conventional and plasmacytoid, which should fully differentiate in the 6-8 day time period. Conventional DCs are larger than plasmacytoid DCs, thus pDCs express low levels of CD11c antigens as well as low MHC class II levels. This separation into two subsets is thought to be influenced by estradiol (E2), but the type of cytokine used to culture the dendritic cells will is also an influence.

There has been clarification within Genome-wide association studies (GWAS) that 50 chromosomal loci are linked to polymorphisms that make SLE more likely. Some of the putative genes include: Three prime exonuclease-1 (TREX1), Interferon Regulatory Factor 5(IRF5), Toll-like Receptor 7 (TLR7), major histocompatibility (MHC) locus, B lymphocyte induced maturation protein 1 (BLIMP1), and PR domain zinc finger protein 1 (PRDM1) (Schur  *et al*, 2014). Gene expression assays such as real time [quantitative] polymerase chain reactions (qPCR) allow insight to the levels of what genes are expressed by target cells and tissues. In this project, interleukin 6 (IL-6) expression from murine dendritic cells was assayed after being cultured for 6-8 days as well as after stimulation of lipopolysaccharide (LPS) to begin an in-vitro immune response. This assay provides an insight to the pro-inflammatory activities of IL-6 and its role in Systemic Lupus Erythematosus.

**Materials and Methods**

***Bone Marrow Preparation***  Mice femurs of both phenotypes (Balb/c and C57BL6) were obtained from the barrier—this is where mice are housed— by a qualified lab technician. I observed the entire bone marrow preparation protocol which occurred under a sterile hood, all tools were autoclaved before use. A gauze pad was dedicated to each sample to remove any remaining muscle on the bones. All bones remained in Hanks Balanced Salt Solution (HBSS) until all muscle was removed from all samples. The HBSS was aspirated out and 70% ethanol was poured to cover the bones for at most 2 minutes. The ethanol was aspirated out and HBSS was poured to cover the bones again. This wash process occurred 2-3 times. All samples were moved to allocated petri dishes depending on what mouse’s sample were used, in HBSS. Scissors, forceps, 26 gauge needles (1 per sample), 10mL syringes, 50mL vials (1 per sample), and vial filters were gathered. All samples were held with forceps to avoid contamination of any bacteria or other cells. Tips of the bones were cut to allow the needle to fit in the bone. The syringe was filled with 10mL of HBSS, and the needle was put into the bone while flushing out the bone marrow, into a 50mL vial,

--**Place Figure 1 Here**--

with the HBSS in the syringe.Once all bone marrow was flushed from the bones, the supernatant from the sample’s petri dish was collected using a p-1000 micropipette and put into the corresponding bone marrow collection vial. Once all vials were completed, HBSS was added to all vials to create equal volumes for centrifugation—1500rpm for 7mins. at 4ºC. During centrifugation, 1X Red Blood Cell (RBC) lysis buffer was taken from -20ºC storage. After centrifugation, the HBSS was aspirated out of all vials and only 1mL of RBC was used to resuspend each cell pellet, for only 2 minutes to avoid the lysis of possible dendritic cells. At least 25mL of HBSS was poured into each vial after no more than 2 minutes exposure to RBC lysis buffer. The vials were checked for equal volumes and then centrifuged again under the same conditions. Again, the fluids were aspirated out and 10mL of HBSS was added to each vial, suspending the cell pellet. This 10mL solution was then pipetted through the vial filter for each sample, into a new 50mL vial. Moving forward, all samples not being used were put on ice until needed. Cell counting with a hemacytometer was performed in order to find which sample produced the minimum numbers of dendritic cell progenitors. From the 10mL of each sample, 10µL of sample were put into eppendorf tubes. Then 10µL of Trypan Blue dye was pipetted to stain the cells. From this 20µL solution in the eppendorft tubes, 10µL was pipetted into the hemacytometer, using a dilution factor of 2. When obtaining cell count, only the center box of the hemacytometer was used.

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Cells were cultured at 2.0x106 cells/mL in RPMI 1640 media (1% L-Glutamine, 1% Pen/strep, and without phenol red) once the minimum cell count was obtained. The volume of the cells cultures determined which well plate was going to be used. All well plates were opened under the hood and then labeled. GM-CSF was added to three wells at 4µL/mL while FLT3L was added to the other three wells at 2µL/mL. Then E2 (1000x) and ICI were added into separate wells of GM-CSF and FLT3L at 1µg/mL. Completed cell cultures were then put into the incubator (37ºC) for one week; however, GM-CSF and FLT3L were added every 3 days.

***Dendritic Cell Stimulation***On day 7, the next cell culture was made for Lipopolysaccharide (LPS) stimulation. Half of the volume from each bone marrow preparation culture was put into a new multi-welled plate. Each cell culture has two wells in the new multi-welled plate, one well for LPS stimulation and the other well as a control for the LPS stimulation. LPS is used at 1µg/mL and was used to stimulate the dendritic cells. This stimulation should activate the dendritic cells which emulates an immune response. During activation, interleukin-6 (IL6) gene expression levels arise. After stimulation, the multi-welled plate was put into the incubator for 24 hours. Flow cytommetry was performed by a certified lab member after this 24 hour incubation. Parameters were gated in order to distinguish the size difference of DC subsets: plasmacytoid DC (pDC) and conventional DC (cDC).

