17β-Estradiol Concentration and Direct β_2-Adrenoceptor Inhibition Determine Estrogen-Mediated Reversal of Adrenergic Immunosuppression

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Abstract

Background: Sympathetic innervation of lymphoid organs, and the presence of 17β-estradiol (estrogen or E_2) and adrenergic receptors (ARs) on lymphocytes, suggests that sympathetic stimulation and hormonal activation may influence immune functions.
Purpose: Modeling and simulating these pathways may help to understand the dynamics of neuroendocrine-immune modulation at the cellular and molecular levels.
Methods: Dose- and receptor-dependent effects of E_2 and AR subtype-specific agonists were established *in vitro* on lymphocytes from young male Sprague-Dawley rats and were modeled *in silico* using the MATLAB Simbiology toolbox. Kinetic principles were assigned to define receptor–ligand dynamics, and concentration/time plots were obtained using Ode15s solvers at different time intervals for key regulatory molecules. Comparisons were drawn between *in silico* and *in vitro* data for validating the constructed model with sensitivity analysis of key regulatory molecules to assess their individual impacts on the dynamics of the system. Finally, docking studies were conducted with key ligands E_2 and norepinephrine (NE) to understand the mechanistic principles underlying their interactions.
Results: Adrenergic activation triggered proapoptotic signals, while E_2 enhanced survival signals, showing opposing effects as observed *in vitro*. Treatment of lymphocytes with E_2 shows a 10-fold increase in survival signals in a dose-dependent manner. Cyclic adenosine monophosphate (cAMP) activation is crucial for the activation of survival signals through extracellular signal-regulated kinase (p-ERK) and cAMP responsive element binding (p-CREB) protein. Docking studies showed the direct inhibition of ERK by NE and β_2-AR by E_2 explaining how estrogen signaling overrides NE-mediated immunosuppression *in vitro*.
Conclusion: The cross-talk between E_2 and adrenergic signaling pathways determines lymphocyte functions in a receptor subtype and coactivation-dependent manner in health and disease.

Keywords

Immunomodulation, IFN-γ, Norepinephrine, P-ERK, T-cell, 17β-estradiol

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Introduction

The neuroendocrine-immune network is a complex interregulatory system with wide plasticity in order to maintain systemic homeostasis.[1–3] In females, the cyclic fluctuations in the levels of gonadal hormones, especially 17β-estradiol (E_2), affect the functioning of immune effector cells by binding to specific estrogen receptors (ERs).[4–7]

In the periphery, *in vitro* and *in vivo* E_2-stimulation has been shown to enhance splenocyte proliferation and cytokine production through the alteration of specific signaling molecules.[2,4,8] E_2 enhances splenocyte proliferation, Interferon-γ (IFN-γ) expression, through extracellular

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signal-regulated kinase (p-ERK) and cAMP responsive element binding (p-CREB) signals; enhances activity of antioxidant enzymes (AOE) including superoxide dismutase (SOD) and catalase (CAT); and increases splenocyte nitric oxide (NO) expression dose dependently.[4] In the resting state, the close apposition of T-lymphocytes in direct synaptic association with sympathetic noradrenergic (NA) nerve fibers that innervate the lymphoid organs renders them highly responsive to NE.[7,9–13] Because both E_2 and NE mediate their effects on lymphocytes through their specific receptors, their downstream effects are dependent upon the kinetic parameters that govern receptor–ligand interactions.[10,14–16] Previous studies from our laboratory and others have shown that both E_2 and NE signaling cascades involve similar molecules (p-ERK, p-CREB, cAMP, and phosphorylated serine/threonine kinase [p-Akt]) in modulating the expression of similar cytokines (IFN-γ and interleukin-2 [IL-2]), thereby altering cell-mediated immune functions.[2,4,17,18] Thus, the scope of cross-talk between the two pathways during multiple ligand–receptor interactions is complex. Considering that NE can bind to α1-/α2- or β1-/β2-/β3-adrenoceptors (AR) on lymphocytes and that E_2 can bind to cytosolic or nuclear estrogen receptors (cER and nER) and initiate signals that target similar down-stream signaling molecules, the underlying kinetic parameters can influence the outcome of the cross-talk through synergistic, additive, or diminutive effects.

Our lab has delineated these effects in splenocytes using AR-specific agonists and antagonists in the presence and absence of E_2.[17,18] α1-AR agonist phenylephrine enhances p-ERK and p-CREB expression, while α2-AR agonist clonidine enhances p-Akt expression and may play a crucial role in the sustenance of naïve cells in the secondary lymphoid organs.[17] β2-AR agonist terbutaline enhances the expression of p-ERK, p-CREB and p-Akt signals, AOE activities including CAT and SOD, and the production of NO in splenocytes.[18] In this study, we have superimposed the four signaling cascades (Estrogen signaling, α1-AR signaling, α2-AR signaling, and β2-AR signaling) from our *in vitro* studies and have created a dynamic model of resting lymphocyte functions. In order to simulate real-time events, we have used kinetic principles that govern receptor–ligand activation based on secondary data from the available literature along with concentration data from *in vitro* studies published from our laboratory.[4,17,18] The present study aims to create a computational model of the pathways studied *in vitro* so that the scope of the intercommunication between these pathways can be better understood in health and disease.

Methods

Model Building

The model was built using both primary and secondary data sources. The dose- and receptor-dependent effects of E_2 and AR subtype-specific agonists were established *in vitro* on lymphocytes from young male Sprague-Dawley rats in previous studies published by our laboratory.[17,18] Briefly, splenic lymphocytes were isolated from young male Sprague-Dawley rats and treated with different doses of E_2,[4] AR-α agonists phenylephrine and clonidine,[17] and AR-β agonist terbutaline[18] for a period of 24 to 72 h. In these studies, the concentration of the signaling molecules, cytokines, and other secondary molecules was determined using enzyme-linked immunosorbent assay (ELISA), and these data were used to construct the model (Tables 1 and 2). The possible cross-talk pathway was mapped using receptor-specific inhibitors and inhibitors of signal transduction molecules, and it was modelled *in silico* using the MATLAB Simbiology toolbox (MATLAB and SimBiology toolbox Release 2019a, The Mathworks, inc., Natick, Massachusetts, United States). Receptor–ligand dynamics were defined based on kinetic principles from the available literature and previous studies from our laboratory.[4,17,18] State-versus-time plots were obtained using Ode15s solvers at 24 h or 72 h for long-term effects on lymphocyte functions. Comparisons were drawn between *in silico* and *in vitro* data for validating the constructed model. Sensitivity analysis was performed on key regulatory molecules to assess their individual impacts on the dynamics of the system. The model was uploaded in the Github database. (https://github.com/Anandt1082/Neuroimmunomodulation-by-estrogen-and-adrenergic-agonists.git).

Model

The model was built to show an interconnected network of four signaling pathways on the basis of *in vitro* studies conducted in our laboratory including the following:

1. α1-adrenoceptor signaling[17]; (Table 1A and Figure 1A) Construction of the α1-adrenoceptor signaling pathway was based on the data from our lab and others which showed that stimulating the α1-AR in splenocytes with 10^{-6} M and 10^{-9} M specific agonist phenylephrine did not alter splenocyte proliferation but enhanced the survival signaling molecules including p-ERK and p-CREB.[17] In order to simulate the receptor–ligand reactions in real-time, the receptor–ligand interactions were defined for α1-AR as shown in Table 2A.[19] The concentrations of ERK and CREB used in the model were based on the data from the published *in vitro* study determined using ELISA.[17] Downstream signaling cascades were assumed to follow the law of mass action. Molecules for which concentrations were not known, such as protein kinase-A (PKA) and CREB-binding protein (CBP), were assigned arbitrary values (257 nM) that did not impose a constraint on the system.
2. α2-adrenoceptor signaling[17]; (Table 1B and Figure 1A) α2-AR signaling was constructed based on the data obtained from our study showing that incubation of α2-AR specific agonist clonidine (10^{-9} M- 10^{-6} M)

enhanced the Akt signaling cascade.[17] Although our study showed a decrease in cellular proliferation in response to clonidine, there was no reversal upon cotreatment with specific antagonist idazoxan. It is possible that α2-AR stimulation is necessary for the sustenance of nerve fibers through the activation of B-cell lymphoma-2 (BCL-2) downstream to Akt signaling.[20] The receptor–ligand interactions for α2-AR are defined in Table 2A.[21] The concentration for Akt was obtained from the *in vitro* study using ELISA.[17] Downstream signaling cascades were assumed to follow the law of mass action. Molecules for which concentrations were not known, such as BCL-2, were assigned arbitrary values (300 nM) that did not impose a constraint on the system.

3. β2-adrenoceptor signaling[18]; (Table 1C, Figure 1A)
   Construction of the β2-adrenoceptor signaling was based on the data obtained from the published *in vitro* study.[18] Stimulation of splenocytes using the β2-adrenoceptor agonist terbutaline significantly enhanced p-ERK and p-CREB expression, and activity of SOD and CAT through PKA. Terbutaline treatment also enhanced IL-2 expression through p-Akt. p-Akt expression also enhances the activity of inducible nitric oxide synthase (iNOS), leading to an increase in NO expression. NO can react with superoxides (O_2-) and form peroxynitrites that may accumulate and lead to apoptosis, or can trigger CAAT, CCAAT-enhancer binding proteins (C/EBP), C/EBP homologous protein (CHOP), or BCL-, leading to dimerization of Bcl-2 Associated X-protein (BCL-2–BAX) complex, in turn leading to apoptosis.[22] The receptor–ligand interactions for β2-AR and IFN-γ are defined in Table 2A.[23,24] The concentration for ERK, CREB, Akt, NO, IFN-γ, and IL-2 were obtained from the *in vitro* study using ELISA.[17] Downstream signaling cascades were assumed to follow the law of mass action. Molecules for which concentrations were not known, such as phosphoinositide 3-kinase (PI3K), PKA, CBP (257 nM), SOD, CAT, iNOS (0 units), CAAT, CEBP, CHOP, BAX, BCL-2, and O2- (0 units), were assigned arbitrary initial values that did not impose a constraint on the system.
4. 17β-Estradiol Signaling
   E_2 signaling through ERs and G-protein coupled receptors (GPCRs[4]; Table 1D and Figure 1B) and their respective kinetic parameters are outlined in Table 2. Estrogen signaling was constructed based on the data from the *in vitro* study published from our lab.[4] Splenocytes stimulated with E_2 showed enhanced

Table 1. Reaction Table and Kinetics for α1-Adrenoceptor Signaling (1A), α2-Adrenoceptor Signaling (1B), β2-Adrenoceptor Signaling (1C), and E_2 Signaling Through ERs and GPCRs (1D)

1A	Reaction	Kinetics	References
1	$[AR-\alpha 1]+[Phenylephrine]\rightarrow[RL1]$	$vm*[Phenylephrine]/(km+[Phenylephrine])$	26–29
2	$[CREB]+[RL1]\rightarrow[p-CREB]$	$nkn.[CREB].[RL1]$	
3	$[p-CREB]+[CBP]\rightarrow[CBP-CREB]$	$ct.[p-CREB].[CBP]$	
4	$[CBP]-[p-CREB]+[DNA]\rightarrow[mRNA1]+[mRNA2]$	$tr.[CBP-p-CREB].[DNA]$	
5	$[mRNA1]\rightarrow[SurvivalSignal]$	$as.[mRNA1]$	
1B	**Reaction**	**Kinetics**	**References**
1	$[AR-\alpha 2]+[Clonidine]\rightarrow[RL2]$	$va*[Clonidine]/(kn+[Clonidine])$	30–33
2	$[PI3K*]+[Akt]+[RL2]\rightarrow[p-Akt]$	$at.[PI3K*].[Akt].[RL2]$	
3	$[RL2]+[PKA*]\rightarrow[PKA]$	$ml.[RL2].[PKA*]$	
4	$[p-Akt]+[BCL2]\rightarrow[BCL2*]$	$bc.[p-Akt].[BCL2]$	
5	$[BCL2*]\rightarrow[SurvivalSignal]$	$aps.[BCL2*]$	

