An Analysis of the Role of Estrogen in Macrophage Biology

Thesis submitted to

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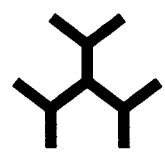

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CERTIFICATE

This is to certify that the thesis titled **"An Analysis of the Role of Estrogen in Macrophage Biology"** submitted by **Manikandan. S** in partial fulfillment of **Ph.D.** degree of Jawaharlal Nehru University embodies the work done by the candidate under my guidance at the National Institute of Immunology. This work is original and has not been submitted in part or in full for any other degree or diploma of any university.

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S. Manikandan

Manikandan

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Abbreviations

°C	degree Celsius
BIM VIII	Bisindoleylmalemide VIII
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
cDNA	Complementary deoxyribonucleic acid
CM-H_2DCFDA	5-(and-6) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester
cNOS	Constitutive nitric oxide synthase
CRE	Cyclic-AMP response element
Da	Daltons
DAF-FM DA	4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E2	17 β-Estradiol
E2-BSA	17 β-Estradiol conjugated to Bovine Serum Albumin
E2-BSA-FITC	17 β-Estradiol conjugated to fluorescein isothiocyanate conjugated BSA
ELISA	Enzyme linked immunosorbent assay
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extracellular signal regulated kinase
ER-α	Estrogen receptor α
ER-β	Estrogen receptor β
FCS	Fetal calf serum

FSC	Forward scatter
h	Hour
HCO_3^-	Bicarbonate ion
hPBMDM	Human peripheral blood monocyte derived macrophages
IFN-γ	Interferon – γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
kb	Kilobase
kDa	Kilo Daltons
LPS	Lipopolysaccharide
M	Molar
MAPK	Mitogen activated protein kinase
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NHE	Sodium-hydrogen exchanger
nm	Nanometre
NO	Nitric oxide
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PKC	Protein kinase C
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute - 1640
RT-PCR	Reverse transcription-Polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
siRNA	Small interfering ribonucleic acid
SNARF-1 AM	5-(and -6)-carboxy SNARF®1-AM
SSC	Side scatter
TNF	Tumor necrosis factor
UV	Ultraviolet
µL	Microlitre
µM	Micromolar

Chapter 1

Introduction

Estrogen is a female sex hormone with profound effects on the reproductive system, central nervous system (1), skeletal system (2), cardiovascular system (3) and the immune system (4). It is a known immunomodulator (4) with both pro- and anti-inflammatory effects on various cell types of the immune system. It is believed to be one of the determinants of sexual dimorphism in the immune responses observed between males and females, for example, a gender bias has been observed in the occurrence of autoimmune diseases with a higher incidence of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), autoimmune thyroiditis, etc. observed in women (5). Also, disorders such as multiple sclerosis & rheumatoid arthritis are ameliorated during pregnancy and estradiol treatment, while SLE is aggravated. In general, it has been observed that humoral mediated autoimmune diseases are aggravated during pregnancy while cell-mediated autoimmune diseases are ameliorated suggesting hormonal modulation of systemic immunity. Sexual dimorphism has also been observed in the incidence and progression of various infectious diseases such as listeriosis (6, 7), toxoplasmosis (8), leishmaniasis (8), pulmonary infections, sepsis (9) etc. indicating the possible role of sex hormones in immune alteration.

Macrophages, the cells of the mononuclear phagocyte system, derived by the differentiation of monocytes play a central role in innate and adaptive immune responses and their activation is central to the outcome of virtually every infectious disease. Failure to properly regulate macrophage function during infection is often itself a major cause of disease. These cells play a major role in diverse processes such as antimicrobial activity, wound healing & repair, scavenging of dead cells (10), morphogenesis and organ development (11), tumor cell lysis, tumor initiation & progression (12), antigen presentation, and modulation of T- & B-lymphocyte activity (13), thus serving as vital adapters between the innate and adaptive immune system. The modulation of macrophage survival and function by estrogen has been linked to the pleiotropic effect of this hormone on the immune system.

Macrophages express estrogen receptors and are therefore capable of responding to increase in estrogens during follicular phase of menstrual cycle (14), at the time of exposure from exogenous sources such as phytoestrogens (15), following administration for prophylactic and therapeutic purposes (16) or during accidental

exposure to estrogenic chemicals (17,18). Estrogen affects a variety of macrophage functions, for example it can reduce accumulation of cholesteryl esters in macrophages (2), stimulate production of nitric oxide (19, 20), increase arachidonic acid release (21), regulate activation related events (2, 22), decrease monocyte adhesion to vasculature (23), enhance macrophage phagocytosis (24) and facilitate Ca^{2+} influx (20) some of which are implicated in mediating the gender bias observed in numerous autoimmune (25), cardiovascular (26) and neurodegenerative disorders (27). In addition, estrogen is able to modulate macrophage death and this event is of great relevance because macrophage survival or death is crucial for disease pathogenesis, however, data available on the influence of the hormone on macrophage cell death process is in itself contradictory. Existing literature show that macrophage-like U937 cells undergo apoptosis when exposed to estrogen (28) but the same cell type is protected from TNF-alpha-induced apoptosis by the hormone (29). According to other reports, estrogen is able to induce apoptosis in undifferentiated U-937 monocytes but macrophages differentiated from these cells are refractory to such effects of estrogen (30). Further examples of cell types where estrogen is reported to induce cell death include bone macrophages like murine osteoclasts, preosteoclastic FLG 29.1 cell line and mouse peritoneal macrophages (2, 31-33).

The apparently paradoxical effect of estrogen on apoptosis in cells of the monocytic lineage could be interpreted to be the result of its ability to differentially modulate anti and pro-apoptotic proteins like the members of the Bcl-2 family that share sequence-homology domains within the group. The various pro and anti-apoptotic Bcl-2 family members are able to heterodimerize and their relative concentrations function as a rheostat for the apoptotic program (34). Certain apoptotic stimuli like exposure to nitric oxide (35), oxysterol (36) and activation inducing agents increase macrophage Bcl-2 or other members of Bcl-2 family of proteins like Bfl-1 (37), but the involvement of Bcl-2 family members in regulating the macrophage death pathway is not understood. In the context of tumor development, mechanisms regulating macrophage death are important because these cells constitute a large proportion of the tumor cells and are evidently important for either progression or regression of tumors (12). Survival of macrophages in estrogen microenvironment is relevant especially in the cells populating estrogen target tissues like uteri, breast, brain and cervix. Also, an understanding of the mechanism of macrophage survival

under altered Bcl-2 level becomes important in the backdrop of development of Bcl-2 small molecule inhibitors, antisense oligonucleotides and RNAi against Bcl-2 intended to be used for treatment of resistant carcinoma and some of which are currently in phase I and phase II clinical trials (38-40). In addition, estrogens and the various selective estrogen receptor modulators (SERM) are in various phases of clinical trial or are already in use for prophylaxis or treatment of various disorders like cancers, osteoporosis, stroke, atherosclerosis, multiple sclerosis, Alzheimer's disease etc., and hence a better understanding of the effect of this hormone on the immune cells is imperative.

Also, the contradictions in the literature regarding the effect of estrogen on macrophage survival is largely due to the fact that the data has been collected from studies performed on mouse or rat macrophages and the various human monocyte-macrophage cell lines overlooking the fact that the effect of estradiol is highly cell type specific and dose dependent and thus cannot be directly extrapolated to human macrophages. While human macrophage cell lines should be used as model systems for investigations because they are amenable to genetic manipulations and available in larger numbers, it is imperative to make comparisons with ex-vivo human peripheral blood monocytes and monocyte-derived macrophages at every step.

Based on the existing literature on estrogen effect on macrophage survival that demonstrate wide gaps in understanding of the influence of estrogen on death pathways in macrophages, the overall theme of the thesis is proposed to understand the role of estrogen in macrophage biology under physiological as well as pathological conditions like infection. In addition, a dissection of the signaling mechanisms involved in estradiol regulation of macrophage function and identification of key players that are vital for modulating human macrophage survival under estrogen exposure would be carried out. Though estradiol is predominantly a female hormone, it does not necessarily restrict the scope of this study to female physiology due to the widespread exposure of mankind to phytoestrogens as well as the fact that there is continuous local biosynthesis of estradiol by the macrophage due to their aromatase activity (41).

The specific objectives of the proposed study are:

1. Investigate the effect of estrogen on human monocyte-macrophage survival and define estrogen receptor involvement in the process.
2. Identify the members of the Bcl-2 family of proteins involved in macrophage response to estrogen and explore their role in modulation of macrophage survival and function.
3. Elucidate the signaling pathways operative under estrogen exposure leading to modulation of the identified Bcl-2 family members.
4. Determine the effects of estrogen on macrophage response to leishmanial infection.

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Chapter 2

Review of Literature

2.1. Introduction

Sex hormones are steroidal hormones that have been categorically associated with sexual development or function in males and females. The male sexual hormones are collectively called androgens of which testosterone plays the major role in modulating male reproduction. In females, estrogen and progesterone are the primary sex hormones. These hormones being steroidal and lipophilic in nature freely diffuse across the plasma membrane (1) and mediate their effects by binding to specific receptors, the androgen receptor (AR) for testosterone (2), estrogen receptor (ER) for estrogen (3) and progesterone receptor (PR) for progesterone (4).

2.2 Estrogen and its receptors

Estrogen, the female sex hormone is synthesized primarily in the developing follicles of the ovary and the placenta upon stimulation mediated by follicle stimulating hormone (FSH) and luteinizing hormone (LH) (5) secreted by the pituitary gland. The biosynthesis involves aromatization of androstenedione (produced in the theca folliculi cells) to estrone, followed by its conversion to estradiol by 17β-hydroxysteroid reductase (6). Small quantities of estrogen are also synthesized in peripheral tissues such as liver, adrenal and adipose tissue by aromatization of testosterone (7). These secondary sites are especially important in post-menopausal females and for the generation of estrogen in males (7). The three major naturally occurring estrogens in women, all of which are synthesized from androgens, are estradiol, estriol and estrone (4). Estradiol is the most active and abundant form of estrogen. However, the levels of estriol are high in pregnant and post-menopausal women. The physiological levels of estrogen fluctuate in women of reproductive age, being highest during late follicular phase and at the time of ovulation (Figure 2.1).

In plasma, estradiol is largely bound to sex hormone binding globulin (4) and albumin, with only a fraction being free and biologically active. Deactivation involves conversion to less active estrogens such as estrone and estriol. Estradiol is conjugated in the liver by sulfate and glucuronide formation and excreted via the kidneys. Some of the water soluble conjugates are excreted via the biliary system, and partly

reabsorbed after hydrolysis from the intestinal tract. This enterohepatic circulation contributes to maintaining estradiol levels (8).

