CHAPTER 5

IMMUNOMODULATORY ROLE OF ESTROGEN (E) ON β1-ADRENERGIC RECEPTOR (AR) AGENTS (AGONISTS AND ANTAGONISTS)-INDUCED LYMPHOCYTE FUNCTIONS

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

5.1 Rationale

The expression of β1-AR is predominantly found on the CD4+ T cells and B cells of rodents and humans and the ability of NE to signal through β-AR on T and B lymphocyte was dependent upon various factors such as type of activation, time of engagement, and expression of co-stimulatory molecules such as CD86. Although the presence of β1-AR on lymphocytes has been characterized, the role of β1-AR signaling in influencing the immune responses has not been delineated in the resting lymphocytes. Hence this study aims to investigate the immunomodulatory role of β1 AR agonists in the presence of estrogen or specific antagonist metoprolol in altering resting lymphocyte proliferation, cytokine production (IL-2, IFN-γ), signaling molecule expression including p-ERK, p-CREB and p-Akt in vitro.
5.2 Methods

5.2.1 Experiment 1

Adrenergic receptor (AR)-specific modulation by 17β-estradiol on lymphoproliferation and cytokines were measured by incubating lymphocytes (2x10^5 cells/ml) with β1-ARspecific agonist, dobutamine, and β1-AR specific antagonist, metoprolol. Dobutamine was co-incubated with lymphocytes at varying concentrations (10^{-3}M, 10^{-6} M and 10^{-9}M) with or without 10^{-5}M of the metoprolol or 17β-estradiol (E210^{-9} M). 17β-estradiol, dobutamine, and metoprolol were purchased from Sigma-Aldrich, St. Louis, MO. Stock solutions of dobutamine (0.1M) and metoprolol (0.1M) were prepared fresh everyday using 10 mMascorbate and serially diluted using media to the aforementioned concentrations. Stock solution of 17β-estradiol (0.1M) was prepared in absolute ethanol and serially diluted using media to the aforementioned concentration.

5.3 Results

5.3.1 In vitro effects of E2 on β1-adrenergic modulation of splenocyte proliferation and cytokine production

There was a significant decline in the proliferation of splenocytes treated with higher doses of β1- AR agonist, dobutamine, (10^{-3} M) which was reversed by co-treatment with β1-AR specific antagonist, metoprolol and estrogen (Fig. 5.1A). Metoprolol (10^{-5} M) and Estrogen (10^{-9} M) treatment alone also significantly enhanced the proliferation of splenocytes.

IFN-γ production was unaltered by dobutaminetreatment in lymphocytes (Fig. 5.1B). Estrogen (10^{-9} M) treatment alone significantly increased IFN-γ production compared to control.

Treatment of lymphocytes with dobutamine did not alter IL-2 production while co-treatment of dobutamine-treated lymphocytes with metoprolol (Dob 10^{-9} M and 10^{-6} M) and estrogen (10^{-6} M) significantly enhanced IL-2 production (Fig. 5.1C).

5.3.2 In vitro effects of β2-adrenergic agonists on splenocyte nitric oxide production

Nitric oxide production was not significantly altered in splenic lymphocytes treated with β1-AR agonist, dobutamine (Dob 10^{-9} M, 10^{-6} M and 10^{-3} M), although there
was a significant increase on co-treatment with β1-AR specific antagonist, metoprolol (Met 10⁻⁵ M). Co-treatment of dobutamine-treated lymphocytes with estrogen (10⁻⁹ M) significantly increased nitric oxide production compared to agonist-treated groups (Table 5.1).

**Figure 5.1**  *In vitro addition of β₁-AR-specific agonist, dobutamine, on splenocyte proliferation and cytokine production.*  Dobutamine treatment decreased splenocyte proliferation (A) and did not alter IFN-γ (B) and IL-2 production (C). Co-treatment of splenocytes with β-AR-specific antagonist, metoprolol or estrogen, reversed dobutamine-mediated decline in splenocyte proliferation and enhanced IL-2 production.  *p<0.05
compared to control, $p<0.05$ compared to respective agonist-treated group, $#p<0.05$ compared to $17\beta$-estradiol-treated group, $\dagger p<0.05$ compared to agonist with $17\beta$-estradiol-treated group, $‡p<0.05$ compared to $17\beta$-estradiol and agonist with $17\beta$-estradiol-treated groups.

**Table 5.1** *In vitro effects of $\beta_1$-adrenergic agonists on splenocyte nitric oxide production* ($\mu$g equivalents of NaNO$_2$/ml).

