CHAPTER 4

SELECTIVE MODULATION OF LYMPHOPROLIFERATION AND CYTOKINE PRODUCTION VIA INTRACELLULAR SIGNALING TARGETS BY $\alpha_1$-AND $\alpha_2$-ADRENOCEPTORS AND ESTROGEN IN THE SPLENOCYTES

Specific Objective 1C Establish the Influence of Estrogen (E) on $\alpha_2$-Adrenergic Receptor (AR) agents (agonists and antagonists)-induced modulation of splenic lymphocyte functions.

4.1 Rationale

The mechanistic implications of the presence of sympathetic noradrenergic innervation in lymphoid organs in synaptic association with lymphocytes open to the influence of hormonal fluctuations throughout reproductive age in females has not been investigated yet. The expression of $\alpha_1$- and $\alpha_2$-AR are have been reported either to be induced by cytokines and hormones or normally expressed on natural killer (NK) cells and T and B lymphocytes. The role of $\alpha_1$- and $\alpha_2$-AR in altering immune reactivity especially, in the presence of estrogen is not known although centrally they are known to modulate the female reproductive behavior, lordosis. Hence, the in vitro effects of different concentrations of $\alpha_1$- and $\alpha_2$-AR agonists, phenylephrine and clonidine,
respectively on proliferation of splenic lymphocytes and cytokine (IL-2 and IFN-γ) production in the absence and presence of 17β-estradiol were examined to establish their effects on cell-mediated immune responses.

To determine the specificity of their actions on cell-mediated immune responses, specific α1- and α2-AR antagonists, prazosin and idazoxan, respectively were co-incubated in the absence and presence of α1- and α2-AR agonists. In addition, the levels of molecular signaling factors such as p-ERK 1/2, p-CREB, p-Akt, and p-NF-κB in the splenocytes were measured to understand the molecular actions of α1- and α2-AR agonists in the absence and presence of estrogen. Simultaneously, the NO production was measured to determine the role of this compensatory factor on α1- and α2-AR and estrogen-induced modulation of immune reactivity.

The results obtained indicate that α1- and α2-AR agonists suppress lymphocyte proliferation and differentially regulate cytokine production and phosphorylation of ERK, CREB, Akt, and NF-κB in the absence and presence of estrogen.

4.2 Treatment

4.2.1 Experiment 1

Splenic lymphocytes (2 x 10^5 cells/ml) were treated with different doses (10^-3 M, 10^-6 M and 10^-9 M) of α1-AR specific agonist, phenylephrine or α2-AR specific agonist, clonidine, in the presence and absence of α1-AR specific antagonist, prazosin (10 µM) or α2-AR specific antagonist, idazoxan (10 µM), in 24-well and 96-well culture plates in the presence and absence of 10^-9 M 17β-estradiol. The doses of α-AR antagonists and 17β-estradiol were selected from a preliminary study in splenic lymphocytes (Fig. A2.1; A2.2). The plates were kept in a humidified chamber with 5% CO2 at 37°C to measure the effects of α-AR and estrogen on cytokines and T lymphocyte proliferation.

Adrenergic agonists and antagonists (Sigma-Aldrich, St. Louis, MO) were freshly prepared every day as 0.1 M stock solutions in 10 mM L-ascorbate and serially diluted to the aforementioned concentrations using media.
4.2.2 Experiment 2:

Adrenergic receptor-specific effects on downstream signaling molecules was assessed by co-incubating different doses (10^{-3} M to 10^{-9} M) of α₁- and α₂-AR specific agonists with or without their specific antagonists (10^{-5} M) in 24-well and 96-well culture plates in the presence of iNOS inhibitor NG-monomethyl-L-arginine (LMMA; 10^{-5} M) for 72 hours. Lymphocyte proliferation was assessed using the MTT assay at the end of 72 hours.

LMMA was prepared as stock solutions of 0.1M in sterile distilled water and serially diluted to the aforementioned concentrations using media. The inhibitors were purchased from Sigma-Aldrich, St. Louis, MO.