***RNA and cDNA*** ***Synthesis*** From the activated dendritic cells, a cell count was performed in order to know which RNA protocol to use. Both the RNeasy®Mini and the RNeasy® Micro kits were from QIAGEN®. Each kit contained a protocol for obtaining RNA samples, which were the protocols used for each trial. The RNA samples were made from the dendritic cells with and without LPS stimulation—these cultures were made the day before—each trial had between 12-24 samples: 1 mouse yielded 12 dendritic cell samples. Using the protocol in the iScript™ cDNA Synthesis Kit from Bio-Rad Laboratories, Inc., cDNA samples were made from each RNA sample. The cDNA samples were then brought to run RT-PCR; trials had 20-40µL volumes depending on RNA sample yields. RT-PCR took an hour to complete.

***qPCR and Obtaining Gene Expression Values*** A reaction mix was created for all samples to be used in qPCR. LightCycler® 480 Probes Master Mix (2X), assay mix (IL-6 gene at 20x), and 2.5µL of water make up the reaction mix, and then this amount was triplicated based on number of samples. The type of IL-6 used was Mm99999064\_m1. The reaction mix was then micro-pipetted into the 384 qPCR- well plate. 2µL of cDNA was then added to the corresponding wells to the qPCR plate. Water is used as a negative control for the samples. A transparent sheet was then put over the plate and brought to the qPCR machine: LightCycler® 480 from Roche. The Roche software that works alongside the LightCycler® 480 was able to obtain the Ct value using a ΔC method. From the Ct value, the levels of IL-6 gene expression were able to be recorded. These levels were then compared amongst all trials, which will be shown in the results section.

**Results**

After full maturation and activation of dendritic cells (DCs), differentiation amongst the DCs should have taken place: cDCs or pDCs. The cytokines that the DC progenitors were exposed to did have an influence on which subset of the DC the progenitor would become; however, the hormone E2 (estradiol) and ICI 182,780 (ICI) did not influence differentiation of the DCs. In order to distinguish the two subsets from each other, flow cytometry was performed by a certified lab member. If a DC had more than 103 expression of Siglec-H antigens, it would fall into the pDC parameter gated off in Figure 3.

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Also represented in Figure 3, is DC differentiation between the two cytokines FLT3L and GM-CSF alongside the addition of E2 and ICI in the culture medias.

Dendritic cells cultured in the presence of GM-CSF had greater geometric mean fluorescence intensity (geoMFI). Siglec-H+ cells exposed to GM-CSF had the greatest MHC II expression; overall DCs cultured in media with FLT3L expressed far less MHC II levels. Siglec-H+, FLT3L DCs had a geoMFI range of 1062-1382 while Siglec-H+ GM-CSF DCs had a geoMFI range of 17825-28014. Siglec-H- DCs cultured in media with FLT3L had a geoMFI range of 1011-1842 whilst Siglec-H- DCs from the GM-CSF cultures had a geoMFI range of 13424-15094. Figures 4 & 5 display the geoMFIs throughout all activated CD11c+ DC cultures.

**--Figures 4 and 5 go Here--**

Obtaining gene expression levels came from the LightCycler® 480 software that used a ΔCt method. The Ct value was calculated by this software based on the graph that was made during the qPCR trials. The main gene of interest was IL-6 after LPS stimulation. Both mouse phenotypes had different gene expression values, and estradiol (E2) and ICI influence on IL-6 gene expression levels was also looked at. Ct values were then graphed with graphpad. As expected, all samples activated by LPS stimulation had greaterIL-6 gene expression values than the samples that weren’t activated by LPS stimulation.

 Figure 6 displays all gene expression levels. Female C57BL6 mice had the greatest levels of IL-6 expression; accordingly the dendritic cells stimulated by LPS from the culture exposed to E2 yielded the maximum expression values, 280 relative units of gene expression with a standard deviation of 200 relative units of gene expression. This correlation may be due to the fact that the concentration of estrogen is greater in females than males.

**--Place Figure 6 Here--**

**Conclusion:**

 After all data has been an analyzed, it can be stated that DCs from C57BL6 mice showed higher gene expression levels of Interleukin-6 when compared to Balb/c mice gene expression levels. More specifically, the dendritic cells that were activated by LPS stimulation and were exposed to E2 yielded the highest IL-6 gene expression levels, averaging 280 relative gene expression units. Comparing both phenotypes, dendritic cells from C57BL6 females expressed higher levels of IL-6 than C57BL6 males, but dendritic cells from male Balb/c mice had higher IL-6 gene expression values than females. The exposure to E2 had a gene expression value for all samples, even though the values are sporadic, this leads to the rejection that E2 or ICI influences the differentiation of DC subsets, but E2 does influence gene expression levels of IL-6 in C57BL6 bone marrow.

Flow cytometry assays visualized the size of DCs and MHC II expression and can be seen in figure 3a-3c. It is evident that DC cultures exposed to GM-CSF yielded no plsmacytoid DCs (pDC), so it has been accepted that pDCs from a GM-CSF DC culture is not a true pDC. GM-CSF generated DCs more efficiently. On the other hand, DC cultures exposed to FLT3L yielded both subsets of DCs. The DCs assayed by flow cytometry were all activated DCs by LPS stimulation.

Limitations faced throughout the study were keeping each DC culture at the same volume because some mice samples had more DCs than others. The DCs were cultured at 2x106 cells/mL in order to work around this limitation; concentrations at which cytokines were put into the DC cultures had to be recalculated for every trial.

Future research on Systemic Lupus Erythematosus should include tracing back multiple genetic loci that would cause symptoms linked to autoimmune diseases, as well as single nucleotide polymorphisms. These future studies should include the examination of estrogen-like effects of other sex hormones such as progesterone. A continuation of this study would include using a mouse hybrid phenotype between C57BL6 and Balb/c mice in order to discover the E2 effect on such mice.

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