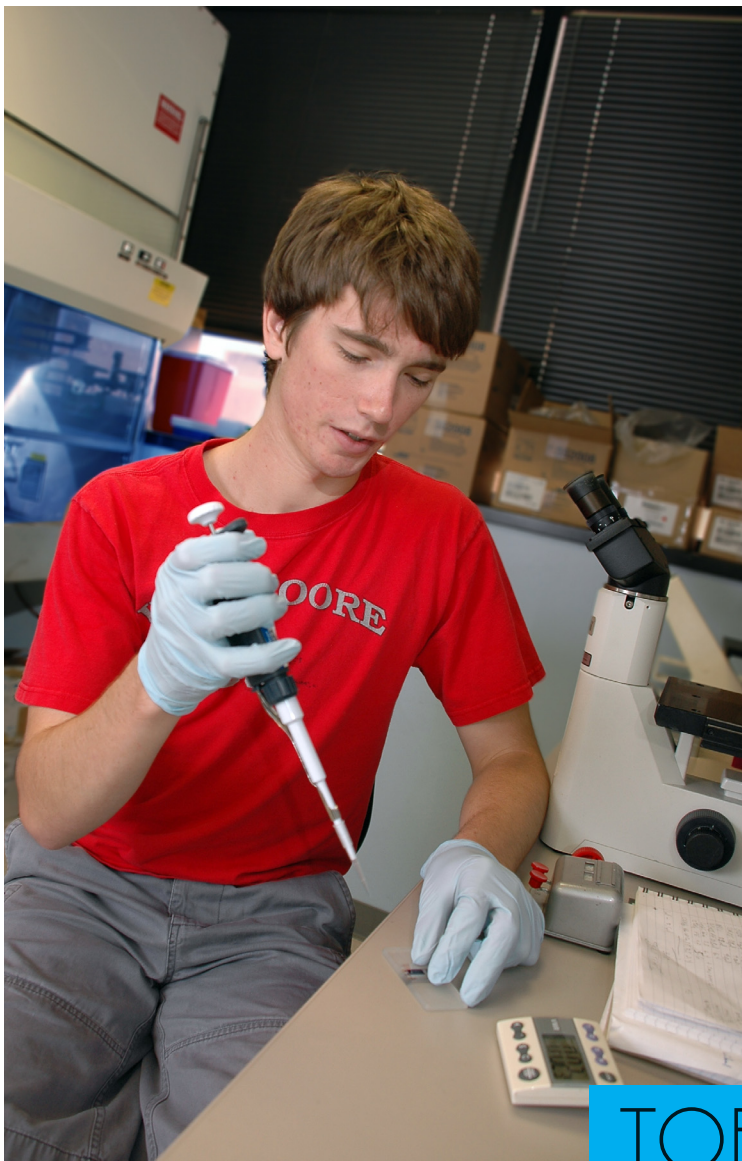


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FINAL RESEARCH MANUSCRIPTS



The Effect of Estradiol and Estrogen Receptors α and β on Bone Marrow and Monocyte Derived Dendritic Cells

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ABSTRACT

Autoimmunity, the attacking of self tissue by the immune system, is a serious set of conditions that often has a higher prevalence in women versus men. In the hopes of understanding both this inherent difference and the way in which these diseases occur, the effects of estradiol acting via estrogen receptors on the immune system are being studied. Dendritic cells (DC) are professional antigen presenting cells that help initiate active immune responses. Looking at DC development as well as function, the lab helps to further the understanding of how estradiol promotes immune responses in inflammation.

Previous work has shown that estradiol promotes differentiation of bone marrow progenitors and monocytes into dendritic cells as well as upregulation of langerin and several costimulatory molecules. Knowing that estradiol promotes signaling through two estrogen receptors, alpha and beta, the question became does either receptor cause these effects individually or through a combination.