4.3 Results

4.3.1 α₁-AR agonist does not alter but α₂-AR agonists inhibit splenocyte proliferation

Treatment of splenic lymphocytes with the ARα₁ agonist phenylephrine in the presence and absence of estrogen (10^{-9} M E₂) did not alter proliferation. However, co-treatment of phenylephrine (10^{-6} M and 10^{-3} M)-treated splenocytes with ARα₁-specific antagonist prazosin (10^{-5} M Pz) significantly (p<0.01) decreased splenocyte proliferation (Fig. 4.1A).

Lymphocytes treated with ARα₂-agonist clonidine (10^{-9} M and 10^{-6} M) showed significant (p<0.05) decrease in proliferation. Co-treatment with 17β-estradiol or ARα₂-specific antagonist idazoxan did not reverse the agonist-mediated decrease in proliferation. However, treatment with ARα₂-specific antagonist idazoxan alone significantly decreased splenocyte proliferation compared with control (Fig. 4.1B).

Proliferation of splenocytes was not significantly altered following 17β-estradiol (10^{-9} M) treatment alone although co-treatment with ARα₂-agonist clonidine (10^{-3} M, 10^{-6} M and 10^{-9} M) significantly (p<0.05) decreased it (Figs. 4.1A and 4.1B).
4.3.2 $\alpha_1$-AR agonist decreases while $\alpha_2$-AR agonist does not alter IFN-\(\gamma\) production by splenocytes

A significant ($p<0.05$) decrease in IFN-\(\gamma\) production was observed in AR$\alpha_1$ agonist phenylephrine-treated ($10^{-3}$ M, $10^{-6}$ M and $10^{-9}$ M) splenocytes and the effect was not reversed by co-incubation with the antagonist prazosin. However, co-treatment of phenylephrine ($10^{-3}$ M, $10^{-6}$ M and $10^{-9}$ M)-treated splenocytes with 17$\beta$-estradiol ($10^{-9}$ M) significantly ($p<0.01$) reversed the agonist-mediated decline (Fig. 4.2A).

On the other hand, IFN-\(\gamma\) production was unaltered in AR$\alpha_2$-agonist clonidine-treated splenocytes with or without estrogen. Co-treatment of clonidine ($10^{-3}$ M)-treated splenocytes with AR$\alpha_2$-antagonist idazoxan ($10^{-5}$ M) significantly decreased IFN-\(\gamma\) production compared with agonist-treated group (Fig. 4.2B).

There was a significant increase in IFN-\(\gamma\) production in splenic lymphocytes treated with 17$\beta$-estradiol ($10^{-9}$ M) alone compared with control which was significantly ($p<0.01$) reversed by co-treatment with AR$\alpha_1$-agonist phenylephrine ($10^{-9}$ M) alone (Figs. 4.2A and 4.2B).

4.3.3 $\alpha_1$- and $\alpha_2$-AR agonists similarly increase IL-2 production by splenocytes

IL-2 production was significantly ($p<0.05$) increased in AR$\alpha_1$ agonist phenylephrine-treated splenocytes ($10^{-3}$ M, $10^{-6}$ M and $10^{-9}$ M) in a dose-dependent manner while co-incubation with the AR$\alpha_1$ agonist antagonist prazosin ($10^{-5}$ M) reversed it. Co-treatment of phenylephrine-treated splenocytes with 17$\beta$-estradiol did not significantly alter the agonist-mediated increase (Fig. 4.3A).