(Table 1 continued)

(Table 1 continued)

IC	Reaction	Kinetics	References
1	$[\beta 2-AR]+[Terbutaline]\rightarrow[RL3]$	$bt.[Terbutaline]/(kbt+[Terbutaline])$	34–43
2	$[RL3]+[PKA]\rightarrow[PKA^*]$	$gh.[RL3].[PKA]$	
3	$[RL3]+[PI3K]\rightarrow[PI3K^*]$	$jk.[RL3].[PI3K]$	
4	$[PKA^*]+[ERK]\rightarrow[p-ERK]$	$fd.[PKA^*].[ERK]$	
5	$[p-ERK]+[CREB]\rightarrow[p-CREB]+[ERK]$	$pe.[p-ERK].[CREB]$	
6	$[p-CREB]+[CBP]\rightarrow[CBP-CREB]$	$ct.[p-CREB].[CBP]$	
7	$[CBP]-[p-CREB]+DNA\rightarrow[mRNA1]+[mRNA2]$	$tr.[CBP-p-CREB].[DNA]$	
8	$[p-Akt]+[DNA]\rightarrow[mRNA2-1-1]$	$kj.[p-Akt].[DNA]$	
9	$[mRNA2-1-1]\rightarrow[iNOS]$	$hg.[mRNA2-1-1]$	
10	$L-Arg+iNOS\rightarrow NO$	$hg.L-Arg/(fd+[L-Arg])$	
11	$[NO]+[CAAT]\rightarrow[C/EBP]$	$vm.[NO].[CAAT]$	
12	$[C/EBP]\rightarrow[CHOP]$	$cvc.[CAAT]/(kc+[CAAT])$	
13	$[DNA]+[CHOP]\rightarrow[mRNA2-1]$	$vch.[CHOP]/(kch+[CHOP]$	
14	$[mRNA2-1]\rightarrow[BAX]$	$fbax.[mRNA2-1]$	
15	$[BAX]+[BCL2]\rightarrow[BCL2-BAX]2'$	$vbax.[BAX]/(kbax+[BAX])$	
16	$[BCL2-BAX]2\rightarrow[ApoptoticSignal]$	$vx.[BCL2-BAX]2$	
17	$[DNA]+[p-Akt]\rightarrow[mRNA3]$	$kj.[DNA].[p-AKt]$	
18	$[mRNA3]\rightarrow[IL-2]$	$kl.[mRNA3]$	
19	$[IL-2]\rightarrow[SurvivalSignal]$	$kj.[IL-2]$	
20	$[p-ERK]+DNA\rightarrow[mRNA6]$	$f1.[p-ERK].[DNA]$	18
21	$[Superoxides]+[CAT]\rightarrow[SurvivalSignal]$	$v1.[Superoxides]/[k1+Superoxides]$	
22	$[p-ERK]+DNA\rightarrow[mRNA7]$	$jh.[p-ERK].[DNA]$	
23	$[Superoxides]+[SOD]\rightarrow[SurvivalSignal]$	$v2.[Superoxides]/[k2+Superoxides]$	

(Table 1 continued)

(Table 1 continued)

24	$[Superoxides]+[NO]\rightarrow [Peroxynitrite]$	$gf.[Superoxides].[NO]$	
25	$[mRNA6]\rightarrow [CAT]$	$kj.[mRNA6]$	
26	$[mRNA7]\rightarrow [SOD]$	$kj.[mRNA7]$	
ID	**Reaction**	**Kinetics**	**References**
1	$[17\beta-Estradiol]+[GPCR]\rightarrow [cAMP]$	$Vm2.[\beta-Estradiol]/(km1+[17\beta-Estradiol]$	44–50
2	$[cAMP]+[PKA]\rightarrow [PKA^*]$	$pk.[cAMP].[PKA]$	
3	$[ER]+[17\beta-Estradiol]\rightarrow [E-ER]$	$erb.[ER1].[17\beta-Estradiol]$	
4	$[E-ER]+[shc-grb2sos]\rightarrow [E-ER-shc-grb2sos]$	$eg.[E-ER].[shc-grb2sos]$	
5	$[E-ER-shc-grb2sos]+[PI3K]\rightarrow [PI3K^*]$	$ep.[E-ER-shc-grb2sos].[PI3K]$	
6	$[E-ER]+[shc-grb2sos]+[RAS]\rightarrow [RAS^*]$	$era.[E-ER].[shc-grb2sos].[RAS]$	
7	$[RAF]+[RAS^*]\rightarrow [RAF^*]$	$kc.RAF^*.RAS^*$	
8	$[MEK]+[RAF^*]\rightarrow [MEK-RAF^*]$	$mek.[MEK].[RAF^*]$	
9	$[MEK-RAF^*]\rightarrow [MEK-p]+[RAF]$	$mekp.[MEK-RAF^*]$	
10	$[MEK-p]+[RAF^*]\rightarrow [MEK-p-RAF^*]2$	$rm.[MEK-p].[RAF^*]$	
11	$[MEK-p-RAF^*]2\rightarrow [MEK-pp]+[RAF]$	$mpp.[MEK-p-RAF^*]2$	
12	$[ERK]+[MEK-pp]\rightarrow [p-ERK]$	$ka2.[ERK].[MEK-pp]$	
13	$[p-ERK]+[p-CREB]\rightarrow [p-CREB]+[ERK]$	$pe.[p-ERK].[CREB]$	
14	$[p-CREB]+[CBP]\rightarrow [CBP-CREB]$	$ct.[p-CREB].[CBP]$	
15	$[CBP]-[p-CREB]+[DNA]\rightarrow [mRNA1]+[mRNA2]$	$tr.[CBP-p-CREB].[DNA]$	
16	$[mRNA2]\rightarrow [IFN-\gamma]$	$ifp.[mRNA2]$	
17	$[mRNA2]\rightarrow [SurvivalSignal]$	$bnb.[mRNA2]$	
18	$[PI3K^*]+[Akt]\rightarrow [p-Akt]$	$at.[PI3K^*].[Akt]$	

(Table 1 continued)

(Table 1 continued)

19	$[p-Akt]+[DNA]\rightarrow[mRNA2-1-1]$	$kj.[p-Akt].[DNA]$	
20	$[mRNA2-1-1]\rightarrow[iNOS]$	$hg.[mRNA2-1-1]$	
21	$L-Arg+iNOS\rightarrow NO$	$hg.L-Arg/(fd+[L-Arg])$	
22	$[NO]+[CAAT]\rightarrow[C/EBP]$	$vm.[NO].[CAAT]$	
23	$[C/EBP]\rightarrow[CHOP]$	$cvc.[CAAT]/(kc+[CAAT])$	
24	$[DNA]+[CHOP]\rightarrow[mRNA2-1]$	$vch.[CHOP]/(kch+[CHOP])$	
25	$[mRNA2-1]\rightarrow[BAX]$	$fbax.[mRNA2-1]$	
26	$[BAX]+[BCL2]\rightarrow[BCL2-BAX]2'$	$vbax.[BAX]/(kbax+[BAX])$	
27	$[BCL2-BAX]2\rightarrow[ApoptoticSignal]$	$vx.[BCL2-BAX]2$	
28	$[17\beta-Estradiol]+[cER]\rightarrow[E-cER]$	$ecr.[17\beta-Estradiol].[cER]$	
29	$E-cER\rightarrow[E-cER]2$	$ed.[E-cER]$	
30	$[E-cER]2+[DNA]\rightarrow[mRNA2]$	$nt.[E-cER]2.[DNA]$	
31	$[mRNA2]\rightarrow[IFN-\gamma]$	$ifn.[mRNA2]$	
32	$[IFNGR]+[IFN-\gamma]\rightarrow[IFN\gamma-IFNGR]$	$vm.[CREB\}/(kd+[CREB])$	
33	$[IFN\gamma-IFNGR]\rightarrow[IFN\gamma-IFNGR]2$	$rd.[IFN\gamma-IFNGR]$	
34	$[IFN\gamma-IFNGR]2\rightarrow[IFN\gamma-IFNGR*]2$	$rc.[IFN\gamma-IFNGR]2$	
35	$[IFN\gamma-IFNGR*]2+[RAS]+[shc-grb2sos]\rightarrow[RAS*]$	$kx.[IFN\gamma-IFNGR*]2.[RAS*].[shc-grb2sos]$	
36	$[p-ERK]+DNA\rightarrow[mRNA6]$	$f1.[p-ERK].[DNA]$	[4,17,18]
37	$[Superoxides]+[CAT]\rightarrow[SurvivalSignal]$	$v1.[Superoxides]/[k1+Superoxides]$	
38	$[p-ERK]+DNA\rightarrow[mRNA7]$	$jh.[p-ERK].[DNA]$	
39	$[Superoxides]+[SOD]\rightarrow[SurvivalSignal]$	$v2.[Superoxides]/[k2+Superoxides]$	
40	$[Superoxides]+[NO]\rightarrow[Peroxynitrite]$	$gf.[Superoxides].[NO]$	
41	$[mRNA6]\rightarrow[CAT]$	$kj.[mRNA6]$	
42	$[mRNA7]\rightarrow[SOD]$	$kj.[mRNA7]$	