To study the murine bone marrow progenitors, estradiol and two estrogen receptor selective agonists were added - estrogen receptor alpha agonist ($ER\alpha$) and estrogen receptor beta agonist ($ER\beta$). Flow cytometry revealed that $ER\alpha$ was responsible for many of the responses to estradiol such as increased DC differentiation. $ER\alpha$ is also responsible for the shift in DC populations to Ly6clowCD11bint as well as increased langerin production.

A similar study with monocytes provided different results. Both $ER\alpha$ and $ER\beta$ resulted in the same results as estradiol, indicating that either can lead to the increased cell survivability and upregulation of langerin that was observed. In contrast to the bone marrow system, treatments upregulated the CD11b phenotype marker in monocytes that was downregulated in the bone marrow.

In conclusion, $ER\alpha$ is specifically responsible for several of estradiol's effects on murine bone marrow progenitors while in monocytes either $ER\alpha$ or $ER\beta$ can induce a response.

INTRODUCTION

The immune system is comprised of two main branches – innate and adaptive immunity. Innate immunity works as the general, first line defense that recognizes certain shared molecular patterns in antigens. Hence, the innate immune system cell is capable of responding against a broad range of antigens in a non specific way. The adaptive immunity responds and trains the cells that later can mount a specific protective response against antigens. A key link between these two responses is the antigen presenting cells, particularly dendritic cells (DC). They act by engulfing antigens, processing them, and then presenting pieces to train T-cells and B-cells to react.¹

Autoimmune responses are a set of dangerous conditions in which the body's immune system attacks self-tissue.² Of specific interest is the sex bias encountered in many of these diseases. Sjogren's syndrome, SLE, and thyroid disease are all examples that affect predominately women.³ This clear bias has led to research involving sex hormones, such as estradiol. Further support

for investigating this link is that bone marrow progenitors and mature immune system cells express estrogen receptors.⁴

In vivo, inflammation leads to an elevation in GM-CSF levels, and as such it is added to in vitro cultures to mimic inflammation and to promote DC differentiation.⁴ Estradiol has been shown to promote a shift towards the Ly6C-CD11bint DC population in GM-CSF driven in vitro cultures from bone marrow cells. In cultures with bone marrow progenitors from $ER\alpha$ knockout mice, this subset is not generated indicating that $ER\alpha$ is mediating this effect. In the same setup, estradiol has also been shown to increase the expression of langerin, a molecule expressed by Langerhans cells and other skin and tissue DC populations recently described.⁶

Monocytes are direct precursors of dendritic cells. Upon inflammation, they differentiate at an increased rate. Estradiol has been shown to increase CD11bhi DC differentiation as well as upregulate langerin expression. Preliminary results in lab experiments using $ER\alpha$ knockout mice suggested that $ER\beta$ could mediate these effects.

One goal of the experiment was to revisit the bone marrow system using agonists for both estrogen receptors. The second goal was to explore which estrogen receptors were responsible for which of estradiol's effects in monocytes.

METHODS

Mice. One or four female C57BL/6 mice were used to harvest the bone marrow by flushing hind legs for bone marrow or monocyte cultures respectively.

Cell Cultures. Cells were cultured in RPMI free of phenol red, with 10% charcoal dextran stripped FBS. Cell medium also contains a 1:25 ratio of GM-CSF.

The cells were cultured in four different conditions: 17 β estradiol at 1nM, estrogen receptor alpha agonist (PPT) at 50nM, estrogen receptor beta agonist (DPN) at 10nM, and a control which only contained the drug vehicles.