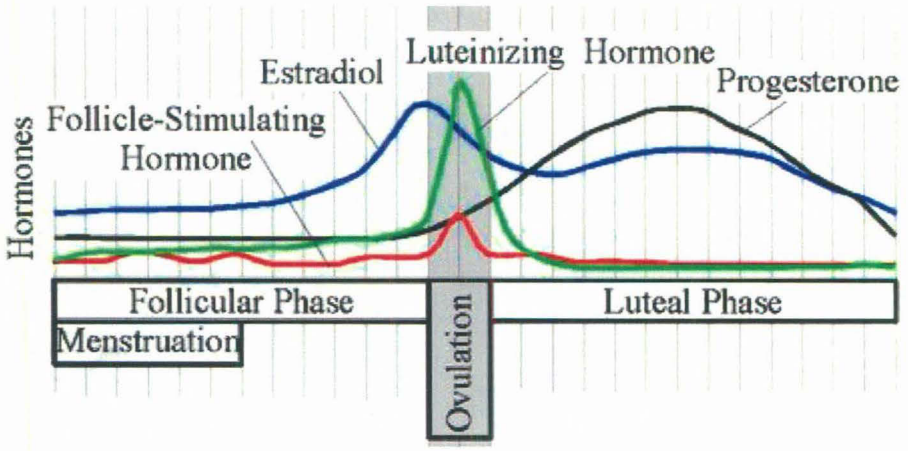


Figure 2.1: Variation of estrogen levels during menstrual cycle in humans. Note that the peak level of estrogen is reached at the time of ovulation following luteinizing hormone (LH) and follicle stimulating hormone surge.

Estrogen mediates its action by binding to estrogen receptors which belong to the nuclear receptor superfamily, a family of ligand regulated transcription factors (9). The classical mechanism of estrogen action involves binding of estrogen to the receptor and migration of the estrogen-estrogen receptor complex into the nucleus, where it activates transcription by binding to estrogen response elements (ERE) on promoter of responsive genes (9) or indirect activation of transcription via activator protein -1 (AP-1) (10) or SP-1 elements (11). Recent literature has estrogen mediated signaling *via* plasma membrane localized estrogen receptors that does not involve translocation of hormone-receptor complex to the nucleus (11). Estrogen receptors are primarily of two sub-types called estrogen receptor – α (ER-α) and estrogen receptor – β (ER-β). ER-α and ER-β are products of genes on different chromosomes, namely chromosome 6 and 14 respectively. ER-α and ER-β contain the evolutionarily conserved structural and functional domains of nuclear receptors including DNA-binding, dimerization, ligand binding and transactivation domains. They share a high degree of sequence identity within the DNA binding domain (12). The domain structure of ER-α and ER-β is shown in Figure2.2. The N- and C-termini contain the ligand independent and ligand dependent activation function domains (AF-1 and AF-2) respectively which mediate the transcriptional activation of estrogen regulated

genes. A comparison of the activity of AF-1 domain has revealed that it is very active in ER-α, while it is minimal in ER-β (13) which may partly explain the distinctive response of these two receptors to estrogen.

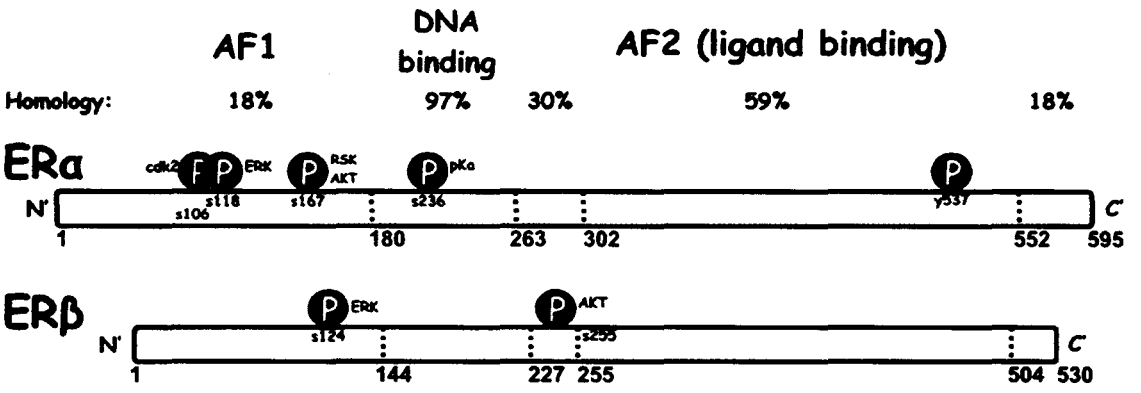


Figure 2.2: Domain structure of ER-α and ER-β. A comparative domain structure of ER-α and ER-β is depicted. The percentage homology in the domains between ER-α and ER-β is depicted in percentages above. The circles marked "P" represent phosphorylation sites. AF, Activation function domain.

ER-α and ER-β show distinctive tissue specific and cell-specific expression, for example ER-α is expressed primarily in uterus, heart, kidney and liver, while ER-β is expressed primarily in ovary, prostate, hematopoietic and central nervous system while others such as thymocytes and neurons co-express both receptor sub-types simultaneously (12). In most systems, ER-β is known to inhibit ER-α mediated transcriptional activation (14, 15) and these differences are attributed to the differences in their respective transactivation regions. Also, ER-α or ER-β knockout mice show distinctive phenotype suggesting non-redundant roles for these receptor sub-types in mediating estrogen action *in-vivo* (16). The classical localization of the estrogen receptor is described to be in the nucleus, but current literature has demonstrated its presence in the cytoplasm (17), mitochondria (18) and the plasma membrane (19) as well. The estrogen receptors in these varied locations have diverse functions different from the classical nuclear localized estrogen receptors. For example, the membrane localized estrogen receptor is known to mediate the rapid non-genomic effects of estrogen such as Ca^{2+} influx (20), ERK phosphorylation (21) etc. The membrane localized estrogen receptor originates from the same transcript as

that of nuclear receptor as demonstrated by the absence of membranous receptor in endothelial cells derived from ER-α and ER-β knockout mice (19) as well as by appearance of membranous receptor in ER-negative breast cancer cells that were transfected with wild-type ER-α. The membranous estrogen receptors are known to activate G-protein coupled receptor signaling either directly or indirectly (22). Moreover, a recent study has revealed that G-protein coupled receptor 30 (GPR-30) is an estrogen binding protein which mediates the rapid membranous effects (23). A number of splice variants have been described for ER-α and ER-β, a few of which such as ER-46 have been shown to be aberrantly expressed in malignant breast cancer cells (24). However, the roles of other splice variants are not known though there are speculations that they may act as negative regulators by heterodimerizing with the full-length estrogen receptors.

The expression of ER-α and ER-β is autoregulated by estrogen, though the molecular mechanisms involved are only poorly understood. Estrogen exposure results in a ligand dependent activation of two distinct promoters in ER-α resulting in its transcriptional up-regulation (25). Moreover, long-term exposure of estrogen is known to enhance the expression of ER-α, but inhibit that of ER-β. In summary, these studies highlight the complexity involved in the interaction of ER-α and ER-β and their autoregulation suggesting cell and tissue specific variation.

2.3. Functional role of estrogen in human physiology and pathology

Estrogen plays a crucial role in the development of both female and male reproductive organs and is required for maintaining fertility in both sexes. It is also required for the development of secondary sexual characteristics in females. Apart from its well known role in reproductive system, it is also essential for the proper functioning of a number of other cellular systems including the hematopoietic system (26), nervous system (27), cardiovascular system (28), musculoskeletal system (29) and the immune system (30).

2.3.1. Estrogen and nervous system:

Estrogen receptors are expressed in various cells of the brain and both ER-α and ER-β have been detected. Estrogen is known to mediate sexual differentiation of

the brain during embryogenesis (31). Estrogens affect the neuronal excitability, it increases firing rate in the pituitary while decreases spontaneous firing in the medial preoptic area (32). Estrogen protects against neurotoxicity from a wide range of toxic insults such as free radicals, excitotoxicity, beta-amyloid induced toxicity, and ischemia (33). Epidemiological evidence suggests estrogen-induced protection against a number of neurodegenerative disorders in pre-menopausal women, for e.g., cerebrovascular accident, Parkinson's and Alzheimer's disease (34). The mechanism of neuroprotection in Parkinson's disease involves regulation of dopaminergic neurotransmission by influencing dopamine synthesis, uptake and release. They inhibit dopaminergic degeneration by a variety of mechanisms, including reducing apoptosis, regulating neurotrophic growth factors, reducing neuroinflammation, regulating NO levels in brain vasculature, and protecting against damage from oxidative stress (35, 36). Nonetheless, several clinical trials have disputed the neuro-protective effect of estradiol leading to the development of "timing hypothesis" which suggests that there is a critical window period during which Hormone Replacement Therapy (HRT) should be initiated in perimenopausal women to derive its beneficial effects (37), which has now been validated by data from the nurses' health study (38).

2.3.2. Estrogen and cardiovascular system

The incidence of cardiovascular diseases is low in pre-menopausal women (38) but increases manifold in post-menopausal women becoming comparable to that of men, suggesting a role for estrogen in cardioprotection (38). Reduced levels of ER-α has been linked to development of coronary artery disease as evidenced by decreased expression of ER-α in atheromatous arteries as compared to normal arteries (39). The Framingham heart study has revealed the *ESR1 PvuII* polymorphism to be associated with increased risk of myocardial infarction in males (40). Also, ER-β polymorphisms are associated with left ventricular wall thickness in women with hypertension and increased blood pressure in men (41, 42). Moreover, mice lacking ER-β develop hypertension on aging and manifest abnormal vascular function (43). The cardio-protective effect of estrogen is attributed to its ability to increase high density lipoprotein (HDL) levels (34), decrease macrophage uptake of oxidized low density lipoprotein (LDL) (44) and by nitric oxide (endothelial derived relaxation factor) mediated vasodilatation (45).