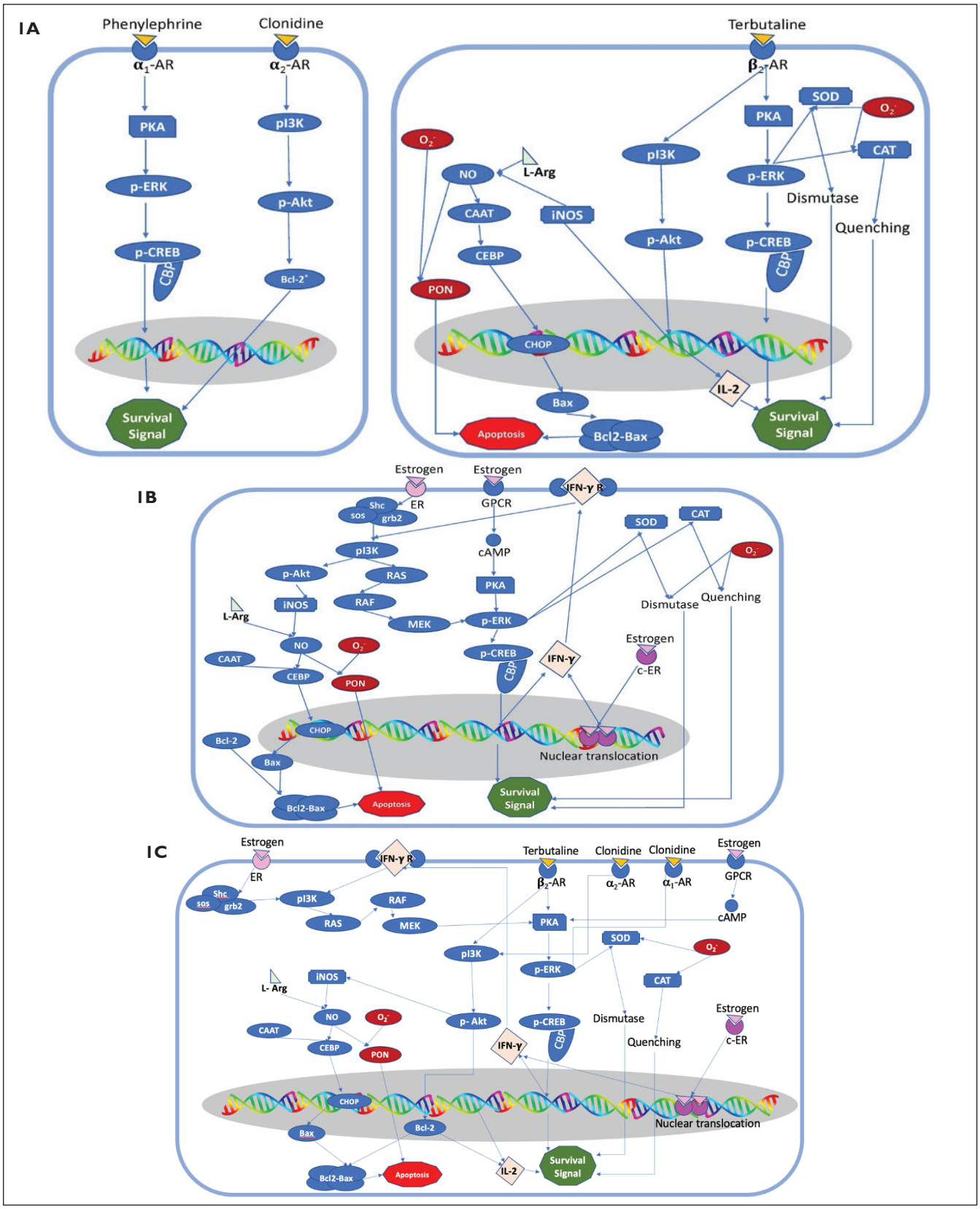


Figure 1. *In silico model of adrenergic [1A] and estrogen [1B] signalling cascades in lymphocytes:* The cross-talk between the adrenergic signals (1A) and estrogen-mediated signals (1B) result in specific immunomodulatory effects depending upon the estrogen concentration. The layout of the signalling pathway is elucidated from receptors to transduction into the cytosol and nuclear translocation of signalling molecules leading to specific outcomes (1C).

Table 2. Receptor Binding Maxima and Kd Values (A), Signaling Molecule Concentrations (B), and Ligand Concentrations (C) as Incorporated in the Model

2A	Receptor	Bmax	Kd (nM)	Reference
1.	α1-AR	175.3 (fM/10^6 cells)	0.65	19
2.	α2-AR	19.9 (fM/10^6 cells)	3.7	21
3.	β2-AR	1222 sites/cell	19.9	23,24
4.	IFN-gR	708 ± 14 receptors/cell	0.9±0.2	24
5.	cER	0.6204 (fM/mg protein)	5.5	25
6.	nER	(0.136 fM/mcg DNA)	-	25
2B	Signaling Molecules		Concentration (nM)	Reference
1.	ERK		251	17
2.	CREB		245.6	
3.	Akt		219	
4.	IFN-g		560	
2C	Signaling Molecules		Concentration	Reference
1.	Phenylephrine		10^{-6} M	17,18
2.	Clonidine		10^{-6} M	
3.	Terbutaline		10^{-6} M	
4.	Estrogen		10^{-8} M	

expression of ERK and CREB signals through PKA and cAMP by estrogen binding to GPCRs, leading to the expression of IFN-γ. IFN-γ can bind to IFN-γ receptor, and can activate PI3K, Ras, Raf, MEK, ERK and CREB cascades, leading to survival signals. Estrogen can bind to ERs; stimulate Shc, Grb-2, and Sos; activate PI3K, Akt, and iNOS leading to NO expression; activate CAAT, CEBP, and CHOP; and induce pro-apoptotic signals through Bcl-2/Bax cascades. NO can also bind with O_2- leading to the production of peroxynitrites that serve as an apoptotic signal. On the other hand, E_2 also promotes the expression of SOD and CAT through p-ERK leading to scavenging of the excess O2-, in turn leading to survival signals.[4] E_2 can also bind to nuclear receptors and lead to the production of IFN-γ. The receptor–ligand interactions for E_2 receptors and IFN-γ receptors are defined in Table 2A.[24,25] The concentration for ERK, CREB, Akt, NO, and IFN-γ were obtained from the *in vitro* study using ELISA.[17] Downstream signaling cascades were assumed to follow the law of mass action. Molecules for which concentrations were not known, such as PI3K, PKA, CBP (257 nM), SOD, CAT, iNOS (0 units), CAAT, CEBP, CHOP, BAX, BCL-2, and O2- (0 units), were assigned arbitrary initial values that did not impose a constraint on the system. Enzyme kinetics for CAT, SOD, and iNOS were defined using the Henri-Michaelis-Menten equation.

Simulations

Simulations were performed using Ode15s solvers for 24 h and 72 h depending upon the signals studied.

1. Estrogen Signaling
   a. Simulation Conditions: (Figure 2A, Figure 3A, Figure 4A, and Figure 5A)
      i. Terbutaline = 0 M
      ii. Phenylephrine = 0 M
      iii. Clonidine = 0 M
      iv. $E_2 = 10^{-6}$ M
      v. Time: 24 h and 72 h
2. Adrenergic Signaling
   a. Simulation Conditions: (Figure 2B, Figure 3B, Figure 4B, and Figure 5B)
      i. Terbutaline = 10^{-6} M
      ii. Phenylephrine = 10^{-6} M
      iii. Clonidine = 10^{-6} M
      iv. E_2 = 0 M
      v. Time: 24 h and 72 h
3. Estrogen and Adrenergic Signaling
   a. Simulation Condition-1: (Figure 2C, Figure 3C, Figure 4C, and Figure 5C)
      i. Terbutaline = 10^{-6} M
      ii. Phenylephrine = 10^{-6} M
      iii. Clonidine = 10^{-6} M
      iv. $E_2 = 10^{-6}$ M
      v. Time: 24 h and 72 h
   b. Simulation Condition-2: (Figure 6)
      i. Terbutaline = 10^{-6} M
      ii. Phenylephrine = 10^{-6} M
      iii. Clonidine = 10^{-6} M
      iv. $E_2 = 10^{-6}$ M, 10^{-5} M, 10^{-4} M, and 10^{-3} M
      v. Time: 24 h and 72 h

The Mcode is included in the supplementary evidence. The model was tested for various other physiologically relevant

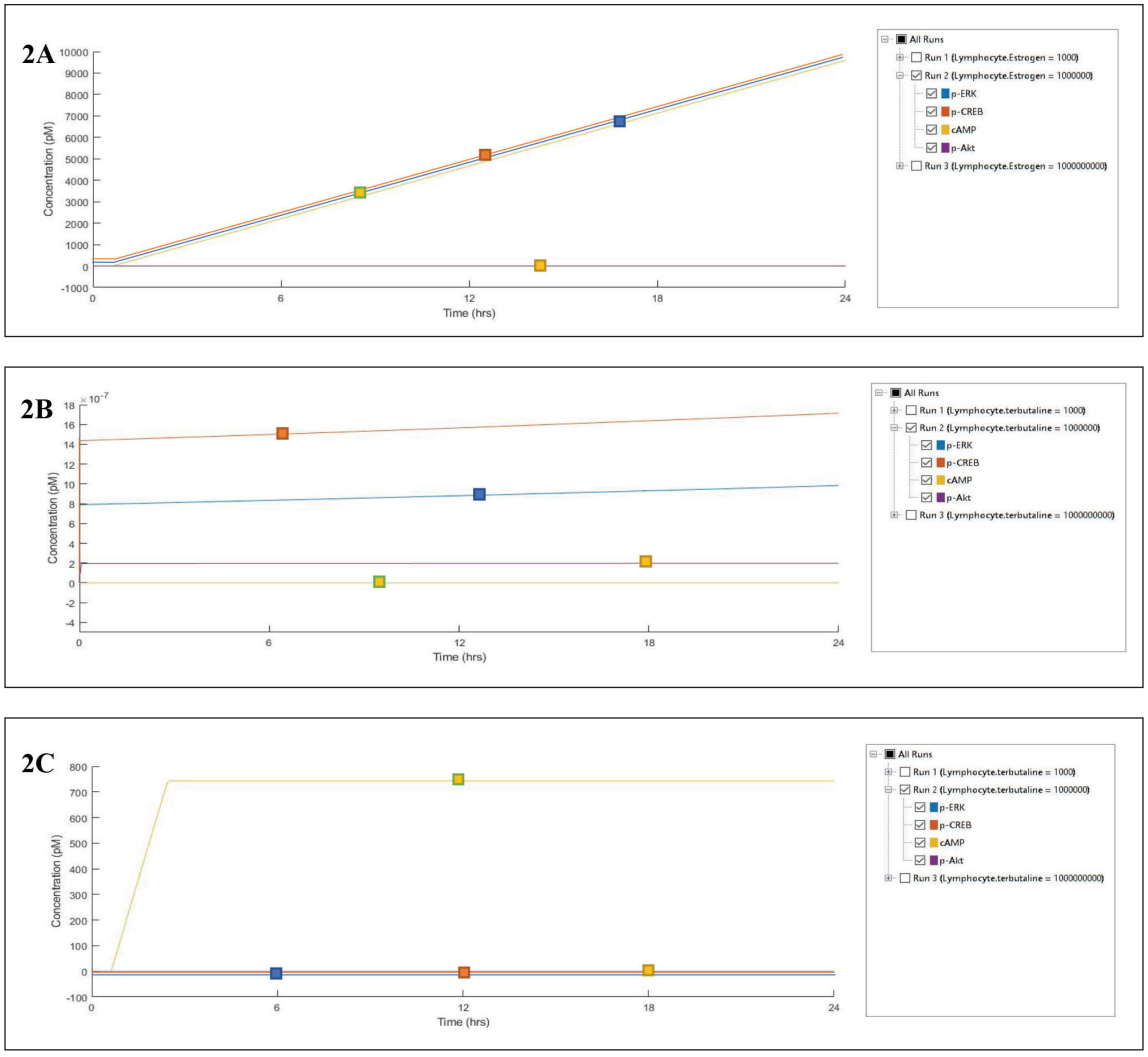


Figure 2. *Expression of molecular markers p-ERK, p-CREB, cAMP and p-Akt by lymphocytes treated with estrogen, adrenergic agonists and in combination:* Simulation of lymphocytes treated with 10^{-6} M Estrogen for 24 hours (2A). Simulation of lymphocytes treated with terbutaline (10^{-6} M), phenylephrine (10^{-6} M) and clonidine (10^{-6} M) for 24 hours (2B). Simulation of lymphocytes treated with terbutaline (10^{-6} M), phenylephrine (10^{-6} M) and clonidine (10^{-6} M) in the presence of estrogen (10^{-6} M) for 24 hours (2C).

combinations, as follows and the figures are shown in supplementary evidence.