On the other hand, IL-2 production was not significantly altered in AR$\alpha_2$ agonist clonidine ($10^{-3}$ M) treated splenocytes while co-treatment with AR$\alpha_2$-specific antagonist idazoxan significantly decreased it. IL-2 production was significantly ($p<0.05$) increased in splenocytes co-treated with 17$\beta$-estradiol ($10^{-9}$ M) and clonidine ($10^{-6}$ M) compared to splenocytes treated with clonidine ($10^{-6}$ M) alone (Fig. 4.3B).
**Figure 4.1**   *In vitro* addition of α₁- and α₂-AR agonists and antagonists in the absence and presence of 10⁻⁹ M 17β-estradiol on T cell proliferation in the spleen. Higher dose of α₁-AR agonist, phenylephrine (Phe), decreased the proliferation of splenocytes irrespective of the antagonist, prazosin (Pz), and 17β-estradiol co-treatment (1A). Treatment of splenic lymphocytes with α₂-AR agonist, clonidine (Clo), decreased proliferation irrespective of antagonist, idazoxan (Iz), co-treatment but co-incubation with 17β-estradiol reversed the agonist-mediated (Clo 10⁻⁶ M) decline (1B). *p<0.05 compared to control. §p<0.05 compared to respective agonist-treated group. †p<0.05 compared to 10⁻⁹ M 17β-estradiol-treated group. ‡p<0.05 compared to respective group without 17β-estradiol.
Figure 4.2  In vitro addition of α₁- and α₂-AR agonists and antagonists in the absence and presence of 10⁻⁹ M 17β-estradiol on IFN-γ production in the spleen. There was a decrease in IFN-γ production following α₁-AR agonist, phenylephrine (Phe), treatment while 17β-estradiol co-treatment reversed the agonist-mediated decline (2A). In contrast to α₁-AR agonist, α₂-AR agonist, clonidine (Clo), did not alter IFN-γ production in the presence and absence of 17β-estradiol (2B). *p<0.05 compared to control. §p<0.05 compared to respective agonist-treated group. †p<0.05 compared to respective group without 17β-estradiol.
Figure 4.3  *In vitro addition of α₁- and α₂-AR agonists and antagonists in the absence and presence of 10⁻⁹ M 17β-estradiol on IL-2 production in the spleen.* Phenylephrine, α₁-AR agonist, enhanced IL-2 production by splenocytes and the effects were reversed by co-treatment with specific antagonist, prazosin (Pz), in the absence and presence of 17β-estradiol (A). Clonidine in the presence of 17β-estradiol did not alter IL-2 production in splenocytes and the effect was reversed by the specific antagonist, idazoxan (Iz; B). *p<0.05 compared to control. §p<0.05 compared to respective agonist-treated group. ‡p<0.01 compared to respective agonist-treated group and 17β-estradiol-treated group.
4.3.4 α-AR specific agonists and estrogen alter splenocyte nitric oxide production in a dose and receptor subtype-dependent manner

Treatment of splenic lymphocytes with ARα1-specific agonist phenylephrine (10^{-9} M) significantly (p<0.05) enhanced splenocyte nitric oxide production compared to control while co-treatment with estrogen significantly (p<0.01) reversed it. Interestingly, splenocytes treated with ARα1-specific antagonist prazosin (10^{-5} M) also showed significantly enhanced nitric oxide production compared to control (Fig. 4.4A).

Co-incubation of AR-α2 agonist clonidine (10^{-6} M and 10^{-9} M) significantly (p<0.05) increased nitric oxide production compared to control while co-treatment with ARα2-specific antagonist idazoxan (10^{-5} M) and estrogen (10^{-9} M) significantly (p<0.01) reversed it. Similar to ARα1-agonist prazosin, treatment of splenocytes with ARα2-specific antagonist idazoxan (10^{-5} M) alone also significantly (p<0.05) enhanced nitric oxide production(Fig. 4.4B).

Treatment with 17β-estradiol (10^{-9} M) alone did not significantly alter splenocyte nitric oxide production compared with control although co-treatment with ARα1-agonist phenylephrine (10^{-3} M and 10^{-9} M) and ARα2-agonist clonidine (10^{-3} M, 10^{-6} M and 10^{-9} M) significantly (p<0.05) decreased it (Figs. 4.4A and 4.4B).

4.3.5 α-AR specific agonists and estrogen differentially modulate p-ERK expression in splenic lymphocytes

Treatment of lymphocytes with ARα1 agonist phenylephrine significantly (p<0.05) enhanced the expression of phosphorylated extracellular signal regulated kinase 1/2 (measured as p-ERK/Total ERK) compared with control and the effect was not reversed by co-treatment with ARα1-specific antagonist prazosin (10^{-5} M) and estrogen (10^{-9} M). Interestingly, splenocytes treated with ARα1-specific antagonist prazosin (10^{-5} M) alone also showed increased p-ERK/Total ERK expression compared to control (Fig. 4.5A).