2.3.3. Estrogen and skeletal system

Estrogen plays a major role in maintaining the skeletal homeostasis in both men and women. Estrogen maintains bone density by inhibiting osteoclast-mediated bone resorption and by enhancing osteoblast-mediated bone formation (34). Estrogen mediates osteoclast apoptosis by a TGF-β mediated mechanism (46). Patients with a mutation in the aromatase gene display reduced bone mineral density due to deficiency in estrogen production (47).Though ER-α and ER-β single nucleotide polymorphisms (SNP) have been associated with risk of osteoporosis, the association remains controversial (48). ER-α and ER-β have been detected in human and murine osteoblasts and osteoclasts and in several osteoclastic cell lines. However, data from ER-α or ER-β knockout mice have been unequivocal regarding its effect on bone mineralization (49, 50).

2.4. Effect of estrogen on the immune system

Cells of the immune system, namely neutrophils (51), T- and B- lymphocytes (52), NK cells (53), monocytes and macrophages (54, 55), eosinophils (56) and basophils (57) have been demonstrated to express estrogen receptors.

2.4.1. T-Lymphocytes: In mice, estrogen treatment decreases both thymic size and cellularity (58). Also, it decreases the secretion of IL-2 from T-cells as well as the IL-2R levels (59). Furthermore, they have been demonstrated to expand the CD25 expressing Treg compartment thereby promoting tolerance (60).

2.4.2. B-Lymphocytes: B-lymphopoiesis is suppressed during pregnancy (61) and elevated in estrogen deficient mice. Estradiol affects the production of B-lineage cells by interfering with the differentiation, proliferation, and survival of early pre-B cell precursors (26). Also, it alters the threshold for B-cell activation and apoptosis by modulating the levels of CD22, SHP-1, and Bcl-2 (62) and blocks tolerance induction of naïve B-cells (63).

2.4.3. NK cells, mast cells and eosinophils: Estrogen suppresses NK-cell cytotoxicity (64), augments histamine and serotonin release from mast cells following stimulation with substance-P (65), and enhances eosinophil degranulation and adhesion to human mucosal microvascular endothelial cells (66).

2.4.4. Estrogen as an immunomodulator

Estrogen is a well known immunomodulator (67) with pleiotropic effects on cells of the immune system. Estrogen treatment of mice results in thymic involution whereas ovariectomy results in thymic enlargement and an increase in immature thymocytes (68). Also, estrogen enhances the antigen presentation by uterine epithelial cells and dendritic cells (69). In humans, the plasma IgM, but not IgG levels are significantly higher in females as compared to males (70). Also, women have a higher CD4/CD8 ratio (71) due to an absolute increase in the number of CD4+ lymphocytes. Estrogen also alters the secretion of a number of cytokines; it has been reported to elevate or suppress LPS-induced secretion of IL-1, IL-6 and TNF-α by murine lymphoid cells (72).

2.4.5. Estrogen regulation of autoimmune response

The gender bias observed in the occurrence of a number of autoimmune and infectious diseases is attributed partly to the immunomodulatory role played by the sex hormones, namely, estrogen, progesterone and androgen (73). Many autoimmune diseases are more prevalent in women as compared to men (Figure 2.3). This sexual dimorphism spans a broad spectrum ranging from organ-specific (such as Grave's disease) to generalized (Systemic lupus erythematosus) autoimmune disorders. The gender bias is very strong in diseases such as SLE where the ratio of affected females to males is as high as 9:1.

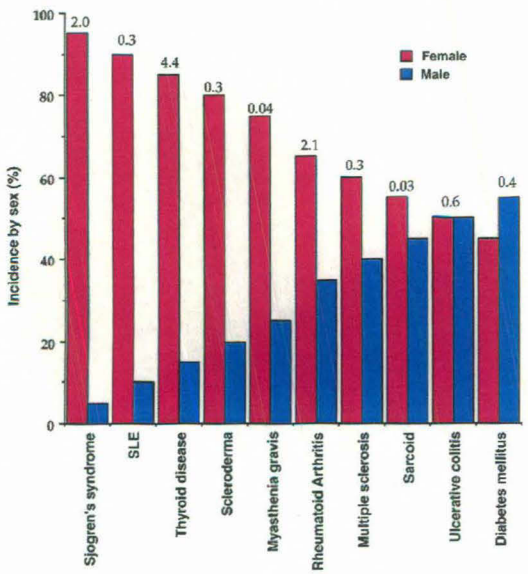


Figure 2.3: Sex-based incidence of various autoimmune diseases. Note the high incidence of autoimmune disorders in females as compared to males.

The reasons for this sex bias is unclear but may be mediated by factors such as sex-related differences in immune responsiveness, response to infection, sex steroid effects, and sex-linked genetic factors (73). In humans, oral contraceptive use is associated with reduced incidence of multiple sclerosis and rheumatoid arthritis (74, 75), while it may exaggerate existing lupus disease or induce the onset of lupus in healthy women. Moreover, studies have indicated that estrogen and progesterone collectively influence autoimmune activity (76). Male and female patients of SLE have been reported to have abnormalities in estrogen metabolism, with abnormal 16α-hydroxylation leading to elevated levels of estrogen metabolites (77).Also, estrogen has been shown to stimulate secretion of autoantibody in SLE- prone and SLE-resistant mice even in the absence of auto-antigen (78). Estrogen and other steroid hormones exhibit dose-dependent biphasic effects on immune cells (79), where high doses inhibit cell mediated immune response whereas low doses facilitate it. Thus, estrogens generally act as a trigger for autoimmune diseases at low concentrations and as an inhibitor at high concentrations. However, in SLE, the pathogenesis of which is dependent on autoantibody production, high dose of estrogen has a detrimental effect (80).

2.4.6. Gender bias in infectious diseases

Sexual dimorphism has been observed in the incidence and progression of a number of infectious diseases such as listeriosis (81), toxoplasmosis (82), leishmaniasis (82), and septic shock with most infections occurring at a higher frequency in men as compared to women. For example, males are more susceptible than females to *Plasmodium berghei*, *Trypanosoma cruzi*, *Trypanosoma brucei*, *Leishmania mexicana*, *Leishmania major*, and *Giardia lamblia* while females are more susceptible to *Leishmania donovani*, *Leishmania tropica*, *Trichomonas vaginalis*, and *Toxoplasma gondii* (82). This gender bias largely depends on the type of host response elicited and the cytokine milieu generated in response to infection. CD4+ T-cell responses are divided into T helper cell type 1 (Th1) or Th2 type responses. The T_H1 lymphocytes establish a pro-inflammatory environment via secretion of IL-2, IFN-γ, and IL-12, while the Th2 cells secrete IL-4 and IL-10 (83) which are essential for mediating humoral immunity. Also, the ability of estrogen to modulate phagocytosis (84), cytokine secretion (85), nitric oxide generation (86, 87),

and reactive oxygen species (87) production impact the onset and progression of a number of infectious diseases.

2.4.7. Effect of estrogen on Leishmanial infection

The consensus regarding protective immunity against leishmaniasis suggests a role for IL-12 secretion from macrophages and dendritic cells which promotes the production of IFN-γ (82) from the innate immune cells such as NK-cells or from cells of the adaptive immune system such as the T-lymphocytes (CD4+ Th1 cell). IFN-γ drives the production of nitric oxide through activation of iNOS which is leishmanicidal. Th1 response mediates clearance of leishmanial infection while a Th2 response results in non-healing disease. The demonstration that males generate a strong Th1 response while females generate a strong Th2 response and antibody production predicts that males would be more resistant to leishmanial infection. This is confirmed in B10/129 and DBA2 male mice which are more resistant than females to *Leishmania major* infection (88, 89) and similarly, *Leishmania tropica* infections are more persistent in human females (90). Also, a study by Krishnan *et al.* has revealed that during pregnancy which is associated with high levels of estrogen and progesterone, women are more susceptible to *Leishmania major* infection (91). Other studies have also demonstrated that females are more susceptible than males to leishmanial infection; for e.g., visceral leishmaniasis in humans caused by *Leishmania donovani* (92, 93), or *L.major* (94) infection in Balb/C and DBA/2 mice. These studies have also demonstrated that orchidectomy increases the resistance of males to infection while testosterone increases the susceptibility in females (94).

2.5. Modulation of macrophage survival and function by estrogen

2.5.1. Macrophages – Origin, distribution and function

Macrophages, which are derived from circulating monocytes, are cells of the mononuclear phagocyte system and play a central role in innate and adaptive immune responses and their activation is central to the outcome of virtually every infectious disease. They perform diverse functions including phagocytosis, chemotaxis, tissue repair, regulation of lipid and iron metabolism, antigen processing and presentation, killing of tumor cells and microbes and cytokine production. Also, they produce a

wide range of biologically active products including several enzymes such as lysozymes, proteases, enzyme inhibitors, coagulation factors, adhesion and binding proteins, complement components, reactive oxygen intermediates such as hydrogen peroxide and superoxide, reactive nitrogen intermediates, eicasanoids such as prostaglandin E2, thromboxane, leukotrienes, and several cytokines such as IL-1, TNF-α, TGF-β, platelet derived growth factor (PDGF), and fibroblast growth factor (FGF) (95).

Macrophages originate in the bone marrow from pluripotent hematopoietic stem cells under the influence of monocyte colony stimulating factor (M-CSF) and granulocyte- monocyte colony stimulating factor (GM-CSF) which stimulates differentiation to monoblasts (96). Monoblasts differentiates to promonocytes and monocytes which leave the bone marrow into the circulation. Once in the circulation, monocytes migrate to virtually every tissue where they differentiate into resident macrophages (97). They are widely distributed in a number of tissues as resident macrophages, for e.g., osteoclasts in bone, microglia in brain, kupffer cells in liver, langerhans cells in skin, peritoneal macrophages in serous cavity, Hofbauer cells in placenta and "dust cells" or alveolar macrophages in the lungs. These tissue-specific macrophages perform highly specialized functions in these tissues and contribute to the overall heterogeneity of the macrophage population (95).

2.5.2. Macrophage expression of estrogen receptors

Estrogen receptor-α and estrogen receptor-β (44, 52, 98) are expressed on murine and human macrophages including a number of tissue specific macrophages such as peritoneal macrophages, osteoclasts, microglial cells, synovial macrophages, alveolar macrophages, langerhans cells etc. However, the relative levels of expression of the two receptor sub-types have been shown to be different among macrophages distributed in different tissues (52). Also, a study by Mor *et al.*, has demonstrated that human U937 monocytes, a promonocytic leukemia cell line expresses ER-β, while differentiated U937 macrophages express both ER-α and ER-β (55) while human THP-1 macrophage cell line expresses both ER-α and ER-β (98). A study analyzing the gene expression levels of ER-α and ER-β in human monocytes has revealed that pre-menopausal females express almost equal quantities of ER-α and ER-β, while post-menopausal females and adult males show several fold increase in the expression of ER-α though the expression of ER-β remains comparable to that of pre-menopausal

females (52). The literature on sub-cellular distribution of estrogen receptors in macrophages is unclear as studies have described them to be nuclear, cytoplasmic and or membranous (20, 55). Although the distribution of these receptors have been reported on human macrophage cell lines (55), there is no study demonstrating the sub-cellular localization of estrogen receptor in primary human macrophages except for an indirect evidence of rapid estrogen effects on human monocytes suggesting a membranous localization (20).