1. Estrogen Signaling: (Supplementary Figures 1–4)
   a. Simulation Conditions
      i. Terbutaline = 0 M
      ii. Phenylephrine = 0 M
      iii. Clonidine = 0 M
      iv. $E_2 = 10^{-3}$ M, 10^{-6} M, 10^{-9} M
      v. Time: 24 h and 72 h
2. Adrenergic Signaling
   a. Simulation Condition-1: Terbutaline Signaling (Supplementary Figures 5–8)
      i. Terbutaline = 10^{-3} M, 10^{-6} M, and 10^{-9} M
      ii. Phenylephrine = 10^{-6} M
      iii. Clonidine = 10^{-6} M
      iv. $E_2 = 0$ M
      v. Time: 24 h and 72 h
   b. Simulation Condition-2: Phenylephrine Signaling (Supplementary Figures 9–12)
      i. Terbutaline = 10^{-6} M
      ii. Phenylephrine = 10^{-3} M, 10^{-6} M, and 10^{-9} M
      iii. Clonidine = 10^{-6} M
      iv. $E_2 = 0$ M
      v. Time: 24 h and 72 h
   c. Simulation Condition-3: Clonidine Signaling (Supplementary Figures 13–16)
      i. Terbutaline = 10^{-6} M
      ii Phenylephrine = 10^{-6} M
      iii. Clonidine = 10^{-3} M, 10^{-6} M, and 10^{-9} M
      iv. $E_2 = 0$ M
      v. Time: 24 h and 72 h
3. Adrenergic + Estrogen Signaling
   a. Simulation Condition-1: Terbutaline + Estrogen Signaling (Supplementary Figures 17–20)

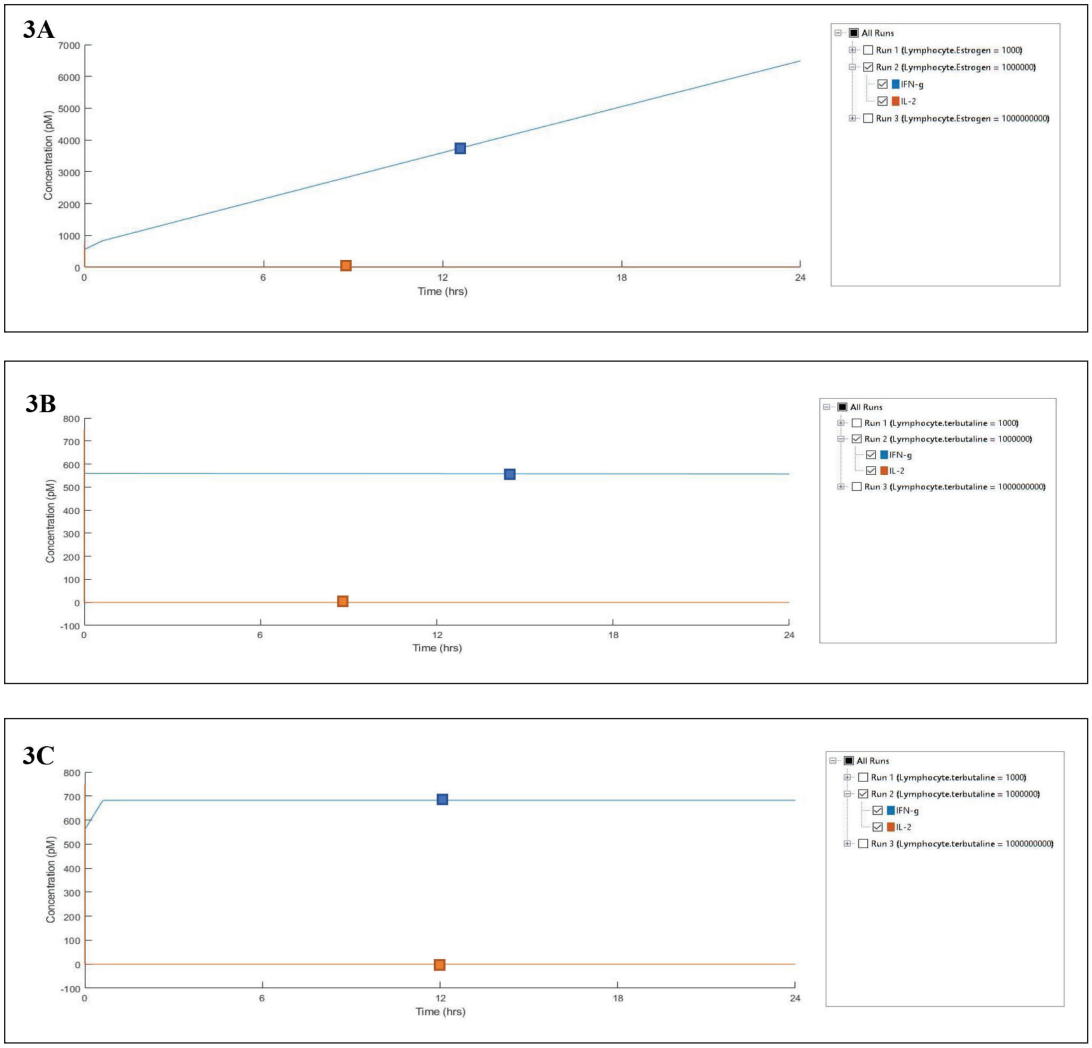


Figure 3. *Expression of cytokines (IL-2 and IFN-g) by lymphocytes treated with estrogen, adrenergic agonists and in combination: Simulation of lymphocytes treated with* 10^{-6} M Estrogen for 24 hours (3A). Simulation of lymphocytes treated with terbutaline (10^{-6} M), phenylephrine (10^{-6} M) and clonidine (10^{-6} M) for 24 hours (3B). Simulation of lymphocytes treated with terbutaline (10^{-6} M), phenylephrine (10^{-6} M) and clonidine (10^{-6} M) in the presence of estrogen (10^{-6} M) for 24 hours (3C).

i. Terbutaline = 10^{-3} M, 10^{-6} M, and 10^{-9} M
ii. Phenylephrine = 10^{-6} M
iii. Clonidine = 10^{-6} M
iv. E_2 = 10^{-8} M (Physiological concentration)
v. Time: 24 h and 72 h

b. Simulation Condition-2: Phenylephrine + Estrogen Signaling (Supplementary Figures 21–24)
i. Terbutaline = 10^{-6} M
ii. Phenylephrine = 10^{-3} M, 10^{-6} M, and 10^{-9} M
iii. Clonidine = 10^{-6} M
iv. E_2 = 10^{-8} M
v. Time: 24 h and 72 h

c. Simulation Condition-3: Clonidine + Estrogen Signaling (Supplementary Figures 24–28)
i. Terbutaline = 10^{-6} M
ii. Phenylephrine = 10^{-6} M
iii. Clonidine = 10^{-3} M, 10^{-6} M, and 10^{-9} M
iv. E_2 = 10^{-8} M
v. Time : 24 h, 72 h

For signaling molecules including p-ERK, p-Akt, p-CREB, and cAMP; cytokines including IL-2 and IFN-γ; AOEs (SOD and CAT); and O2-/peroxynitrites, the simulations were performed up to 24 h, similar to the *in vitro* study. Finally, for survival vs. apoptotic signals, the simulations were carried out for 72 h. Because all the ARs are activated by the same ligand NE, physiologically, the simulations were run for different doses of one receptor subtype (e.g., terbutaline 10^{-3} M, 10^{-6} M, or 10^{-9} M) while allowing for a mid-range activity in the other two (clonidine 10^{-6} M and phenylephrine 10^{-6} M) in the presence (10^{-6} M or 10^{-8} M) and absence (0 M) of E_2. Key regulatory molecules were scanned, and their sensitivities were analyzed at 24 h for each of the ligands used (supplementary Figures 1D–28D; M code attached as supplementary Index-2).

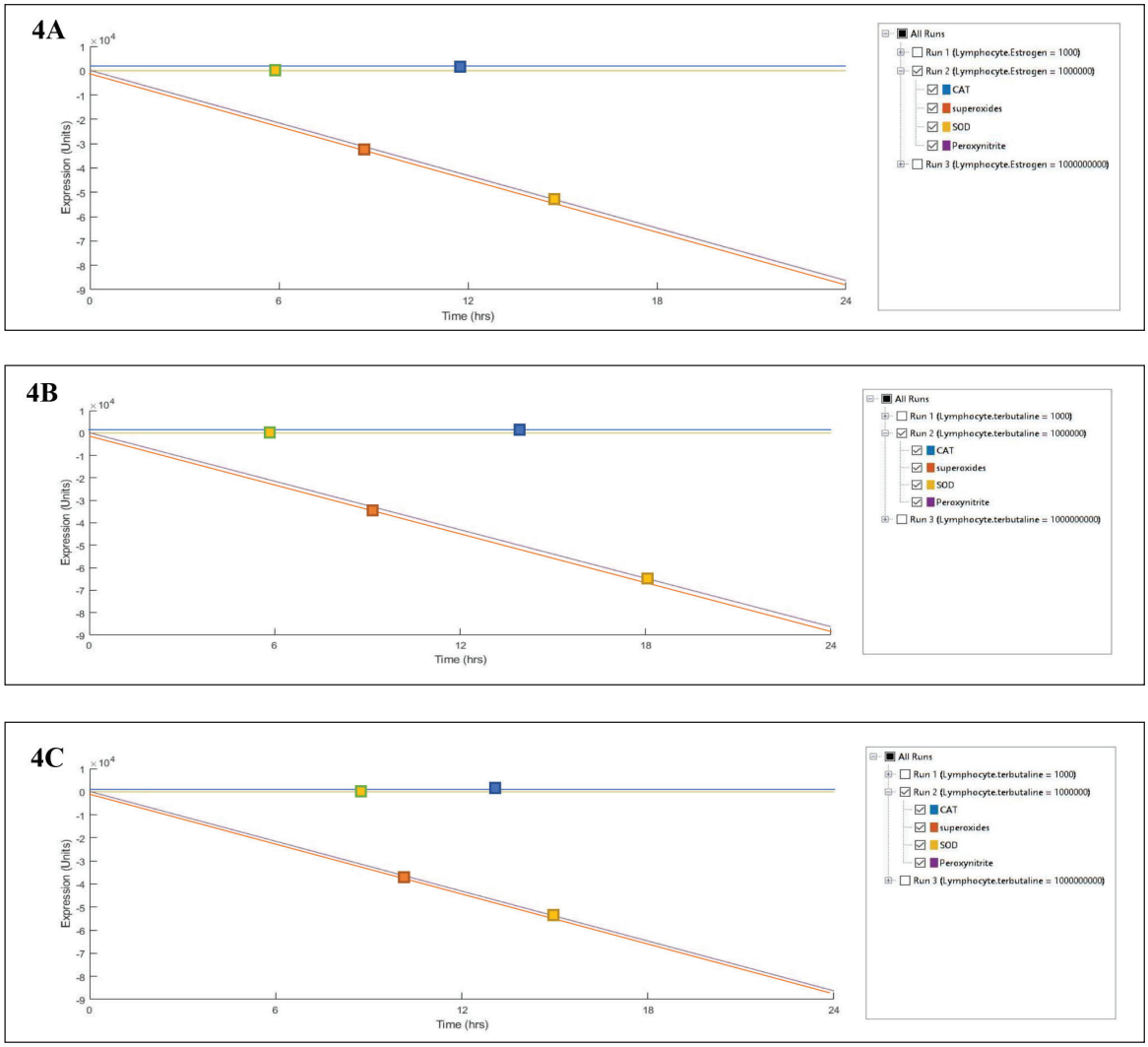


Figure 4. *Expression of antioxidant enzymes (CAT and SOD), superoxides and peroxynitrites by lymphocytes treated with estrogen, adrenergic agonists and in combination: Simulation of lymphocytes treated with* 10^{-6} M Estrogen for 24 hours (4A). Simulation of lymphocytes treated with terbutaline (10^{-6} M), phenylephrine (10^{-6} M) and clonidine (10^{-6} M) for 24 hours (4B). Simulation of lymphocytes treated with terbutaline (10^{-6} M), phenylephrine (10^{-6} M) and clonidine (10^{-6} M) in the presence of estrogen (10^{-6} M) for 24 hours (4C).