<table>
<thead>
<tr>
<th>Nitric oxide production</th>
<th>$(-) E_2$ (-) Met</th>
<th>$(+) Met10^{-5} \text{ M}$</th>
<th>$(+) E_2 10^{-9} \text{ M}$</th>
</tr>
</thead>
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<tr>
<td>Control</td>
<td>$2.0\pm0.1$</td>
<td>$3.5\pm0.2^{*}$</td>
<td>$2.0\pm0.1$</td>
</tr>
<tr>
<td>Dob $10^{-9} \text{ M}$</td>
<td>$2.1\pm0.3$</td>
<td>$3.5\pm0.1^{¥}$</td>
<td>$2.8\pm0.3^{‡}$</td>
</tr>
<tr>
<td>Dob $10^{-6} \text{ M}$</td>
<td>$2.0\pm0.2$</td>
<td>$2.6\pm0.1^{¥}$</td>
<td>$1.9\pm0.2$</td>
</tr>
<tr>
<td>Dob $10^{-3} \text{ M}$</td>
<td>$2.3\pm0.1$</td>
<td>$2.6\pm0.1^{¥}$</td>
<td>$2.0\pm0.02$</td>
</tr>
</tbody>
</table>

*$p<0.05$ compared to control, $¥p<0.05$ compared to respective agonist-treated group, $\dagger p<0.05$ compared to agonist with $17\beta$-estradiol-treated group.

### 5.3.3 In vitro effects of estrogen on $\beta_1$-adrenergic modulation of p-ERK expression in splenocytes

Treatment with $\beta_2$-AR agonist, dobutamine, significantly increased splenocyte p-ERK/Total ERK expression (Dob $10^{-3}$ M, $10^{-6}$ M, $10^{-9}$ M; Fig. 5.2A) and p-ERK expression (Dob $10^{-3}$ M, $10^{-6}$ M, $10^{-9}$ M; Fig. 5.2C) compared to control. Co-incubation with the antagonist metoprolol (Dob $10^{-9}$M) or estrogen (Dob $10^{-3}$ M, $10^{-6}$ M, $10^{-9}$ M) significantly reversed the agonist-mediated increase. Estrogen ($10^{-9}$ M) treatment alone significantly increased p-ERK/total ERK expression compared to control. There was no significant alteration in Total ERK expression indicative of equal loading (Fig. 5.2B).

### 5.3.4 In vitro effects of estrogen on $\beta_1$-adrenergic modulation of p-CREB expression in splenocytes

No significant alteration in p-CREB/total CREB expression and p-CREB expression was observed in $\beta_1$-AR agonist dobutamine-treated splenic lymphocytes with or without $\beta_1$-AR antagonist metoprolol (Fig. 5.3A, 5.3C). Co-incubation with estrogen ($10^{-9}$M) however, increased p-CREB/total CREB expression and p-CREB expression.
There was no significant alteration in Total CREB expression indicative of equal loading (Fig. 5.3B).

**Figure 5.2**  In vitro addition of β₁-AR-specific agonist, dobutamine, modulates the expression of p-ERK/Total ERK (A), Total ERK (B) and p-ERK (C) in cell lysates of splenocytes by ELISA. Dobutamine treatment enhanced p-ERK/Total ERK expression (A, C). Estrogen alone and along with dobutamine reduced the increase in dobutamine-induced p-ERK/Total ERK expression (A, C). *p<0.05 compared to control, §p<0.05 compared to respective agonist-treated group, †p<0.05 compared to agonist with 17β-estradiol-treated group.
Figure 5.3  In vitro addition of β1-AR-specific agonist, dobutamine, on the expression of p-CREB in cell lysates of splenocytes by ELISA. Dobutamine treatment did not alter p-CREB/Total CREB expression (A). Estrogen alone increased p-CREB/Total CREB expression. *p<0.05 compared to control

5.3.5 In vitro effects of estrogen on β1-adrenergic modulation of p-Akt expression in splenocytes

Treatment of splenocytes with dobutamine enhanced of p-Akt/total Akt expression and p-Akt expression in the absence and presence of antagonist, metoprolol. Estrogen co-
incubation alone enhanced p-Akt/Total Akt expression and p-Akt expression in splenocytes but there was no effect with co-treatment with terbutaline(Fig. 5.4A, 5.4C). There was no significant alteration in Total Akt expression indicative of equal loading (Fig. 5.4B).

**Figure 5.4**  *In vitro addition of β1-AR-specific agonist dobutamine, on p-Akt expression in cell lysates of splenocytes by ELISA.* Dobutamine treatment enhanced p-Akt/Total Akt expression (A). Estrogen alone also enhanced p-Akt/Total Akt expression. *p<0.05 compared to control.*
5.4 Key Findings

Dobutamine treatment decreased splenocyte proliferation and did not alter IFN-γ and IL-2 production. Co-treatment of splenocytes with β-AR-specific antagonist, metoprolol or estrogen, reversed dobutamine-mediated decline in splenocyte proliferation and enhanced IL-2 production. Although dobutamine treatment did not alter splenocyte nitric oxide production, estrogen co-treatment significantly enhance nitric oxide production by dobutamine-treated cells.

Dobutamine treatment enhanced p-ERK/Total ERK expression and p-Akt/Total Akt expression although it did not alter p-CREB expression. Co-treatment with estrogen significantly reversed the dobutamine-induced p-ERK/Total ERK expression. Thus taken together these results indicate that estrogen modulates β1AR-mediated cell signaling pathways in resting lymphocytes and reverses dobutamine-mediated immunosuppressive effects.