2.5.3 Effect of estrogen on macrophage function

Estrogen has been demonstrated to affect the development of monocytes and macrophages; it increases colony forming unit-granulocyte/monocyte (CFU-GM) (99), which are the progenitors of human monocytes, and similarly, diethylstilbestrol (DES), a synthetic estrogen, increases the number of circulating murine monocytes and peritoneal macrophages (100) as well as human kupffer cells (101).

2.5.3.1. Estrogen effect on macrophage cytokine production

The non-linear effect of estrogen is classically demonstrated by its effect on cytokine secretion from macrophages. It is known to increase or decrease TNF-α (102, 103) and IL-1 (103) production depending on the concentration, species tested as well as the presence or absence of LPS. For e.g., in murine macrophages, estrogen at physiological concentrations enhances IL-1 mRNA and IL-1 production, while at higher concentrations it is inhibitory (103, 104, 105). The enhancement of IL-1 production is mediated by an increase in Ia molecule *in-vivo* (106). It has also been demonstrated that estrogen increases or decreases LPS-induced generation of IL-6, IL-8, IL-12, and IFN-γ (95). Also, it is shown to inhibit human monocyte chemoattractant protein-1 (MCP-1) and its murine homologue JE probably by binding to estrogen response elements and their consequent interaction with a silencer element (107, 108). Another study by Deshpande *et al.*, has demonstrated that estrogen down-regulates LPS-induced secretion of several cytokines by an NF-κB mediated mechanism (85). There is only one study of the effect of estrogen on human subjects which demonstrated that the secretion of IL-1, and TNF-α increase 2 weeks following oophorectomy, while women who underwent oophorectomy but were supplemented with estrogen did not show an increase in these cytokines demonstrating a direct effect of estrogen on cytokine secretion from human blood mononuclear cells (109).

2.5.3.2. Effect of estrogen on reactive oxygen and reactive nitrogen intermediate generation

Estrogen has been shown to increase macrophage nitric oxide (NO) production by up-regulation of inducible nitric oxide synthase (iNOS) (86), which is an inducible enzyme involved in NO production from L-arginine, or by an acute stimulation of constitutive nitric oxide synthase (cNOS) (20), or by stimulation of endothelial nitric oxide synthase (110). The modulation of iNOS can be mediated by both a direct binding of estrogen-estrogen receptor complex to estrogen response elements on iNOS promoter and transcriptional up-regulation (111), or indirectly *via* elevation of intracellular calcium concentration (20) or by modulating cytokines such as IFN-γ which control iNOS induction (112). A recent study (20) has demonstrated that estrogen increases NO via a membranous estrogen receptor signaling in human monocytes by acutely stimulating cNOS via calcium transients. The cardioprotective effect of estrogen is mediated partly by generation of NO which acts as a vasodilator in peripheral arteries (113).

Estrogen also modulates the generation of reactive oxygen species (ROS), a few studies demonstrate an increased (87) ROS production while others show an inhibition (114). The cardio- and neuro-protective effects of estrogen are partly attributed to their anti-oxidant activity (115). It modulates the anti-oxidant activity by increasing in a concentration dependent manner the expression of manganese superoxide dismutase (MnSOD) and extracellular superoxide dismutase (ecSOD) and their activity (116). It induces a transcriptional up-regulation of MnSOD, while it stabilizes the transcript of ecSOD. Also, oophorectomized mice showed enhanced production of free radicals in the vascular tissue associated with a down-regulation of MnSOD and ecSOD (116). Estrogen inhibits NADPH-oxidase activity and hence superoxide generation by inhibiting $p47^{phox}$ (the cytosolic component of NADPH-oxidase) via an NFκB dependent mechanism (117). It is also known to lower mitochondrial oxidative load by preserving ATP levels *via* enhanced oxidative phosphorylation as shown by activation of cytochrome C oxidase (COX) (115, 118), a key enzyme involved in oxidative metabolism. Also, it reduces the mitochondrial F_0-F_1 ATP-ase activity by direct binding and inhibition (119). An imbalance in Ca^{2+} homeostasis can also trigger nitric oxide and ROS generation (120), while estrogen

ensures Ca^{2+} homeostasis by sequestering Ca^{2+} in the mitochondrial matrix and endoplasmic reticulum (115) thereby reducing endogenous ROS levels.

2.5.3.3. Effect of estrogen on monocyte adhesion, migration and phagocytosis

Estrogen reduces monocyte adhesion to vasculature by inhibiting the expression of adhesion molecules such as vascular cell adhesion molecule (VCAM-1) (121) and intercellular adhesion molecule (ICAM-1) (122) on endothelial cells. The down-regulation of VCAM-1 mRNA involves inhibition of NF-κB, AP-1 and GATA transcription factors (123). Also, estrogen acts directly on monocytes to inhibit adhesion by decreasing the expression of ICAM-1/β2 and VCAM-1/β1 integrins by a Rac-1 GTPase dependent mechanism (124). The accumulation of monocytes is dependent on interaction of CCR2 with monocyte chemoattractant protein-1 (MCP-1) at inflammatory sites. Postmenopausal women show enhanced expression of CCR2 on monocytes which is reduced upon hormone replacement therapy (125) suggesting the cardio-protective role for estrogen in reducing monocyte adhesion and transmigration across the vascular endothelium by decreasing the CCR2 expression. Estrogen also enhances the ability of macrophages to phagocytose bacteria, dead cells, etc., by up-regulation of FcγRIII receptors and complement receptor CR3 (84).

2.5.4. Effect of estrogen on macrophage survival

The literature on effect of estrogen on monocyte-macrophage survival is highly contradictory. Estrogen induces apoptosis in primary osteoclasts and pre-osteoclastic FLG 29.1 cell line by a TGF-β mediated mechanism (46) or by up-regulation of FasL (126, 127). Furthermore, it has been demonstrated that ER-α deficient osteoclasts do not undergo apoptosis upon estrogen exposure suggesting that signaling *via* ER-α plays a major role in preventing osteoporosis (127). Murine peritoneal macrophages also undergo apoptosis upon exposure to estrogen in a dose-dependent manner (128). Human U-937 promonocytic leukemia cell line which has been demonstrated to express ER-β undergoes apoptosis upon exposure to estrogen, but is refractory to death upon differentiation to macrophages wherein these cells express both ER-α and ER-β (55). Further, the authors of this study have shown that the mechanism of estrogen-mediated death involves up-regulation of FasL on monocytes by binding of estrogen-estrogen receptor complex to the estrogen response element on FasL promoter. In contrast, study by Carruba *et al.*, (129) demonstrate that

U-937 macrophages undergo apoptotic death when exposed to estrogen, while Vegeto *et al.*, (130) have reported protection of U-937 cells by estrogen from TNF-α induced death by decreasing the levels of pro-apoptotic Nip-2 protein. Estrogen is also known to induce anti-apoptotic signaling in microglial cells by reducing the expression of Fas and FasL by an ER-α mediated mechanism (131). The effect of estrogen on survival of human THP-1 cells, an acute monocytic leukemia cell line is contradictory as Thongngarm *et al.*, (132) showed that estrogen inhibits monocyte cell cycle progression and induces apoptosis whereas Cutolo *et al.*, demonstrated that estrogen does not affect the survival of THP-1 cells (133). However, it has to be noted that the study by Thongngarm *et al.*, (132) was performed using supraphysiological concentrations of estrogen and hence cannot be interpreted meaningfully in an *in vivo* scenario. There is one clinical study (134) which demonstrates that menopause is associated with an increase in blood monocyte number as well as a relative decrease in the expression of estrogen receptors providing indirect evidence that estrogen may mediate human monocyte apoptosis.

2.6. An introduction to cell death

The process and mechanisms of cell death is an area of active investigation as dysregulation of this process results in developmental disorders, cancers, persistence of infection etc. Traditionally, death is classified as necrotic or apoptotic, though in recent years several more forms of death with overlapping features yet distinct from either apoptosis or necrosis have been described. The term programmed cell death (PCD) was suggested by Lockshin and Williams in 1964 (135) and has been used to describe the form of cell death where the cells follow a sequence of genetically controlled steps towards their own destruction. PCD has now been further classified as apoptotic and non-apoptotic programmed cell death (136). The various forms of non-apoptotic programmed cell death include autophagy, paraptosis, dark cell death and necrosis-like PCD (136).

2.6.1. Necrosis

Necrosis is an energy-independent mode of death characterized by swelling of cells, formation of cytoplasmic vacuoles, distension of endoplasmic reticulum ,

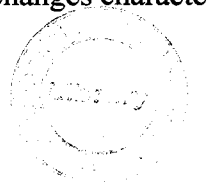
swelling or condensation of mitochondria, cytoplasmic blebs, disaggregation and detachment of ribosomes, disruption of organellar membranes, swollen and ruptured lysosomes with eventual rupture of the plasma membrane resulting in the elicitation of an inflammatory response (136).

2.6.2 Apoptosis

Apoptosis is a form of programmed cell death and the term was proposed by Currie *et al.,* (137) to describe a form of death that was different from necrosis. Apoptosis is an energy dependent process and hence maintenance of intact mitochondrial function is a pre-requisite (138, 139). Morphological changes that characterize apoptosis are cell size shrinkage, membrane blebbing, and chromatin condensation. With cell shrinkage, the cytoplasm becomes dense with tight packaging of organelles. Chromatin condensation is well demonstrated by electron microscopy as electron-dense nuclear material which characteristically aggregates at the periphery of the nuclear membrane (136). Plasma membrane blebbing is followed by karyorrhexis and packaging of cytoplasmic and organellar material into apoptotic bodies which are enclosed in an intact plasma membrane. Also, upon receipt of an apoptotic signal, phosphatidylserine which is normally located on the inner surface of the plasma membrane is exposed to the outer surface (140) acting as a signal for phagocytosis by adjacent cells or professional phagocytes such as macrophages which results in the clearance of an apoptotic cell without the elicitation of an inflammatory response.