Docking

Molecular docking was performed using Auto Dock 4.2.6 for

1. ERK molecule (PDB ID: 2ERK; *Rattus rattus)* and NE (ligand).
2. β2-AR molecule (PDB ID: 3SN6; *Rattus norvegicus)* and E_2 (ligand).

The enzyme/receptor molecule was loaded after assigning hydrogen bonds and kollman charges. The investigation ligands were loaded and their torsions along with rotatable bonds were assigned. The map files were selected directly with setting up grid points with 80 X 106 X 90 dimensions for the searching of ligand within the active site of the enzyme molecule. The docking parameter files were then set up with the search parameter as genetic algorithm and docking parameter utilizing Lamarckian genetic algorithm. The docked structures were generated after maximum number of evaluations using UCSF Chimera for the best conformer which fits with lowest binding energy (kcal/mol).

Results

Modulation of Molecular Markers With E_2 and Adrenergic Agonists in Silico

Simulation of lymphocytes with 10^{-6} M E_2 for 24 h enhanced the expression of p-ERK, p-CREB, and cAMP, compared to simulation with adrenergic agonists alone or in combination with E_2 (Figure 2). cAMP alone was significantly ($P < .01$) enhanced upon simulation with adrenergic agonists and E_2 for 24 h, compared to adrenergic agonists alone (Figure 2C).

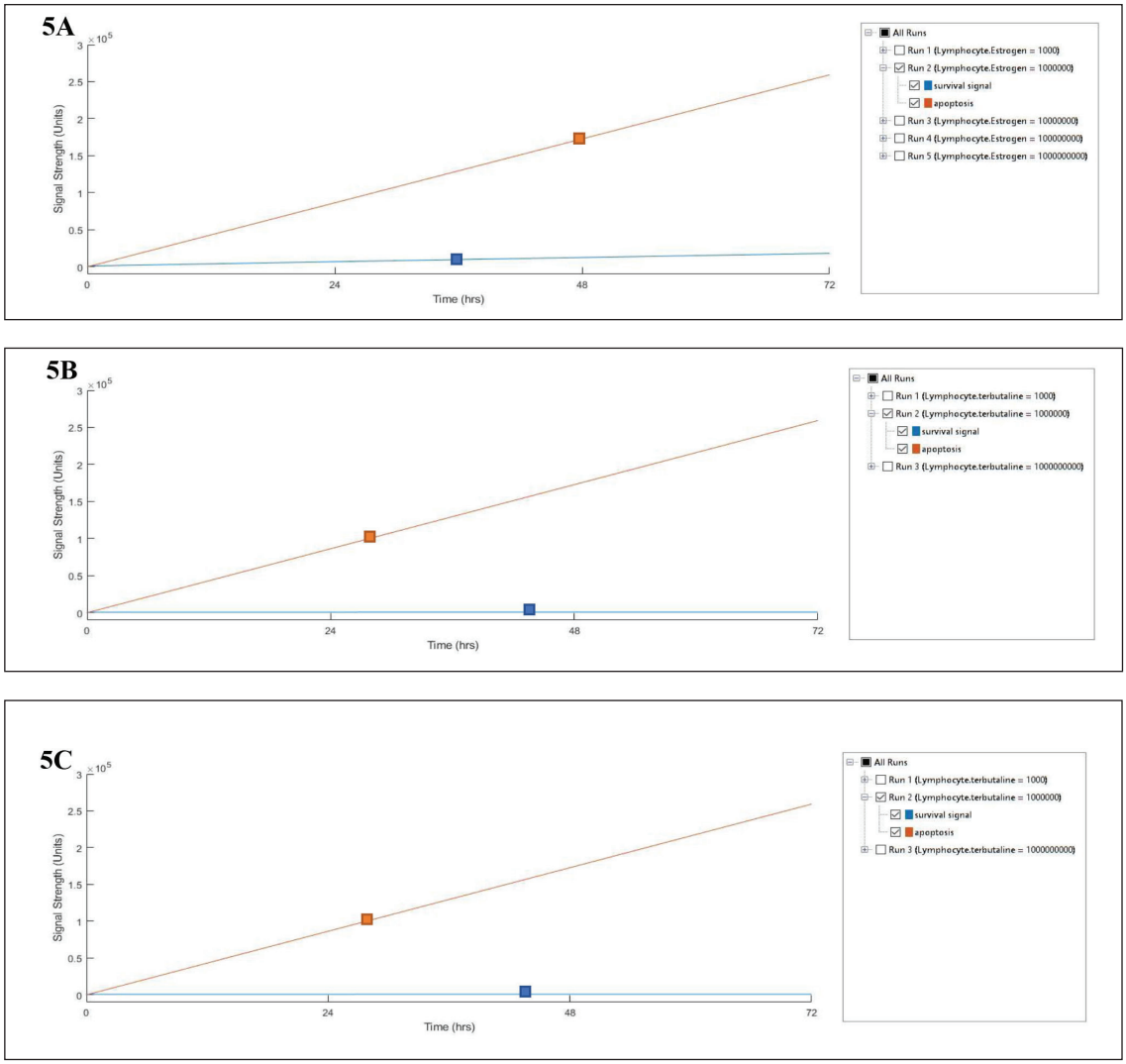


Figure 5. *Expression of survival/apoptosis signals by lymphocytes treated with estrogen, adrenergic agonists and in combination: Simulation of lymphocytes treated with 10-6 M Estrogen for 24 hours (5A).* Simulation of lymphocytes treated with terbutaline (10^{-6} M), phenylephrine (10^{-6} M) and clonidine (10^{-6} M) for 24 hours (5B). Simulation of lymphocytes treated with terbutaline (10^{-6} M), phenylephrine (10^{-6} M) and clonidine (10^{-6} M) in the presence of estrogen (10^{-6} M) for 24 hours (5C).

Modulation of Cytokines With E_2 and Adrenergic Agonists in Silico

IFN-γ expression was significantly enhanced in lymphocytes simulated with E_2 (Figure 3A), compared to simulation with adrenergic agonists (Figure 3B) and adrenergic agonists with E_2 (Figure 3C). Simulation with adrenergic agonists and E_2 showed increased IL-2 expression, compared with adrenergic agonists alone. IL-2 expression declined with time in all three treatment groups.

Modulation of Antioxidant Enzymes, Superoxides and Peroxynitrites With E_2 and Adrenergic Agonists in Silico

AOE expression and decline in peroxynitrites and O2- were similar in lymphocytes simulated with E_2 (Figure 4A), adrenergic agonists (Figure 4B), and adrenergic agonists with E_2 (Figure 4C) for 24 h.

Modulation of Survival/Apoptosis Signals With E_2 and Adrenergic Agonists in Silico

While apoptotic signal expression was similar in all three groups, survival signal was enhanced in lymphocytes simulated with E_2 (Figure 5A), compared to simulations run with adrenergic agonists (Figure 5B) and adrenergic agonists with E_2 (Figure 5C).

Dose-Dependent Modulation of Survival Signals in Lymphocytes Simulated With E_2

Survival signal was significantly elevated with every 10-fold increase in E_2 concentration compared to simulation with 10^{-6}M E_2 (Figure 6).

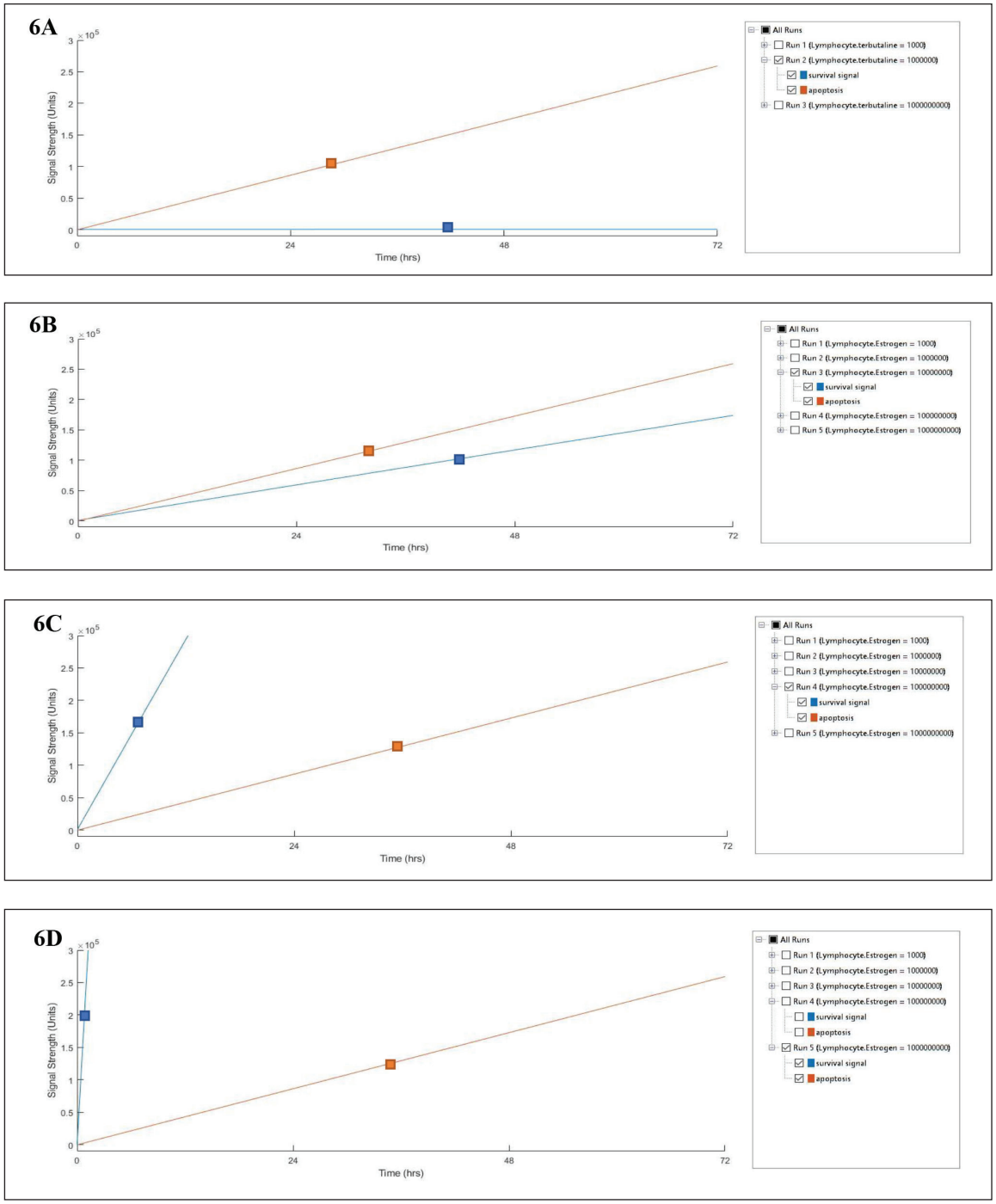


Figure 6. *Effects of increasing doses of estrogen on survival/apoptosis signals by terbutaline, phenylephrine and clonidine:* Simulation of lymphocytes treated with terbutaline (10^{-6} M), phenylephrine (10^{-6} M) and clonidine (10^{-6} M) in the presence of increasing doses of estrogen (10^{-6} M (6A), 10^{-5} M (6B), 10^{-4} M (6C), 10^{-3} M (6D)) for 72 hours.