For the bone marrow experiments the cells were plated at 1.5×10^5 cells per mL. 6 mL of the aforementioned medium mixture were added as well as the drugs in

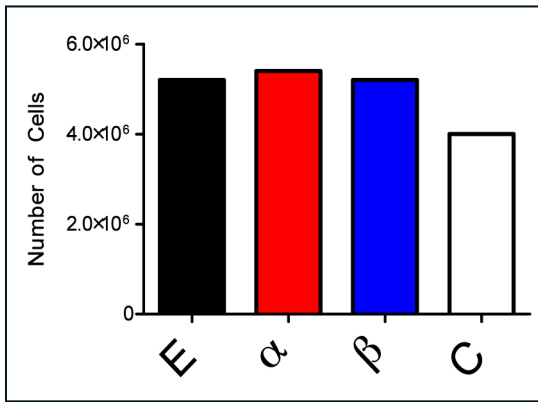


Figure 1. Numbers of bone marrow cells counted at day 7 from bone marrow cultures in the presence of Estradiol (E), ER α agonist (α), ER β agonist (β) and control (C).

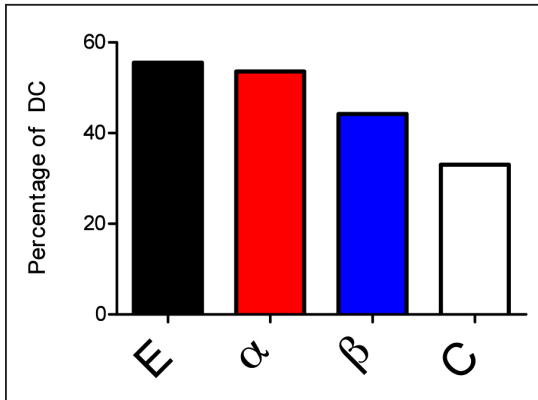


Figure 2b. Percentages of Ly6C-CD11b int DC detected in the presence of Estradiol (E), ER α agonist (α), ER β agonist (β) and control (C).

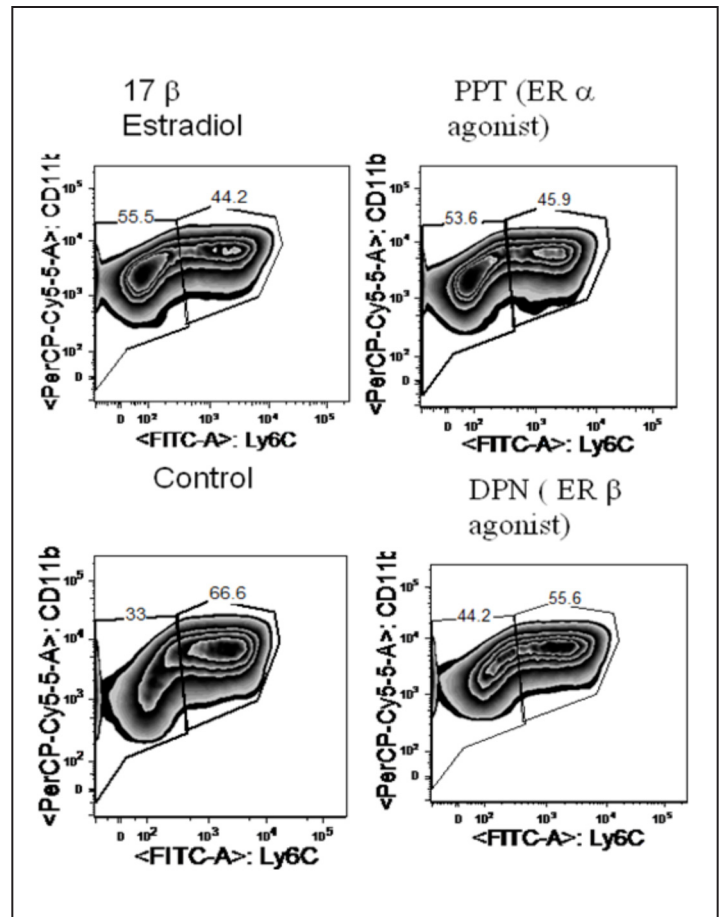


Figure 2a. Zebra plot analysis of Dendritic cells based on Ly6C and CD11b expression.

the concentration described. On day three, 6 mL of new medium were added as well as new drugs in the prescribed concentrations. On day six, 6 mL of medium were removed; the cells were pelleted, resuspended, and added back to the dishes along with 6mL of medium and new drugs.