2.6.2.1 Biochemistry of Apoptosis

Apoptotic process is broadly divided into three phases; Initiation phase, Effector phase, and Degradation phase. The initiation phase depends on the cell type and the nature of the stimulus, for e.g., death receptor mediated signals, genotoxic stress, oxidative damage, radiation damage etc., which results in the activation of the apoptotic program. The effector phase comprises of amplification of these signals and commitment to apoptotic death in the form of activation of proteases, nucleases and other biochemical components involved in the final execution stage. The degradation phase is characterized by enzymatic cleavage of several substrates and genomic DNA resulting in the morphological changes characteristic of apoptosis as described above.



The initiation phase signals can be mediated by death receptor pathway or the extrinsic pathway, the mitochondrial pathway or the intrinsic pathway or the granzyme/perforin mediated pathway. The effector and degradation phase of all these pathways involve the activation of a family of cysteine proteases called caspases which are the executors of the death pathway (136).

2.6.2.2. Caspases – Role in apoptosis

Caspases are so named because they are cysteine proteases that cleave their substrates after aspartic acid residues. The caspases are present in the cell in a pro-enzyme form which is inactive and requires proteolytic cleavage for activation (141). The substrate specificity of individual caspases is determined by four residues amino-terminal to the cleavage site (141). The caspases are divided into two sub-families; initiator caspases and executioner or effector caspases (141).

The initiator caspases are those that mediate activation of the executioner caspases. They possess a long pro-domain with which they interact with various adaptor molecules and their activation is dependent on oligomerization mediated autoproteolytic cleavage. The initiator caspases include caspase-2, -8, -9, and -10.

The executioner caspases are activated by the initiator caspases (141), or calpain (142), or by granzyme B (143). These caspases possess short pro-domains and include caspases-3, -6, and -7. The activation of these caspases and the subsequent cleavage of their specific substrates result in the characteristic morphological changes associated with apoptosis. For e.g., loss of cellular shape is mediated by cleavage of cytoskeletal proteins, pyknosis or nuclear condensation is mediated by degradation of nuclear lamin, DNA fragmentation requires inactivation of inhibitor of caspase-activated DNase (ICAD) (144) and cleavage of poly (ADP-ribose) polymerase (PARP) which impairs DNA repair mechanism (145).

2.6.2.3 Extrinsic pathway of apoptosis

Apoptosis induced by signaling *via* death receptors localized on the cell membrane is called the extrinsic pathway of apoptosis. The well studied death receptors are the tumor necrosis factor receptor (TNFR) and the Fas (CD95) receptor. The members of the TNFR family contain a cysteine-rich extracellular domain and an intracellular death domain (146, 147). The binding of ligand to the cognate receptor results in the trimerization of TNFR or Fas resulting in the recruitment of adaptor

proteins TNFR-associated death domain (TRADD) or Fas-associated death domain (FADD) forming a complex called DISC (Death inducing signaling complex) (148). The death effector domain of the adaptor proteins then recruit pro-caspase-8 to the DISC resulting in its proteolytic cleavage and activation which subsequently activates caspase-3 (141). Alternatively, caspase-8 may mediate cleavage of Bid, a member of the Bcl-2 family of protein, which results in its translocation to the mitochondria followed by release of cytochrome c and caspase-9 activation. Hence, the intrinsic pathway of apoptosis may be initiated by a signal originating from the death receptors demonstrating an integration of these two pathways (149). A schematic of the pathways of apoptosis is shown in Figure 2.4 (136).

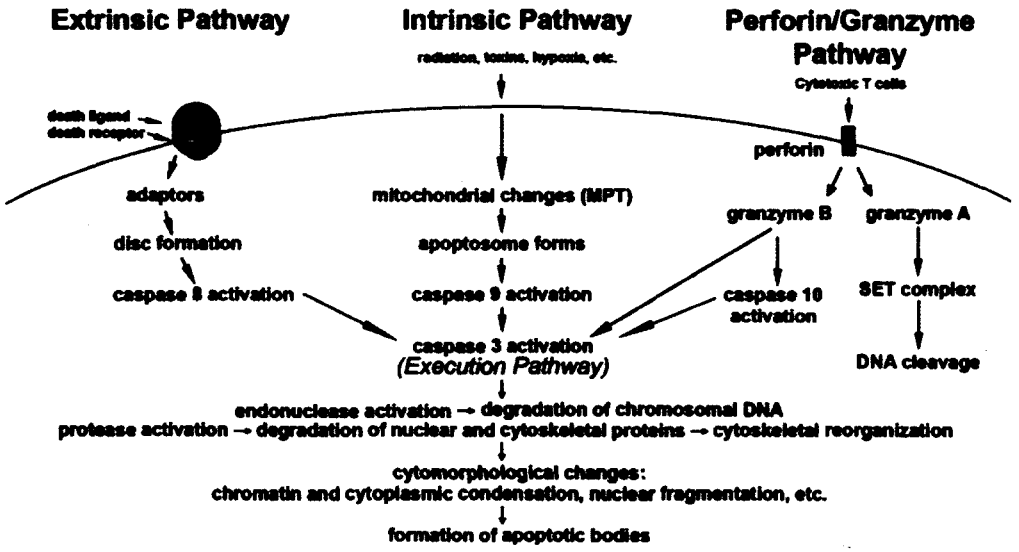


Figure 2.4: Pathways of apoptosis. Schematic showing the different mechanisms of activation of apoptosis, namely the extrinsic, intrinsic, and the perforin/granzyme pathway all of which result in activation of caspases and characteristic apoptosis morphology.

2.6.2.4. Perforin/Granzyme pathway:

This pathway has been reported to be used by CD8+ cytotoxic T-lymphocytes to mediate killing of antigen-bearing cells. It involves secretion of a pore-forming molecule called peforin which creates transmembrane pores on the target cell membrane following which there is an exophytic release of cytoplasmic granules into the target cell (150). The granules contain two important serine proteases Granzyme A and Granzyme B. Granzyme B cleaves proteins at aspartate residues and activates

procaspase-10, cleaves ICAD (151), and may also cleave Bid amplifying signals *via* the mitochondrial pathway (152). They may also directly cleave caspase-3 activating the execution phase of apoptosis (152). In contrast, Granzyme A mediates apoptosis by a caspase-independent mechanism. It cleaves the nucleosome assembly protein SET, which is part of the SET complex, the role of which is inhibiting NM23-H1, a DNase (153). The activation of NM23-H1 results in the cleavage of DNA and apoptosis.

2.6.2.5. The intrinsic pathway of apoptosis

The intrinsic pathway of apoptosis involves mitochondria-initiated events and hence this pathway is also called the mitochondrial pathway of apoptosis. Several stimuli which either directly or indirectly damage the mitochondria initiate this process. The culmination of all these events results in the permeabilization of the mitochondrial outer membrane leading to release of pro-apoptotic proteins such as Cytochrome c, Smac/DIABLO, Omi/HtrA2, etc., into the cytosol (154). Upon release, Cytochrome c forms a caspase activating complex with Apoptosis protease activating factor-1 (Apaf-1) called apoptosome. The apoptosome activates pro-caspase-9 by proteolytic cleavage which further activates the executioner caspases-3 and -7 (136). In contrast, Smac/DIABLO and Omi/HtrA2 interact with cytosolic X-linked inhibitor of apoptosis protein (XIAP) and prevent the inactivation of caspase-3 and caspase-9 (155). Apoptosis may also be induced by release of endonuclease G or Apoptosis inducing factor (AIF) from the mitochondria which then translocates to the nucleus and mediates DNA fragmentation in a caspase-independent manner (156).

2.6.2.6. The Bcl-2 family of proteins

The Bcl-2 family of proteins is named after its first identified member, Bcl-2, which was discovered in patients with human B-cell leukemia carrying the t (14:18) translocation (157). As of date more than 25 genes have been identified in the Bcl-2 family which comprises of both pro- and anti-apoptotic members. The members belonging to the pro-apoptotic group include Bcl-10, Bax, Bak, Bid, Bad, Bcl-XS, Bim, Bik, and Blk, while the anti-apoptotic members include Bcl-2, Bcl-x, Bcl-XL, Bcl-w, and BAG. The anti-apoptotic members are characterized by four short Bcl-2 homology domains (BH1-BH4) (Figure 2.5) (158). Also, they possess a hydrophobic

tail at the C-terminus which allows them to anchor to mitochondrial and nuclear membranes.

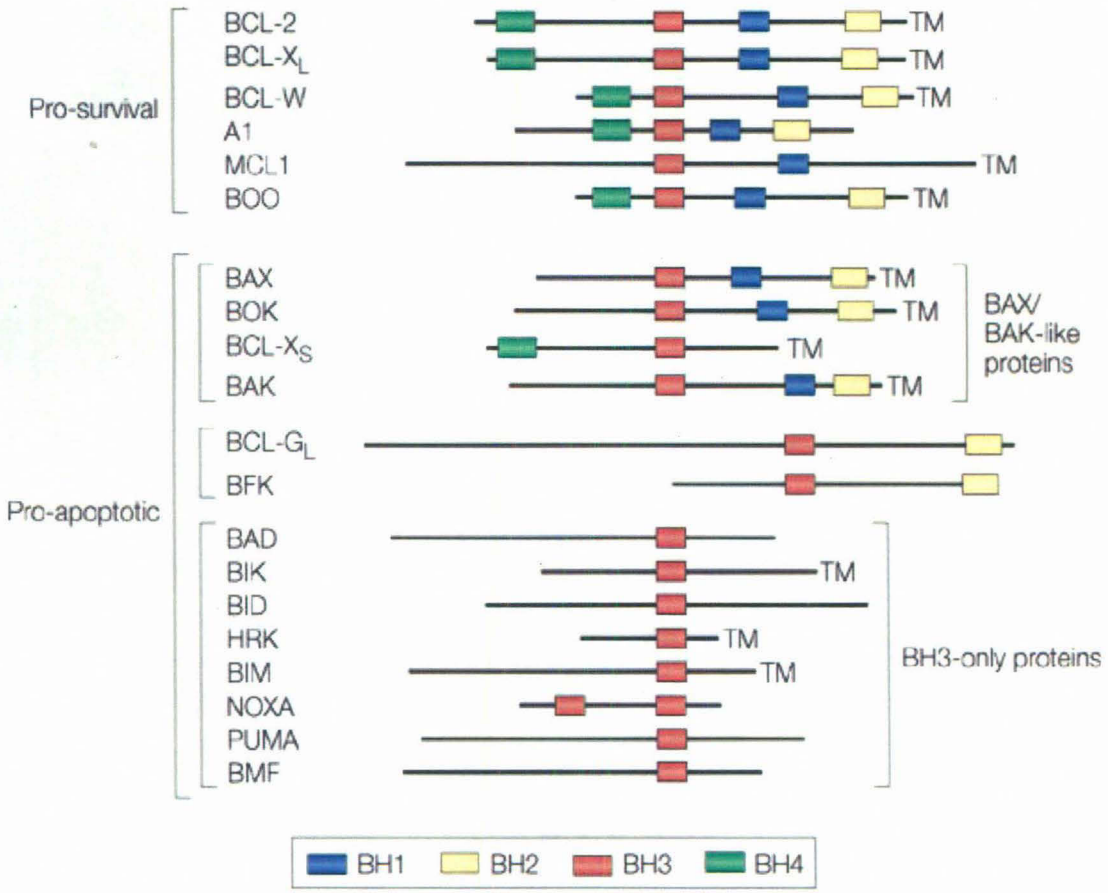


Figure 2.5: Domain architecture of Bcl-2 family of proteins. The BH1-4 domain distribution is shown for various Bcl-2 family of proteins. Note that all of them possess the BH3 domain, while the pro-apoptotic proteins generally lack the BH4 domain.