Docking Studies With Norepinephrine and p-ERK

Docking studies show that NE can directly inhibit ERK and thereby affect subsequent signal transduction processes. NE binds directly to the catalytic pocket with Asp 147 and Glu 69, preventing the Lys 52 residue in the phosphate binding site from forming an ion pair (Ki = 207.69 µM, bond distance = 2Å, and energy = –5.02Kcal/mol; Figure 7A). Close to the same catalytic pocket, NE also binds to residues Asp 177 and His 178 with a Ki = 365.7µM and an energy of –4.69Kcal/mol (Figure 7B). However, the inhibition concentrations are not physiologically significant. Near the active site, NE binds Thr 179, Tyr 203 (Ki = 523.7µM and an energy of –4.48Kcal/mol) and Tyr 203, and Asp177 (Ki = 528.44µM and an energy of –4.47Kcal/mol) in the β9 ribbon strand (Figure 7C).

Docking Studies With 17β-Estradiol and β2-AR Receptor

Docking studies show that E_2 binds directly to the β2-AR receptor (PDB ID: 3SN6; *Rattus norvegicus)* at the Asp 173 residue with a Ki of 22.09 μM, bond distance of 1.99 Å and an energy of –6.35Kcal/mol (Figure 8A), the Met 61 residue with a Ki = 11.0 μM and an energy of –6.76Kcal/mol (Figure 8B), and the Arg 314 residue with a Ki = 8.77 μM, and an energy of –6.9Kcal/mol (Figure 8C), indicating potent inhibitory effect at physiological levels.

Discussion

Immune functions of innate, humoral, or cell-mediated origin are modulated by sympathetic signals and circulating hormone levels during health and disease in a dose- and receptor-type dependent manner.[1,10,12,51] *In vitro* studies from our laboratory have shown that adrenergic stimulation through α1-, α2-, or β2- ARs using specific agonists nonspecifically inhibit lymphoproliferation through distinct signaling pathways: α1-AR mediated immunosuppressive effects by inhibiting IFN-γ production; α2-AR activated the NF-κB, p-Akt; and NO pathways and β2-AR activation involved IL-6, NO, and NF-κB signaling cascades mediating immunosuppression (Figure 1A).[17,18] On the other hand, treatment of lymphocytes with E_2 enhanced proliferation in a dose and receptor subtype-specific manner through p-ERK, p-CREB, and p-Akt involving IFN-γ and compensatory mechanisms including AOEs (Figure 1B).[4,52] Our studies have shown that cotreatment of lymphocytes with E_2 and adrenergic agonists lead to E_2-mediated override of adrenergic immunosuppression in a dose-dependent, AR-subtype independent manner.[17,18]

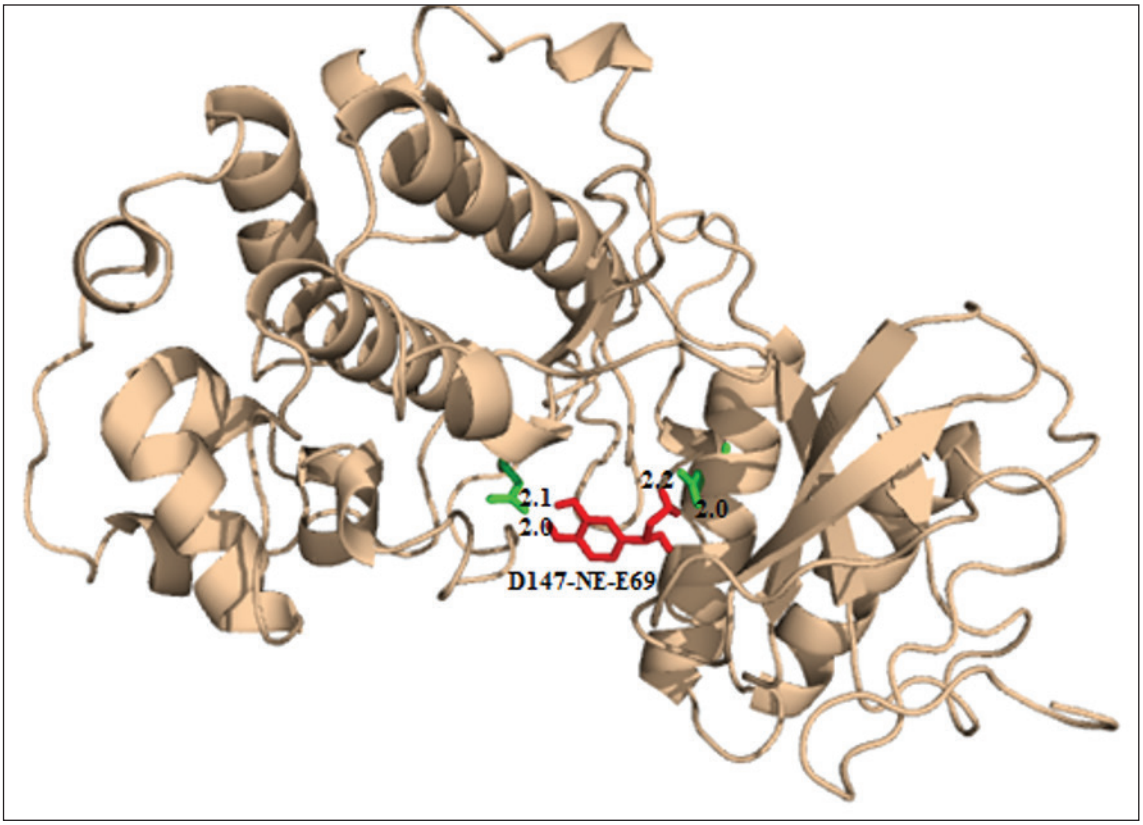
In the present study, we have modelled these signaling pathways *in silico* using the MATLAB Simbiology toolbox. Because of difficulties in conducting the experiments using NE, the *in vitro* studies were conducted using receptor-specific agonists and antagonists. While these experiments are highly beneficial in elucidating the dose-dependent effects on specific adrenoceptor subtypes, they cannot be considered as absolute indicators of NE-mediated effects. This is because when NE is added to the cells, depending on its relative affinity for the individual receptor subtypes, it may bind proportionally to all the Ars, triggering a wide variety of down-stream signals. The cumulative effects of these signals are likely to be different from the outcomes of receptor-subtype-specific signaling fates. In order to overcome this problem, the computational model was constructed on the basis of the data obtained from *in vitro* studies, and all three ARs studied were simultaneously activated to study the outcome. Based on previous findings, an attempt was made to incorporate cross-talks between these pathways by using common signaling molecule pools (Figure 1C).

Simulation of lymphocytes with E_2 enhanced cAMP, p-ERK, and p-CREB expression while adrenergic agonists, phenylephrine, clonidine, and terbutaline enhanced p-CREB and p-ERK expression alone (Figure 2A and 2B). However, E_2 and adrenergic agonists together enhanced the expression of cAMP, suggesting that activation of cAMP may be crucial to E_2-mediated override of adrenergic immunosuppression (Figure 2A and 2C).

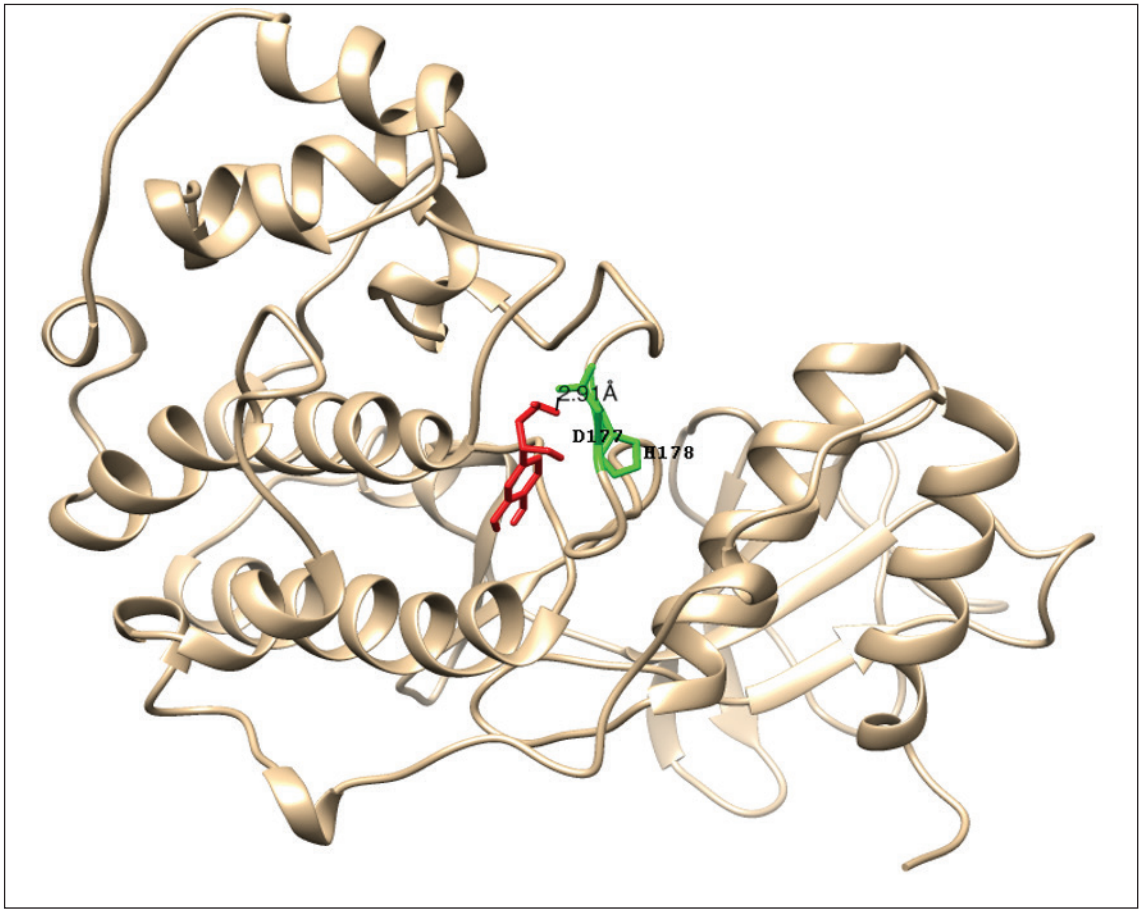
Cytokines play a crucial role in influencing immune functions, thereby affecting survival/apoptosis. Previously we have shown that while activation of α1-ARs decrease IFN-γ and increase IL-2 production, α2- and β2-AR activation did not alter either IFN-γ and IL-2 production.[17,18] However, treatment with E_2 alone or E_2 with adrenergic agonists significantly enhance IFN-γ production alone after 24 h of treatment. We have reported dose-dependent increase in IFN-γ production with E_2 alone or co-treated with α1- and β2-AR agonists. In agreement with these findings, simulation of lymphocytes with α1-AR agonist, phenylephrine, showed increased sensitivity to IFN-γ and p-CREB expression (supplementary Figures 9 and 10) while β2-AR agonist terbutaline showed increased sensitivity to IFN-γ, p-ERK, and p-CREB expression (supplementary Figures 5 and 6). Interestingly, E_2 signaling alone and in combination with adrenergic agonists showed increased sensitivity to IFN-γ and cAMP expression suggesting a probable cause in the dynamics of E_2-mediated signals that predispose it to override adrenergic signals (supplementary Figures 1–28).