For the monocyte experiments cells were plated at 1×10^6 /ml.

On day 0, 2 mL of medium and the drugs and vehicles were added. Medium and drugs were added on day 2 for 2 additional mL of medium. 2 mL of medium were removed and 2 mL of new medium and drugs were added on day four. Cells from the medium were pelleted, resuspended, and placed back in the dishes.

As a control, ICI 182,780, an ER antagonist, was added in a concentration of 100 nM to the control culture in the monocyte experiment.

Flow cytometric analysis. Most of the cells were harvested with PBS. Different cocktail combinations included antibodies to Ly6C, CD11b, CD11c, MHCII, CD86 and CD40. Langerin was stained after cell permeabilization.

For analysis of apoptosis, live cells were stained with a specific kit that contains a reagent conjugated with carboxyfluorescein that penetrates the cells and inhibits active caspases by forming a covalent bond with them. This was used in combination with 7-Actinomycin D (7-AAD), which penetrates dying or dead cells and bind to DNA.

Real-time PCR analysis. RNA extraction and cDNA synthesis was performed, followed by real time PCR using the specific primers for bcl-2 gene.

Magnetic Cell Sorting. Monocytes were negatively selected using Easy Sep. Essentially, biotinylated antibodies were added to the non-monocytes in order to deplete bone marrow progenitors, granulocytes, T and B cells, by using sequential steps in which finally the magnetic beads bound to the magnetic column retain the undesired cells allowing the pouring off of the monocytes.

RESULTS AND DISCUSSION

In the bone marrow system, treatment with estradiol or ER agonists increased the bone marrow cell count over the control (Figure 1). DC differentiation was also increased by

treatment. These effects seem to be produced by both estrogen receptors.

The Ly6C-CD11b int DC population was also increased in the presence of estradiol or ER agonists over the control. ER α agonist and estradiol promoted a higher percentage of this subset than the control. ER β had the same effect although to a lesser extent, suggesting ER α is likely the main ER responsible for producing that effect (Figure 2). However, future research could involve drug titrations to clarify which concentration is specific for each receptor.

In the Ly6C- DC population the number of cells expressing langerin is increased (Figure 3). Furthermore, both estrogen receptors are inducing a response from DC but once again the alpha agonist stimulated higher langerin expression in comparison to beta agonist.

Noticing the increased cell count, PCR was performed to test for the gene Bcl-2. This gene is an anti-apoptotic gene, suggesting that an increase in its expression would suggest increased survivability. It was found that all ER agonists and estradiol treatments showed over a 20 fold increase in Bcl-2 expression when compared to the control (Figure 4).