The pro-apoptotic members are further divided into the Bax sub-family, which include Bax, Bak, and Bok and possess the BH1-BH3 domain, and the "BH3 only" proteins which include Bad, Bid, Bim, Bik, Blk, Puma, Noxa, and Hrk, which possess only the BH3 domain (158). The pro-apoptotic members are usually localized in the cytosol or are loosely attached to the membranes, where upon receipt of an apoptotic stimuli translocate to the outer mitochondrial membrane where they get inserted or interact with other members of the Bcl-2 family. Most members of the Bcl-2 family of proteins can interact with each other forming homodimers, heterodimers or oligomers thereby potentially regulating their activity (136).

2.6.2.6.1 Role of Bcl-2 in apoptosis

Bcl-2 is an anti-apoptotic protein with ability to suppress both caspase dependent and caspase independent cell death pathways. It is localized to the outer membrane of the mitochondria, endoplasmic reticulum, and nuclear membrane (159) acting to guard these organelles. Bcl-2 protects cells from death by several mechanisms including binding to pro-apoptotic Bax and Bak which are involved in mitochondrial outer membrane permeabilization (MOMP) (136), regulation of endoplasmic reticulum and mitochondrial Ca^{2+} homeostasis (160), modulation of anti-oxidant pathways (161), and promotion of glutathione sequestration to the nucleus (162). In addition, Bcl-2 inhibits Ire1, a serine-threonine kinase involved in endoplasmic reticulum stress response which mediates death by activation of Ask1, JNK, and p38 MAPK (163). Also, Bcl-2 can bind to non-homologous proteins such as Raf-1 and calcineurin (164, 165). It mediates translocation of Raf-1 to mitochondrial membrane where it facilitates the phosphorylation of Bad by Raf-1 thereby abrogating its pro-apoptotic effect (166). Moreover, Bcl-2 binding to calcineurin, a Ca^{2+} activated serine-threonine phosphatase may inhibit dephosphorylation of Bad (167) and prevent its apoptotic effect. An alternate model of Bcl-2 action suggests that it inhibits activation of Bax and Bak by sequestering BH3 only proteins (136). Post-translational modifications such as phosphorylation and caspase-mediated cleavage also play a role in modulating the activity of Bcl-2 (168, 169). It has been reported that phosphorylation within the loop region of Bcl-2 induces a conformational change which abrogates its anti-apoptotic effect as well as making it susceptible to cleavage by multiple proteases (170). In contrast to the well known anti-apoptotic role of Bcl-2, several recent studies have suggested a phenotypic conversion in Bcl-2 whereby it is transformed into a pro-apoptotic protein. For e.g., proteolytic removal of N-terminal sequences by caspase-mediated cleavage (169) or by binding of the orphan nuclear receptor Nur77 (171) which induces a conformational change leading to the exposure of the buried BH3 domain converting it into a pro-apoptotic protein.

2.6.2.6.2 Role of Bax in apoptosis

In healthy cells, Bax is localized in the cytosol, but upon receipt of an apoptotic stimulus, it translocates to the mitochondria presumably due to a conformational change resulting in the exposure of its hydrophobic C-terminal domain (172) with which it inserts into the mitochondrial membrane. Bax is also

known to interact with voltage dependent anion channel (VDAC) (173) and adenine nucleotide translocators (ANT) (174) in the mitochondria suggesting a role for Bax in the opening of the permeability transition pore (PTP). The binding of Bax to VDAC mediates a conformational change of the latter resulting in the enlargement of the PTP allowing passage of Cytochrome C (175). Moreover, cleavage of Bax by calpain generates an 18 kDa fragment which is more potent than Bax in mediating cytochrome c release (176) presumably due to the inability of Bcl-2 to interact with the cleaved Bax. The mechanism of activation of Bax is an area of active of investigation and several hypotheses have been proposed including neutralization of several anti-apoptotic proteins by BH3 only proteins such as Noxa and Puma (177), phosphorylation events mediated by kinases (178), down-modulation of scaffold proteins such as clusterin which hold Bax in an inactive conformation (179), intracellular acidification (180), intracellular alkalinization (181), and caspase-mediated cleavage of Bax (182).

2.6.2.6.3. Role of Mitogen activated protein kinases (MAPK) in apoptosis

Several survival and stress signals mediate activation of a family of kinases called the mitogen activated protein kinases (MAPK). They consist of a module of three cytoplasmic kinases: mitogen activated protein (MAP) kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK). They are dual specificity serine-threonine kinases (183) which phosphorylates substrates in both the cytoplasm as well as in the nucleus. When translocated to the nucleus, they are involved in control of gene expression by direct activation of transcription factors.

There are three well characterized sub-families of MAPKs namely extracellular signal regulated kinase (ERK), p38, and c-jun NH2-terminal protein kinase/stress activated protein kinase (JNK/SAPK) (184). p38 and JNK are activated predominantly by chemical and environmental stress and by inflammatory cytokines, and recognized to be part of death-promoting pathway (185). In contrast, the ERK signaling pathway is activated by growth factors and mitogens, and transduces survival, proliferation, and differentiation signals (185).

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Chapter 3

Materials and Methods

3.1. Materials

Roswell Park Memorial Institute medium (RPMI-1640) (with and without phenol red) and Dulbecco's modified Eagle's medium (DMEM) (with and without phenol red) were purchased from Sigma Chemical Company (St. Louis, MO). Fetal calf serum and dextran-coated charcoal stripped fetal calf serum (DCC-FCS) were procured from Biological Industries (Kibbutz Beit Haemek, Israel). 0.22 μm membrane filters were obtained from Millipore (Billerica, MA).

Deoxy-ribonucleotide (dNTP) mix, magnesium chloride ($MgCl_2$), and pGEM-T_{Easy} sequencing vector were purchased from Promega (Madison, WI). Taq DNA polymerase was obtained from New England Biolabs (Beverly, MA), while Superscript II First strand synthesis kit and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). 100 bp DNA ladder, 1 kb DNA ladder, and 6X DNA loading dye were obtained from MBI Fermentas (Ontario, Canada). Synthetic oligonucleotides were obtained from Sigma GENOSYS (Bangalore, India) or Microsynth (Germany). MinElute™ Gel extraction kit was purchased from Qiagen (GmbH, Hilden).

CB-X protein assay kit was purchased from G-Biosciences (St. Louis, MO). Ammonium persulphate (APS) and N, N, N′, N′ - tetramethylene-diamine (TEMED) were obtained from Sigma Chemical Company (St. Louis, MO). Rainbow™ protein markers, nitrocellular membranes, and enhanced chemiluminescnence detection reagent were procured from Amersham Biosciences (Piscataway, NJ). Anti-estrogen receptor α/β, anti-estrogen receptor-α, and anti-actin antibodies were purchased from Calbiochem (Darmstadt, Germany). Anti-phospho CREB, anti-total CREB, anti-phospho ERK, and anti-total ERK were obtained from StressGen Biotechnologies (Victoria, BC). Anti-Bcl-2, anti-Bax, anti-Bad, and anti-Cytochrome C antibodies were purchased from SantaCruz Biotechnology (Santa Cruz, CA). Anti-histone dimethyl lysine antibody was procured from Upstate (VA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Ambion (Austin, TX). Anti-Clusterin antibody was a kind gift from Dr.C.Yan Cheng of the Population Council, New York, USA. Secondary anti-mouse antibody conjugated to Alexa fluor 488 was purchased from Molecular Probes (Eugene, OR). Secondary anti-

mouse and anti-rabbit antibodies conjugated to horseradish peroxidase (HRP) were obtained from Jackson Immunoresearch (Cambridgeshire, UK).

Chloroform, isopropyl alcohol, di-sodium hydrogen phosphate, sodium di-hydrogen phosphate, sodium chloride, glycine, acetic acid, hydrochloric acid, sulphuric acid, Tris, Tris-HCl, potassium chloride, di-potassium hydrogen phosphate, formaldehyde, phenol, hydrogen peroxide, and methanol were obtained from Merck (Mumbai, India). Ethanol was purchased from Fluka Chemie GmbH (Buchs, Switzerland).

17 β-Estradiol (cyclodextrin-encapsulated), estradiol conjugated to BSA (E2-BSA), E2-BSA conjugated to FITC (E2-BSA-FITC), BSA-FITC, propidium iodide, Lipopolysaccharide from *S.typhosa*, Histopaque 1077, PD 98,059, Bisiondoleylmaleimide (BIM VIII), Verapamil, Pimozide, EGTA, EDTA, Phorbol myristate acetate (PMA), nigericin, amiloride, and aminoguanidine were purchased from Sigma Chemical Company (St. Louis, MO). Fluo-3acetoxymethyl ester (Fluo-3-AM), 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM), 5-(and -6) chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), SNARF (5-(and -6)-carboxy SNARF®1-AM), Sodium Green™ tetracetate, and Hoechst 33342 were obtained from Molecular Probes (Eugene, OR). Ketamine was purchased from Neon Pharma (Mumbai, India), while Xylocaine 2% was purchased from AstraZeneca (Bangalore, India).

siRNA against ER-α, ER-β, and Bcl-2 were obtained from Dharmacon (Lafayette, CO), while the Cy3-labeled negative control siRNA was purchase from Ambion (Austin, TX). The siRNA transfection reagent, TranspassR2 was procured from New England Biolabs (Ipswich, MA). The Vybrant apoptosis detection system was purchased from Promega (Madison, WI). Alexa fluor 488 labeled dead *E.coli* particles were obtained from Molecular probes (Eugene, OR). Enzyme linked immunosorbent assay (ELISA) kits for detection of IL-1β, IL-4, IL-6, IL-8, IL-12, IFN-γ, and TNF were obtained from BD Biosciences (NJ, USA).