However incubation with AR α2 agonist clonidine (10^{-9} M and 10^{-6} M) significantly (p<0.05) decreased the p-ERK/Total ERK expression compared with control and the effect was significantly (p<0.01) reversed by co-treatment with the α2-specific antagonist idazoxan (10^{-5} M) and estrogen (10^{-9} M; Fig. 4.5B).
Splenocytes treated with estrogen \((10^{-9} \text{ M})\) alone also showed significantly \((p<0.05)\) enhanced splenocyte p-ERK/Total ERK expression compared with control which was reversed \((p<0.01)\) by co-treatment with AR\(\alpha_2\)-agonist clonidine \((10^{-6} \text{ M})\) and increased \((p<0.01)\) by co-treatment with AR\(\alpha_1\)-agonist phenylephrine \((10^{-6} \text{ M}; \text{Figs. 4.5A and 4.5B}).\)

4.3.6 \(\alpha_1\)-AR agonist enhances while \(\alpha_2\)-AR agonist suppresses p-CREB expression in splenocytes

AR\(\alpha_1\) agonist phenylephrine \((10^{-3} \text{ M})\) significantly enhanced p-CREB/Total CREB expression although neither co-treatment with AR\(\alpha_1\)-specific antagonist prazosin nor estrogen significantly altered it (Fig. 4.6A).

On the other hand, treatment with AR\(\alpha_2\) agonist clonidine significantly \((p<0.05)\) decreased the expression of p-CREB/Total CREB in splenic lymphocytes while co-incubation with AR\(\alpha_2\)-specific antagonist idazoxan \((10^{-5} \text{ M}; p<0.01)\) or estrogen \((10^{-9} \text{ M}; p<0.05)\) significantly reversed it (Fig. 4.6B).

Splenocytes incubated with estrogen \((10^{-9} \text{ M})\) showed significantly enhanced p-CREB/Total CREB expression compared to controls which was reversed \((p<0.05)\) by co-treatment with AR\(\alpha_2\)-agonist clonidine \((10^{-3} \text{ M}, 10^{-6} \text{ M and } 10^{-9} \text{ M})\) alone (Figs. 4.6A and 4.6B).

4.3.7 \(\alpha_1\)-AR agonist had no effect while \(\alpha_2\)-AR agonist increases p-Akt expression in splenocytes

The p-Akt/Total Akt expression in AR\(\alpha_1\)-agonist phenylephrine-treated splenic lymphocytes was unaltered compared with control. Co-treatment of phenylephrine \((10^{-6} \text{ M and } 10^{-9} \text{ M})\)-treated splenocytes with AR\(\alpha_1\) antagonist prazosin \((10^{-5} \text{ M})\) significantly \((p<0.05)\) decreased p-Akt/Total Akt expression compared to agonist-treated group (Fig. 4.7A).

On the other hand, a significant \((p<0.05)\) increase in p-Akt/Total Akt expression was observed in clonidine \((10^{-3} \text{ M, } 10^{-6} \text{ M and } 10^{-9} \text{ M})\)-treated splenocytes and the effects were reversed by co-treatment with AR\(\alpha_2\) antagonist idazoxan \((10^{-5} \text{ M})\). Co-incubation of clonidine \((10^{-9} \text{ M and } 10^{-3} \text{ M})\)-treated splenocytes with 17\(\beta\)-estradiol \((10^{-9} \text{ M})\) significantly \((p<0.01)\) decreased p-Akt/Total Akt expression compared with agonist-
treated groups. Interestingly, treatment of splenocytes with ARα<sub>2</sub>-antagonist idazoxan (10<sup>-5</sup> M) alone also significantly (p<0.05) enhanced p-Akt/Total Akt expression (Fig. 4.7B).