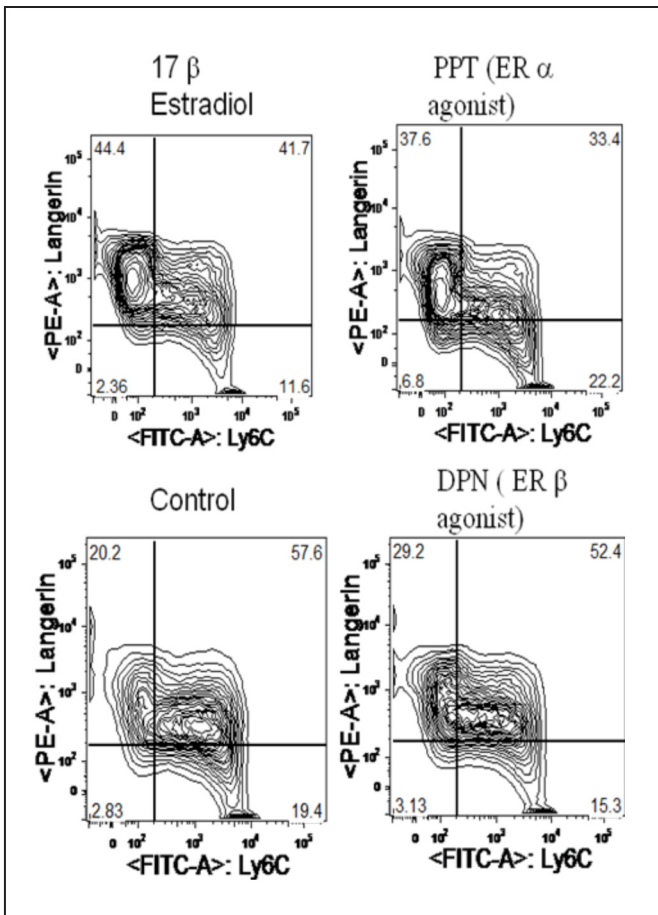


Figure 3. Contour plot analysis of Langerin expression in the Ly6C – DC population in the absence or in the presence of estradiol and the ER agonists.

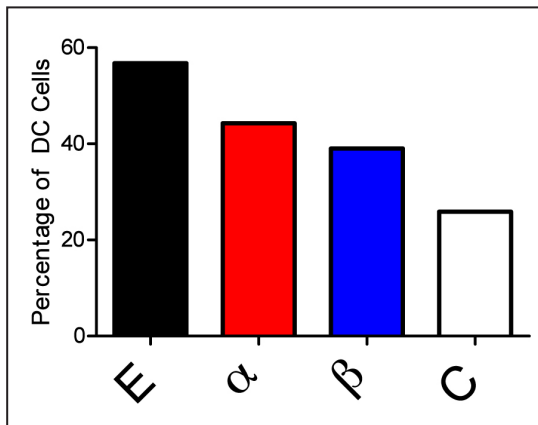


Figure 6. Langerin expression in monocyte-derived DC at day 6 in the presence of Estradiol (E), ER α agonist (α), ER β agonist (β) and control (C). All cells are CD11c+.

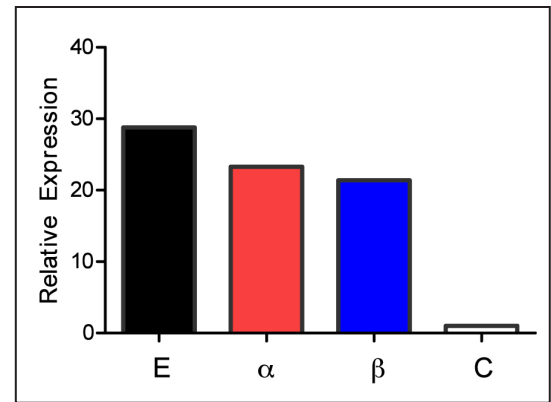


Figure 4. Bcl-2 expression in the presence of Estradiol (E), ER α agonist (α), ER β agonist (β) and control (C) in bone marrow derived cells at day 7.

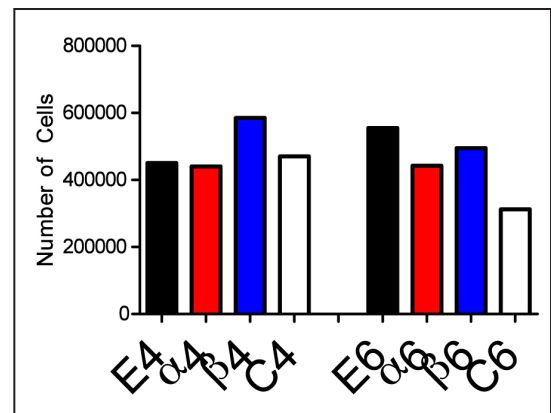


Figure 5. Number of Monocyte-derived cells in GM-CSF cultures with Estradiol (E), ER α agonist (α), ER β agonist (β) and control (C) on days four and six.