All other chemicals used in this study unless otherwise mentioned were purchased from Sigma Chemical Company (St. Louis, MO).

3.2. Methods

3.2.1. Cell culture techniques

3.2.1.1. Cell lines and cell culture

THP-1 acute monocytic leukemia cell line (TIB-202) was purchased from American type culture collection (ATCC) (Manassas, VA). These suspension cells were maintained in culture at 37°C in RPMI-1640 medium supplemented with 10% FCS. They were sub-cultured when the cell density reached ~1X10^6 per mL. To induce differentiation of these monocytic suspension cell cultures to adherent macrophage phenotype, they were subjected to treatment with PMA at a concentration of 10 ng/mL for 36 h. Forty eight hours prior to experimentation, the cells were transferred to phenol-red free RPMI-1640 medium supplemented with 10% dextran-coated charcoal stripped FCS. This was performed to remove all traces of exogenous estrogens as phenol red in culture medium is known to be a weak estrogen (1) and FCS contains multiple steroid hormones which are removed upon stripping with dextran-coated charcoal.

MCF-7, a breast carcinoma cell line was obtained from ATCC (Manassas, VA). They were maintained in culture at 37°C in RPMI-1640 medium supplemented with 10% FCS and were routinely sub-cultured when the cells reached a confluency of around 80%.

3.2.1.2. Peripheral blood monocyte isolation and macrophage differentiation

Peripheral blood (30 mL) was collected by venipuncture from healthy male volunteers after obtaining an informed consent and in accordance to the regulations of the Institutional Human Ethics Committee (National Institute of Immunology, New Delhi, India). The peripheral blood mononuclear cell (PBMC) population was isolated by density gradient centrifugation using Histopaque 1077, where, human whole blood was layered on Histopaque 1077 and centrifuged at 400 x *g* for 35 min at 25°C. The mononuclear cell population was isolated from the plasma-histopaque interface, and the monocytes were further purified by washing off the non-adherent cells after incubating the total PBMC for 1 h at 37°C. The homogeneity of the obtained

population was determined by analyzing cells immunostained with an antibody against CD14 conjugated to FITC and the purity obtained was approximately 85% monocytes, the remaining being lymphocytes. The monocytes were further cultured in the presence of human AB serum for 7 days to allow differentiation to macrophages. At the end of 7 days post-isolation, greater than 95% of cells in culture are monocytes, with the majority of lymphocytes undergoing neglect induced death.

3.2.1.3. Protocol for propagation and maintenance of *Leishmania major* promastigotes

Leishmania major strain (MHOM/Su73/5ASKH) was a kind gift from Dr. Satyajit Rath, Immunobiology Laboratory, National Institute of Immunology, India. *L.major* promastigotes were cultured at 23°C in modified DMEM (DMEM (1 L) supplemented with sodium bicarbonate (3.7 g), HEPES (5.96 g), hemin (5 mg), biotin (1 mg), adenine (13.36 mg), xanthine (7.6 mg), triethanolamine (0.5 mL), and tween 80 (40 mg)) supplemented with 10% FCS. It is known that long term culture of *L.*major promastigotes results in loss of their virulence (2). Hence, to maintain the virulence of these parasites, they were propagated in mice footpad. Towards this end, the stationary phase *L.major* promastigotes were resuspended in Hank's balanced salt solution and $2x10^6$ promastigotes were injected into the footpad of female BALB/c mice. 6 weeks post-infection, the infected footpad was dissected and the lesion harvested. The obtained lesion was minced and resuspended in modified DMEM supplemented with 10% FCS and placed in 23°C incubator to allow differentiation of intracellular amastigotes to promastigotes. This cycle of harvesting promastigotes from footpad lesions was performed every 6 weeks to maintain the virulent phenotype of this parasite.

3.2.2. Biochemical and cell biology techniques

3.2.2.1. Assay for cell viability by propidium iodide dye exclusion method

Propidium iodide (PI) is a DNA intercalating fluorescent dye which is excluded by viable cells with intact membranes, however, dead and dying cells with damaged membranes take up the dye. To assess viability, cells after appropriate

treatment were harvested by centrifugation at 250 x *g* for 5 min following which they were resuspended in 1x PBS (pH 7.5). PI was added at a final concentration of 1 μg/mL and incubated for 5 minutes following which the cells were pelleted by centrifugation and washed once with PBS. These cells were analyzed for uptake of PI by either flow cytometry in FL2 channel (570 nm) or by fluorescence microscopy using a G2A filter block.

3.2.2.2. Assay for cell viability by Trypan blue dye exclusion method

Trypan blue is a diazo vital stain which selectively colours the dead cells blue that can be visualized under light microscope. Equal volumes of cell suspension and 0.4% trypan blue dye were mixed and incubated at room temperature for 5 min. 10 μL of stained cells were loaded on to a hemocytometer and a count of the number of viable and dead cells were made. This procedure was carried out routinely to ensure that cell viability is >95% before plating cells for experiments.

3.2.2.3. Assay for detection of apoptosis by Annexin-V/PI staining

The Vybrant apoptosis assay kit was used to perform Annexin-V/PI staining as described previously (3). The assay is based on the principle that apoptotic cells show loss of membrane asymmetry by exposing phosphatidylserine on the outer surface of the plasma membrane for which Annexin-V, a phosphlipid binding protein, shows high affinity. Hence, Annexin-V conjugated to Alexa fluor 488 binds to phosphatidylserine exposed on apoptotic cells, while propidium iodide binds to nucleic acids of all non-viable cells including necrotic and apoptotic cells. Thus, flow-cytometric analysis of Annexin-V/PI stained cells reveals distinct cellular populations, with the viable cells displaying little or no fluorescence; the early apoptotic cells show green fluorescence of Annexin-V conjugated to Alexa fluor 488; the late apoptotic cells display both green and red fluorescence, while necrotic cells show red fluorescence.

The cells after appropriate treatment were harvested by centrifugation at 250 x *g* for 5 min and were given two washes with ice-cold 1X PBS following which they were resuspended in 100 μL of ice-cold 1X Annexin binding buffer (50 mM HEPES,

700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4). 5 µL of Annexin-V conjugated to Alexa fluor 488 and 1 µL of working solution of PI (100 µg/mL) were added to the 100 µL cell suspension. Cells were incubated for 15 min at room temperature. Following this, 400 µL of 1X Annexin binding buffer was added to dilute the sample. The samples were placed on ice. The fluorescence was measured by flow cytometry in FL1 and FL2 channels for Annexin-V-Alexa fluor 488 and PI fluorescence respectively.

3.2.2.4. Immunocytochemistry

a. Immunostaining in fixed cells:

The cells were fixed with 4% formaldehyde for 20 min, following which two washes were given with ice-cold PBS. Permeabilization and blocking were performed simultaneously by incubating the formaldehyde fixed cells in PBS containing 0.1% saponin and 3% normal goat serum for 30 min. The cells were washed once with ice-cold PBS. The permeabilized cells were incubated with the primary antibody at an appropriate dilution for 1 h at room temperature following which three washes with ice-cold PBS was given. These cells were then incubated with fluorophore conjugated secondary antibody (IgG) for 1 h at room temperature following which three washes with ice-cold PBS were given. The nuclei were stained with Hoechst 33342 at a concentration of 1 µg/mL for 2 min at room temperature. The staining was then visualized under a Nikon TE2000E fluorescence microscope using appropriate filter blocks. Image acquisition was carried out using a high-resolution Retiga *Exi* camera (Q-imaging, Surrey, BC, Canada) and subsequent image analysis was performed on Image-Pro Plus software v5.5 (Media Cybernetics, Silver Spring, MD). Alternatively, the fluorescence staining was detected by flow-cytometry (BD-LSR, Beckton Dickinson, NJ, USA) using an air-cooled argon ion laser (488 nm) at appropriate florescence channels. Subsequent data analysis was performed on WinMdi software (Microsoft, v 2.9).

b. Immunostaining in Live cells:

Immunostaining on live cells was performed by harvesting and resuspending cells in ice-cold PBS. The cells were then incubated with an appropriate dilution of the primary antibody for 1 h at 4°C following which two washes were given with ice-

cold PBS followed by incubation with fluorophore labeled secondary antibody at appropriate dilution for 30 min at 4°C. The fluorescence was then visualized under a fluorescence microscope or analyzed by flow cytometry.

3.2.2.5. siRNA transfection

THP-1 macrophages and human peripheral blood monocyte derived macrophages were transfected with SMARTpool Bcl-2 siRNA (15 pmol), or ER-α siRNA (100 pmol), or ER-β siRNA (100 pmol), or with negative control siRNA (15 pmol or 100 pmol) using TranspassR2 transfection reagent. Prior to transfection, the cells were depleted of serum by washing 2x with serum-free media. The transfection complex was prepared by diluting 0.5 µL of transfection reagent A and 1.0 µL of transfection reagent B to 400 µL of serum-free media and siRNA's were added to the mix at an appropriate concentration and incubated for 20 min at room temperature. The formed transfection complexes were transferred gently using a large bore pipette tip to 10^5 cells/well grown in 24 well plates and incubated for 6 h, following which fresh complete medium was added. Transfection efficiency was estimated by observing Cy3-fluorescence of the negative control siRNA with a Nikon TE2000E fluorescence microscope using a tetramethyl rhodamine filter (530-580 nm). For all transfections, target protein knockdown was assessed 24 h after transfection by probing extracts of transfected cells on Western blots using appropriate antibodies.

3.2.2.6. SDS-PAGE and Western blot

Whole cell extracts were prepared by treating cells with lysis buffer (0.125M Tris, 4% SDS, 20% glycerol, and 10% β-mercaptoethanol), and protein estimation was performed using CB-X protein assay kit as per manufacturer's protocol. Lysates were resolved on 12% SDS-PAGE gel, following which Western transfer was performed onto nitrocellular membranes using a BioRad Western transfer apparatus. The blots were incubated with 5% blotto (non-fat dry skimmed milk) in 0.05% PBS-Tween 20 for 1 h to block non-specific binding sites following which they were incubated for 1 h with primary antibody at an appropriate dilution prepared in 1% blotto in 0.05% PBS-Tween-20. The blots were washed 3x with 0.05% PBS-Tween-20 at 5 min intervals following which they were incubated for 1 h with secondary

antibody at an appropriate dilution. The immunoreactivity was detected by enhanced chemiluminescence using an ECL detection kit (Amersham Biosciences) and were recorded on X-ray films after appropriate exposure and development. It is important to note that the blots for probing phosphorylated proteins were performed using 1% BSA as blocking agent instead of blotto.