A significant role is played by compensatory mechanisms such as AOE apart from signaling molecules, to influence the proliferative/apoptotic milieu of a cell by balancing free radical load including O2- and peroxynitrites to name a few. These free radicals released as byproducts following immune responses or during neurotransmitter release in secondary lymphoid organs may accumulate over the years and contribute to the denervation of sympathetic NA fibers leading to impaired neuroendocrine-immune homeostasis, setting the stage for age-associated diseases.[7] Studies from our lab and others have documented ER-subtype dependent increase in SOD and GPx activities in lymphocytes stimulated with E_2.[4,53–58] Adrenergic stimulation also enhanced SOD and catalase activities *in vitro*, which were suppressed upon coincubation with E_2 indicating cross-talk between the two pathways leading to diminutive effects.[18] Along these lines, previous studies have implicated the involvement of PKA and ERK pathways, which, when inhibited, reversed terbutaline-mediated increase in SOD and CAT activities.[4,17,18] It is possible that these signaling molecules play a regulatory role in the expression and activity of these enzymes, thereby exerting immunomodulatory effects.[59,60] In our model, adrenergic activation, E_2 treatment, and their co-treatment decrease O_2- formation but enhance peroxynitrites, although E_2 treatment, adrenergic simulation, or co-treatment similarly affected AOE activities (SOD and CAT; Figure 4).

7A



7B



7C

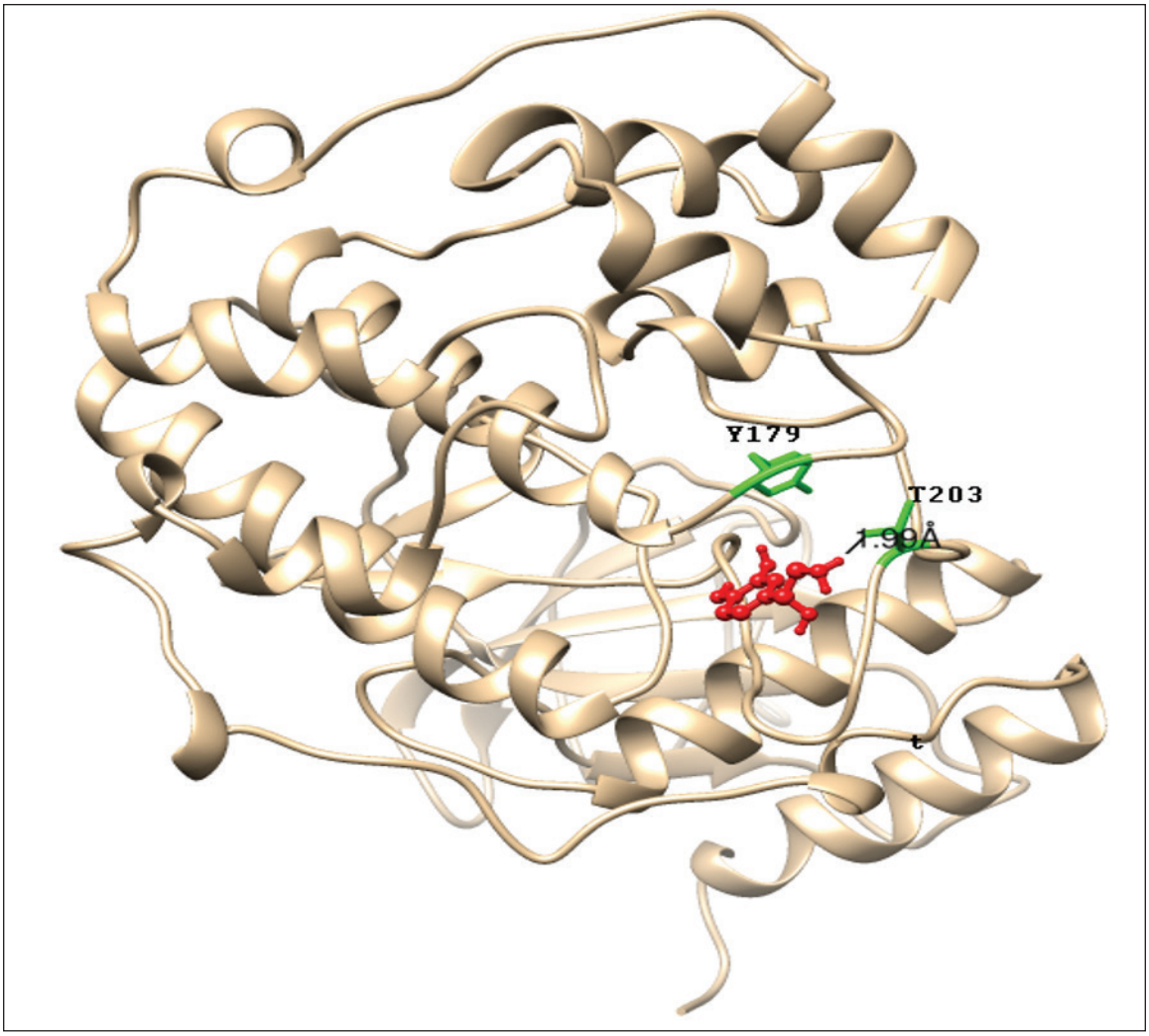
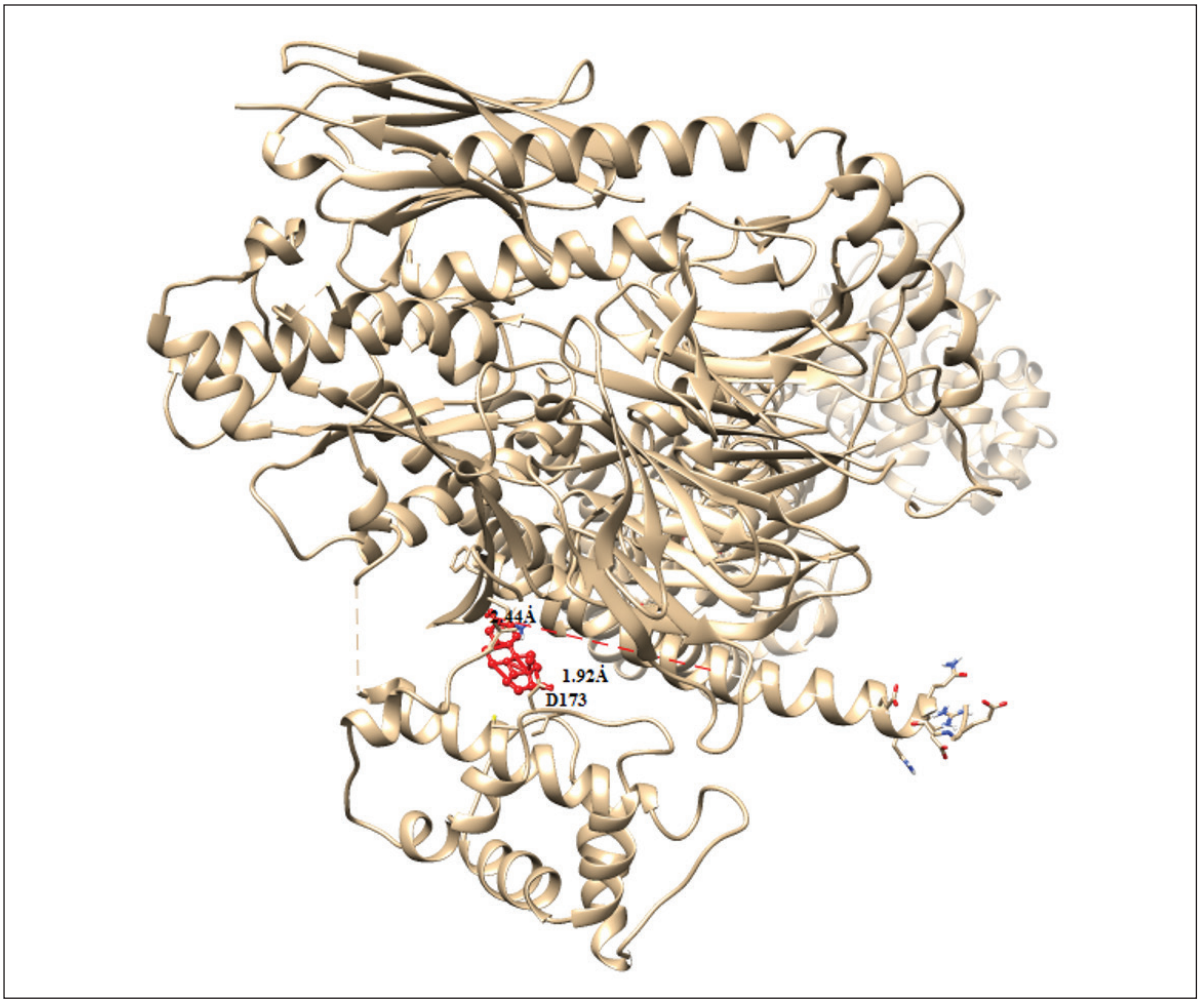


Figure 7. *Docking of norepinephrine with 2-ERK:* NE can bind to 2-ERK on the following possible binding sites shown below in the order of lowest Ki and binding energy Asp 147 and Glu 69 (7A), His 178 and Asp 177 (7B), Thr 179 and Tyr 203, Tyr 203 and Asp 177 (7C).

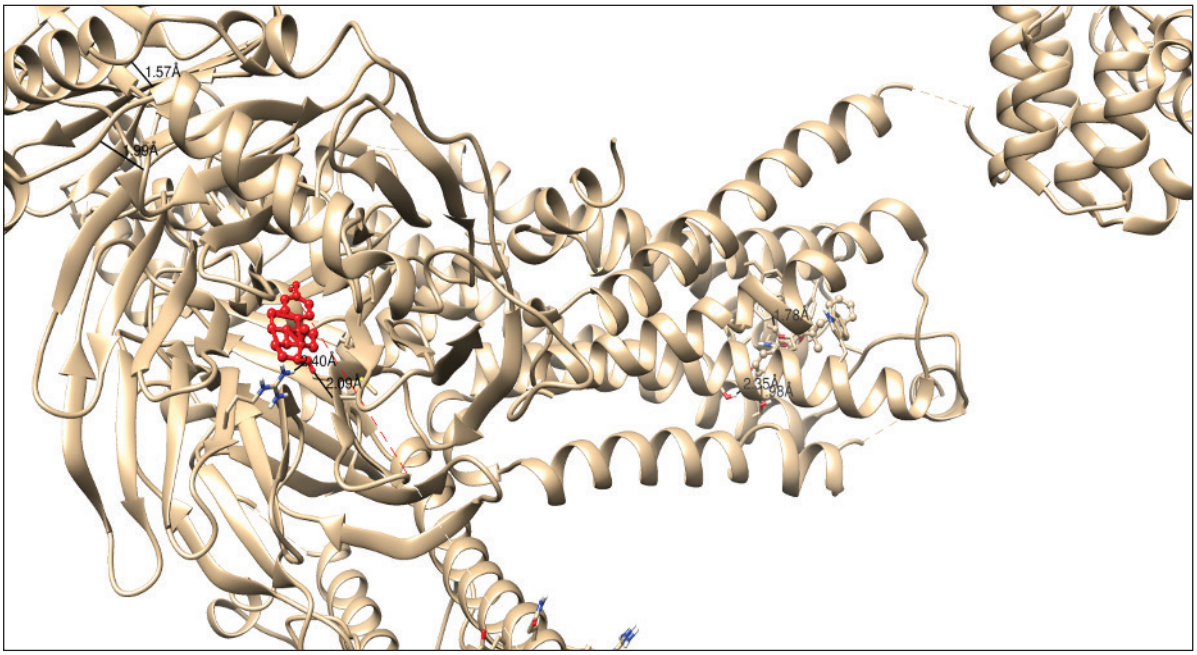
In order to assess the likely effects of the signals and their cross-talks on proliferation, end-point signals were classified as either pro-apoptotic or survival signals. Plots obtained at the end of 72 h indicate that while adrenergic simulation was predominantly pro-apoptotic, E_2 signals were dose-dependently pro-survival (Figure 5). The dose-dependent role of E_2 could be seen by increasing the E_2 concentration by 10-fold leading to further increase in proliferation signals reversing the balance in favor of proliferation over apoptosis after 72 h (Figure 6). This is in accordance with the *in vitro* evidence generated in previous studies where all the adrenergic agonists decreased proliferation while E_2 played a dose-dependent role in increasing proliferation of lymphocytes.