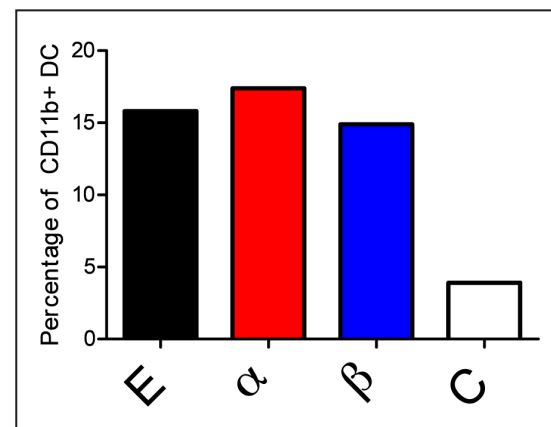


Figure 7. Percentages of CD11b+CD86hi in monocyte-derived DC analyzed at day 6 in the presence of Estradiol (E), ER α agonist (α), ER β agonist (β) and control (C).

In the GM-CSF driven cultures, the number of monocyte-derived cells at day 4 and 6 were also increased upon treatments compared to controls as seen in figure 5. However, there were no differences in DC differentiation.

Meanwhile, the percentage of cells expressing langerin is increased by all treatments. It appears that both alpha and

beta receptors are responsible for the effects of estradiol in relation to langerin as neither alone was equal to the estradiol dish (Figure 6).

While CD11b was downregulated in the bone marrow cultures, it was upregulated in the monocyte-derived dendritic cells upon treatment. Furthermore, in this CD11b+ subset, CD86 expression was also upregulated

over the control, which suggest that estradiol promotes the differentiation into a CD11bhi subset with a more mature phenotype. Both agonists performed similarly to the estradiol culture suggesting both receptors are capable of mediating this effect by estradiol (Figure 7).

To determine the percentage of DC undergoing apoptosis, an assay was used

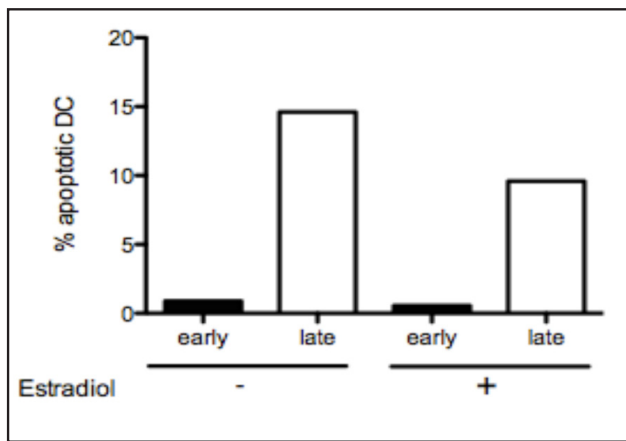


Figure 8. Analysis at day 6 of percentages of DC in early or late stages of apoptosis in the presence or absence of estradiol.

where the detection of active caspases corresponds to cells in early stages of this process whereas the double staining for DNA and caspases corresponds to late stages. The results show that estradiol resulted in a significant decrease in cells experiencing late apoptosis (Figure 8). This suggests that estradiol also has a positive effect on DC survival in GM-CSF driven cultures with monocytes.

In summary, our results support the effect of estradiol in the generation of the Ly6C-CD11b^{int}langerin⁺ DC population which seems to be predominantly mediated by estrogen receptor alpha although to a lesser extent estrogen receptor beta might also contribute. Meanwhile, in the monocytes it appears both receptors either work together or are equally capable of mediating the effects of estradiol.

Future titration experiments with both alpha and beta agonists would allow the determination of the range of concentration that would trigger a selective activation of

one particular estrogen receptor. The effect of estradiol increasing DC differentiation into populations with a more mature phenotype characterized by higher expression of co-stimulatory molecules and increased survival, could lead to exacerbated immune responses. These mechanisms may help to understand the sex bias in autoimmune diseases.

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