**Figure 4.4**  Nitric oxide (NO) production by α<sub>1</sub>- and α<sub>2</sub>-AR specific agonists-treated splenocytes in the absence and presence of 10<sup>-9</sup> M 17β-estradiol. Phenylephrine (Phe; 10<sup>-9</sup> M) augmented splenocyte nitric oxide production in the presence and absence of specific α<sub>1</sub>-AR antagonist, prazosin (Pz), while co-treatment with 17β-estradiol decreased its production (4A). Clonidine (Clo)-treated lymphocytes increased and 17β-estradiol decreased nitric oxide production and the effects were reversed by co-treatment with α<sub>2</sub>-
AR antagonist, idazoxan (Iz; 4B). *p<0.05 compared to control. §p<0.05 compared to respective agonist-treated group. ‡p<0.05 compared to Control+17β-estradiol and respective group without 17β-estradiol.

Figure 4.5  In vitro addition of α₁- and α₂-AR specific agonists, phenylephrine (Phe) and clonidine (Clo), to splenocytes treated with or without α₁- and α₂-AR specific antagonists, prazosin (Pz) and idazoxan (Iz), and 17β-estradiol on p-ERK expression measured by ELISA. p-ERK measured against Total ERK expression in cell lysates was significantly enhanced byphenylephrine in the absence and presence of 17β-estradiol (5A). α₂-AR agonist, clonidine, significantly decreased p-ERK/Total ERK expression while co-treatment with the specific antagonist, idazoxan, and 17β-estradiol reversed it
(5B). *p<0.05 compared to control. §p<0.05 compared to respective agonist-treated group. ‡p<0.05 compared to Control+17β-estradiol and respective group without 17β-estradiol. #p<0.05 compared to Control+17β-estradiol. †p<0.05 compared to respective group without 17β-estradiol.

**Figure 4.6** In vitro addition of α₁- and α₂-AR specific agonists, phenylephrine (Phe) and clonidine (Clo), to splenocytes treated with or without α₁- and α₂-AR specific antagonists, prazosin (Pz) and idazoxan (Iz), and 17β-estradiol on p-CREB expression measured by ELISA. There was an increase in p-CREB expression against Total CREB expression in cell lysates of splenocytes treated with α₁-AR agonist, phenylephrine, and the effects were not reversed by co-treatment with α₁-AR specific antagonist, prazosin,
and 17β-estradiol (6A). α₂-AR specific agonist, clonidine, significantly decreased p-CREB/Total CREB expression while co-incubation with α₂-AR antagonist, idazoxan, and 17β-estradiol reversed it (6B). *p<0.05 compared to control. §p<0.05 compared to respective agonist-treated group. ‡p<0.05 compared to Control+17β-estradiol and respective group without 17β-estradiol.

Figure 4.7 In vitro addition of α₁- and α₂-AR specific agonists, phenylephrine (Phe) and clonidine (Clo), to splenocytes treated with or without α₁- and α₂-AR specific antagonists, prazosin (Pz) and idazoxan (Iz), and 17β-estradiol on p-Akt expression measured by ELISA. Phenylephrine treatment in combination with 17β-estradiol
decreased p-Akt/Total Akt expression in splenocytes compared with 17β-estradiol-treated controls (7A). Clonidine treatment enhanced p-Akt/Total Akt expression and the effects were reversed by co-treatment with idazoxan and 17β-estradiol (6B). *p<0.05 compared to control. §p<0.05 compared to respective agonist-treated group. ‡p<0.05 compared to Control+17β-estradiol and respective group without 17β-estradiol. #p<0.05 compared to Control+17β-estradiol. †p<0.05 compared to respective group without 17β-estradiol.

Further, 17β-estradiol-treated splenocytes also showed significantly (p<0.05) elevated p-Akt/Total Akt expression compared to control which was reversed (p<0.01) by co-treatment with ARα1-agonist phenylephrine (10⁻³ M, 10⁻⁶ M and 10⁻⁹ M) and ARα2-agonist clonidine (10⁻³ M; Figs. 4.7A and 4.7B).

4.3.8 α₁-AR agonist-mediated immunosuppression is iNOS-independent.