Finally, docking studies show that apart from modulating signaling mechanisms, NE can directly inhibit ERK and E_2 can directly bind to key residues on β_2-AR, inhibiting its subsequent signal transduction processes. The structure of 2-ERK shows that the phosphorylation lip contains two phosphorylation sites, Thr 183 and Tyr 185, and an essential phosphate binding residue, **Lys 52**. The active site is held open by the interaction of the C-helix and the conserved glycine of the **Asp-Phe-Gly** sequence **(165–167)**. N terminal of the C-helix lies on top of Gly 167. Arg 65 points toward the active site and Gly 167 interdigitates with Arg 65 and Gln 64. The interdigitation of the Asp-Phe-Gly sequence with Arg 65 may be responsible for domain motions providing a switch that can support a closed active conformation of the molecule or an open inactive conformation, thereby playing a role in regulation. The C-terminal domain has two putative catalytic bases, **Asp 147** and the Mg2+ binding **Asp 165.** The H-bonding network of **Asp 147, Asp 165,** Asn 152, Lys 149, and Thr 188 is vital for catalytic function.[61] ERK (PDB ID: 2ERK; *Rattus rattus)*; was inhibited by NE by binding to the catalytic residue **Asp 147** and Glu 69—the residue that forms an ion pair with **Lys 52** in the phosphate binding site of ERK

8A



8B



8C

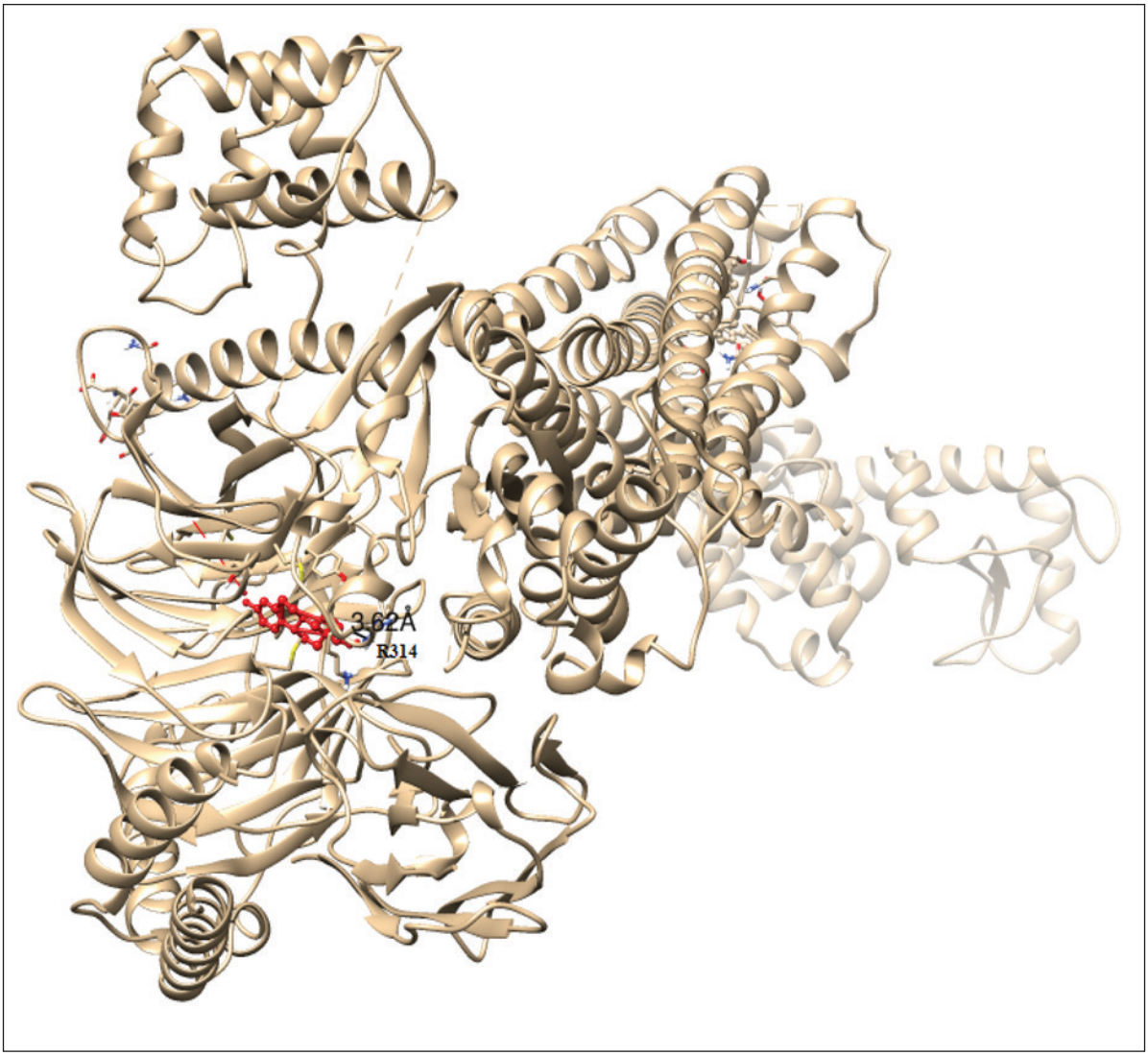


Figure 8. *Docking of 17β-estradiol with β2-AR:* E_2 can bind to β2-AR on the following possible binding sites shown below in the order lowest Ki and binding energy Arg 314 (8A), Met 61 (8B), and Asp 173 (8C)

with a Ki=207.69 μM, bond distance of 2A, and an energy of –5.02Kcal/mol (Figure 7A) —and to Asp 177, which is close to the catalytic pocket with a Ki = 365.7μM and an energy of –4.69Kcal/mol (Figure 7B). NE also inhibits **Asp 177** (binding energy = –4.47Kcal/mol and Ki = 528.44) and **Thr 179 (**binding energy = –4.48Kcal/mol and Ki = 523.7) in the β9 ribbon of the domain interface close to the active site (7C). However, it is important to note that μM concentrations of adrenergic agonists are not physiologically or pharmacologically achievable.

The β2-AR structure comprises of cytoplasmic (CP), transmembrane (TM), and extracellular (EC) domains. The CP domain of β2-AR containing the three CP loops (CL1–CL3) and the EC domain consisting of the N-terminus and three interhelical extracellular loops (EC1–EC3) connect the TM domain containing a bundle of seven helices (H1–H7). EC2 (between H4 and H5) contains a short helix at a position above the ligand binding cavity at the TM region. The aspartate residue at position 79 within the second putative transmembrane segment of the human β2-AR is part of a conserved sequence domain in all GPCRs and has been identified as the key NE binding site in β2-adrenoceptor.[62] In the CP loop of β2-AR, E_2 binds to **Met 61** residue with a Ki = 11.0 μMand an energy of –6.76Kcal/mol (Figure 8B), indicating potent inhibitory effect at physiological levels. Also, E_2 binds to β2-AR receptor at the **Asp 173** residue in EC2 close to the ligand binding cavity (Ki = 22.09 μM, bond distance = 1.99 Å and energy = –6.35Kcal/mol; Figure 8C). **Met 61** residue falls in the first CP loop in the transmembrane receptor, while **Asp 173** falls within the extracellular loop-2.

The most probable binding site is with the **Arg 314** residue with the lowest Ki = 8.77 μM and an energy of –6.9Kcal/mol (Figure 8A). **Arg 314** falls within the seventh transmembrane helix close to the orthosteric binding site consisting of Asn312 that binds salmeterol (within a bond distance of 3.5A) and epinephrine.[63] There is a highly rigid hydrogen bond network among helixes 1, 2, and 7 on the CP side, because of the water molecules that exist between the (Asn-Asp) N-D pair and the conserved NPXXY motif on H7. Tyr 326 and Asn 322 in H7 are connected to Asn51 on H1 and Asp 79 at H2 through four water molecules located in the cavity between H1, H2, and H7, forming the network that is crucial for β2-AR function.[63,64] It is possible that estrogen may inhibit β2-AR through noncompetitive or uncompetitive inhibition, although further studies are required to delineate the functional implications of these binding sites.

Previously published *in vitro* studies from our lab show that NE-mediated immunosuppression is reversed upon treatment with E_2 in peripheral blood mono-nuclear cells (PBMCs).[4,17,18] Concomitantly, our findings indicate that E_2 at physiological levels can potently inhibit an β2-AR receptor by directly binding to it, apart from the dose-dependent effects of E_2 on the signaling network. E_2-mediated reversal of adrenergic immunosuppression may also be mediated through cAMP-dependent signaling cascades which needs to be explored further.

Conclusion

Age associated decline in the number of sympathetic NA fibers, NE availability, reproductive age- associated increase, and decline in E_2 levels, all contribute to immunosenescence—the progression and onset of age-related diseases. Modeling E_2 and adrenergic stimulation-mediated signals will help us to understand variations in the expression of downstream molecular markers with age- and disease-associated variations in kinetic parameters and concentrations, hereby providing an effective tool for understanding the alterations in the cross talk between the pathways in health, aging, and diseases.

Authors' Contribution

HPP: In vitro and In silico Studies, Model Building, Analysis, Manuscript Writing, Concept and Communication. AT: In silico model building using Matlab; GK: Docking Studies. RSN: Manuscript writing. WH: Bioinformatics Work Concept, Proof Reading and Editing. ST: In vitro and In silico work conceptualisation, Proof Reading and Editing.

Statement of Ethics

This study was approved by the Institutional Animal Ethics Committee at SRM University (IAEC Clearance No.:56/IAEC/2011).

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article: This paper has been previously deposited in a preprint repository https://www.biorxiv.org in 2018 and in 2020.

1. Hannah Priyanka, Anand Thiyagaraj, Rahul S Nair, Gokulnath Krithika, Lalji Hima, Waheeta Hopper, Srinivasan ThyagaRajan. "In silico modeling and simulation of neuroendocrine-immune modulation through adrenergic and E2 receptors in lymphocytes show differential activation of cyclic adenosine monophosphate (cAMP)," Cold Spring Harbor Laboratory, 2020.
2. H.P. Priyanka, A. Thiyagaraj, G. Krithika, L. Hima, W. Hopper, S. ThyagaRajan. Modelling and Simulation of neuroendocrine-immune modulation through adrenergic and 17β-estradiol receptors in lymphocytes show differential activation of cyclic adenosine monophosphate (cAMP)", Cold Spring Harbor Laboratory, 2018.

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Supplemental Material

Supplemental material for this article is available online.

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