Co-incubation of iNOS inhibitor LMMA (10⁻⁵ M) with ARα₁-agonist phenylephrine significantly (p<0.01) decreased splenocyte proliferation compared with control (Fig. 4.8A). Treatment of splenocytes with phenylephrine alone did not significantly alter splenocyte proliferation (Fig. 4.1A). Treatment with ARα₁-agonist phenylephrine significantly decreased splenocyte IFN-γ production with or without co-incubation with the iNOS inhibitor LMMA (Fig. 4.8B and 4.2A). However, while treatment with ARα₁-agonist phenylephrine significantly enhanced splenocyte IL-2 production (Fig. 4.3A), co-incubation with iNOS inhibitor LMMA significantly (p<0.001) decreased it (Fig. 4.8C).

4.3.9 α₂-AR agonist-mediated immunosuppression is iNOS-dependent.

Splenocytes treated with α₂-AR agonist clonidine significantly decreased splenocyte proliferation although co-incubation of clonidine-treated splenocytes with the iNOS inhibitor LMMA significantly (p<0.01) enhanced it (Fig. 4.9A). Although clonidine-treatment alone did not significantly alter splenocyte IFN-γ production (Fig. 4.2B), co-incubation with LMMA significantly (p<0.01) enhanced it (Fig. 4.9B). ARα₂-agonist clonidine significantly enhanced splenocyte IL-2 production (Fig. 4.3B), while, co-treatment with LMMA significantly (p<0.01) decreased it (Fig. 4.9C).
Figure 4.8  *In vitro addition of α₁-AR-agonist phenylephrine and iNOS inhibitor LMMA on splenocyte proliferation and cytokine production.* LMMA (10⁻⁵ M) treatment with ARα₁-agonist phenylephrine decreased splenocyte proliferation (A), IFN-γ (B) and IL-2 production (C). *p<0.01 compared to control.
Figure 4.9  *In vitro addition of α₂-AR-agonist clonidine iNOS inhibitor LMMA on splenocyte proliferation and cytokine production.* Splenocytes co-treated with α₂-AR agonist clonidine and the iNOS inhibitor LMMA showed significantly enhanced proliferation (A), IFN-γ production (B) and significantly decreased IL-2 production (C). *p<0.01 compared to control.
4.3.10 $\alpha_1$ and $\alpha_2$-AR agonists differentially modulate p-NF-κB (p50) expression

The expression of p-NF-κB (p50) was assessed against β-actin expression in splenocytes treated with $\alpha_1$-AR agonist phenylephrine and $\alpha_2$-AR agonist clonidine. There was no significant change in β-actin expression indicative of uniform loading. Although treatment with phenylephrine did not significantly affect p-NF-κB (p50)/β-actin expression (4.10A and 4.10B), splenocytes treated with $\alpha_2$-AR agonist clonidine ($10^{-9}$ M) showed significantly enhanced p-NF-κB (p50)/β-actin expression compared to control (4.10C and 4.10D).

Figure 4.10 In vitro addition of $\alpha_1$- and $\alpha_2$-AR –agonists phenylephrine and clonidine on splenocyte p-NF-κB (p50) expression. There was no significant change in β-actin
expression indicative of uniform loading. Phenylephrine treatment did not significantly affect p-NF-κB (p50)/β-actin expression (A and B). Splenocytes co-treated with α2-AR agonist clonidine (10^-9 M) alone showed significantly enhanced p-NF-κB (p50)/β-actin expression compared to control (C and D) *p<0.01 compared to control.

4.4 Key findings:

α1-AR stimulation inhibited lymphocyte proliferation and IFN-γ production and enhanced IL-2, p-ERK and p-CREB expression. Co-stimulation using estrogen enhanced cytokine production and suppressed p-Akt expression. α1-AR blockade reversed agonist-induced IL-2 production alone.

α2-AR stimulation inhibited lymphocyte proliferation, p-ERK and p-CREB expression, and increased p-NF-κB and p-Akt expression. Co-stimulation with estrogen increased IL-2 and suppressed p-CREB expression. α2-AR Idazoxan prevented IL-2 production in the absence and presence of estrogen, and reversed clonidine-induced increase in NO production and p-ERK and p-Akt expression in the presence of estrogen.

These results suggest that the cell-mediated immune responses are selectively modulated depending upon the subtypes of α-AR and further, these effects are differentially regulated in the presence of estrogen mediated through selective alteration in the intracellular signaling pathways involving ERK, CREB, Akt, and NF-κB.