3.2.2.7. Sub-cellular fractionation

Sub-cellular fractionation of THP-1 macrophages was performed after lysis with hypotonic buffer. THP-1 macrophages after appropriate treatment were allowed to swell for 10 min in hypotonic buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5) followed by homogenization with 50 strokes using a Dounce homogenizer. More than 90% cellular lysis was ensured by visualizing under a light microscope, and immediately after lysis, the mitochondrial membranes were stabilized by addition of 2.5x mitochondrial stabilization buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, 2.5 mM EDTA, pH 7.5) to a final concentration of 1x. The homogenate was centrifuged at 1300 x *g* for 15 min to isolate the nuclear fraction. The post-nuclear fraction was further centrifuged at 17,000 x *g* for 15 min in an ultracentrifuge (Beckman Optima XL-100K ultracentrifuge) to isolate the mitochondria. The post-mitochondrial supernatant was centrifuged at 100,000 x *g* for 1 h to obtain the membranous fraction as a pellet and the supernatant obtained was the cytosol. The homogeneity of the obtained fractions was determined by probing for fraction specific proteins by Western blotting.

3.2.2.8. Intracellular free Ca^{2+} assay

Cytosolic free Ca^{2+} was measured using the fluorescent Ca^{2+} indicator Fluo3-AM. THP-1 macrophages were harvested and resuspended in Kreb's buffer (118 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM glucose, 1.5 mM $CaCl_2.2H_2O$). Fluo3-AM was added at a final concentration of 0.5 μM alongwith 1 μM Pluronic acid F-127 to aid in dispersal of the dye. The cells were subjected to constant mixing by end-to-end rotation and incubated with the dye for 20 min at room temperature following which the cells were pelleted and resuspended in fresh Kreb's buffer and incubated for further 15 min to allow complete de-

esterification of the dye. Basal fluorescence was measured in a fluorimeter (BMG Fluostar Optima spectrofluorimeter) at an excitation of 480 nm and an emission of 520 nm. Appropriate treatments were initiated and kinetic fluorescence measurements were performed with the temperature being maintained at 37°C. At the end of each experiment, a calibration was performed to convert the fluorescence values into absolute calcium concentration using the following formula:

$$[Ca^{2+}]_i = K_d\,[(F-F_{min})/(F_{max}-F)]$$

where, K_d is the dissociation constant of Ca^{2+}-Fluo3-AM complex (325 nM), and F represents the fluorescence intensity of cells, F_{max} represents the maximum fluorescence (obtained by treating cells with 1 μM Ca^{2+} ionophore A234187 in the presence of 4 mM $CaCl_2$), and F_{min} corresponds to the minimum fluorescence (obtained by treating cells with 4 mM EGTA).

3.2.2.9. Measurement of intracellular pH

Intracellular pH measurement was performed using the long-wavelength fluorescent pH indicator carboxy SNARF-1 AM. THP-1 macrophages were resuspended in serum-free and phenol-red free RPMI-1640 medium (10^6 cells/mL) and incubated at room temperature for 15 min with SNARF-1 AM at a final concentration of 1 μM. The cells were washed once in fresh serum-free media and incubated for 20 min for complete de-esterification of intracellular acetoxymethyl esters. *In situ* calibration of SNARF-1 AM was performed to determine the pK_a of the dye at 37°C by using the ionophore Nigericin (10 μM), which maintains the intracellular pH the same as that of the controlled extracellular medium in a buffer containing high-K^+. Appropriate groups were subjected to different treatments and fluorescence measurements were commenced in a spectrofluorimeter (Perkin Elmer, Waltham, MA, USA) followed by kinetic analysis. The pH was calculated from the fluorescence measurements using the following formula:

$$pH = pK_a - \log\,[\{(R-R_B)/(R_A-R)\}\ X\ (F_{B(\lambda 2)}/F_{A(\lambda 2)})]$$

where pK_a of carboxy SNARF-1 AM is 7.5 at 37 °C. R is the ratio of fluorescence intensities (F) measured at two emission wavelengths, 580 nm (λ1) and 640 nm (λ2), with fixed excitation at 514 nm. The subscripts A and B represent the limiting values

at the acidic and basic endpoints of the titrations. Na^+ free and HCO_3^- free buffer were prepared as described by Khaled et al., (4).

3.2.2.10. Assay for intracellular Na^+ measurement

Intracellular Na^+ measurement was performed using the fluorescent Na^+ indicator Sodium Green™ tetracetate. THP-1 macrophages were resuspended in phenol-red free RPMI-1640 medium and incubated with Sodium Green™ at a final concentration of 1 μM for 20 min at room temperature. The cells were washed once with fresh serum-free media to remove excess probe following which kinetic fluorescent measurements were commenced in a spectrofluorimeter (BMG Fluostar Optima) at an excitation of 480 nm and emission of 520 nm. *In situ* calibration to determine the dissociation constant (K_d) of the dye at 37°C was accomplished by using the indicator dye in solutions of precisely known free Na^+ concentration in the presence of the pore forming antibiotic gramicidin (10 μM). Intracellular Na^+ was calculated using the following formula:

$$[Na^+]_{free} = K_d \{(F-F_{min})/(F_{max}-F)\}$$

where, K_d of the dye is 5.7 mM at 37°C, F is the fluorescence of the experimental sample, F_{min} is the fluorescence in the absence of Na^+ and F_{max} is the fluorescence under saturating concentrations of Na^+ in the presence of gramicidin (10 μM).

3.2.2.11. Detection of intracellular reactive oxygen species generation

The generation of reactive oxygen species in macrophages was detected by fluorimetry using the fluorescent dye CM-H_2DCFDA, which can detect hydrogen peroxide, hydroxyl radical, peroxyl radical, and peroxynitrite anion (5, 6). To perform the assay, THP-1 macrophages were washed and resuspended in serum and phenol-red free RPMI-1640 medium and incubated at room temperature for 30 min in the presence of CM-H_2DCFDA at a final concentration of 1 μM. Subsequently the cells were washed once with fresh media to remove the excess probe and fluorescence measurements were commenced on a spectrofluorimeter (BMG Fluostar Optima) at an excitation of 480 nm and an emission of 520 nm. Appropriate treatments were initiated and time-kinetic measurements were carried out and the values obtained

were represented as arbitrary fluorescence units and comparisons were made against the untreated control samples. Exogenous addition of hydrogen peroxide to cells was used as a positive control for the assay.

3.2.2.12. Measurement of intracellular nitric oxide (NO) generation

Nitric oxide (NO) generation within the macrophage was detected using the fluorescent NO-sensitive probe DAF-FM diacetate (7). THP-1 macrophages were harvested and resuspended in serum and phenol-red free RPMI-1640 medium and incubated at room temperature for 30 min in the presence of 1 μM DAF-FM diacetate dye. The cells were washed once with fresh medium to remove the excess probe and kinetic fluorescent measurements were performed on a spectrofluorimeter (BMG Fluostar Optima) at an excitation of 480 nm and emission of 520 nm. Time kinetic measurements were performed after appropriate treatment and the values were represented as arbitrary fluorescence units with the comparisons being made against the fluorescence of the control cells. SNAP (S-nitroso-N-acetylpenicillamine), a photoactivatable nitric oxide donor (8) was used as positive control in the assay.

3.2.2.13. Measurement of phagocytic ability of macrophages

The phagocytic ability of macrophages was determined by monitoring the uptake of Bioparticles® Alexa fluor 488 labeled dead *E.coli* (Molecular Probes, Eugene, OR). $2X10^5$ THP-1 macrophages were plated per well in a 24 well plate. Alexa fluor 488 labeled dead *E.coli* particles were opsonized with an opsonizing reagent obtained from Molecular Probes. These opsonizing reagents are derived from purified rabbit polyclonal IgG antibodies that are specific for *E.coli*. These opsonized bioparticles® were transferred to the macrophage culture at an multiplicity of infection (MOI) of 1:10, i.e., 10 bacteria per macrophage. The plates were briefly centrifuged at 250 x *g* to allow the bacteria to settle at the bottom of the plate and were then transferred to an incubator maintained at 37°C and 5% CO_2 in air for 1 h. The culture medium was aspirated to remove excess unbound bacteria and the cells were washed 3x with ice-cold PBS. To eliminate fluorescence from non-phagocytosed bacteria adhering to the macrophage membrane, 0.25 mg/mL Trypan blue was added and incubated for 10 min to quench the fluorescence of extracellular

bacteria. The cells were washed once with ice-cold PBS and the uptake of labeled bacteria was analyzed by flow-cytometry.

3.2.2.14. Cytokine ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed to detect cytokine secretion from human THP-1 macrophages upon activation with LPS. The ELISAs were performed according to manufacturer's protocol. Briefly, THP-1 macrophages were subjected to various treatments and after appropriate time interval the cell culture supernatants were harvested. The capture antibodies for the individual cytokines were diluted 1:250 in coating buffer (0.1 M Sodium carbonate, pH 9.5) and 100 µL was aliquoted into each well of a 96 well ELISA plate (BD biosciences). The plates were incubated at 4°C for 16 h following which three washes with 0.05% PBS-Tween-20 were given. Blocking was performed using 200 µL of assay diluent (PBS with 10% FCS) per well for 1 h at room temperature following which 100 µL of appropriately diluted standards and samples were added and incubated for 2 h at room temperature. A total of 5 washes with 0.05% PBS-Tween-20 were given and the plates were subsequently incubated with 100 µL of detection antibody and streptavidin-HRP for 1 h at room temperature following which 5 washes were given. 100 µL of tetramethylbenzidine (TMB) substrate was aliquoted into each well and incubated for 15 min at room temperature in dark following which 50 µL of stop solution (2N H_2SO_4) was added to terminate the reaction. The absorbance was read at 450 nm and the cytokine levels in the samples were derived based on the OD_{450} values obtained with standards of known concentration.

3.2.2.15. *Leishmania major* infection of human macrophages *in vitro*

Human THP-1 macrophages were plated at a density of $2X10^5$ cells per well in a 24 well plate and appropriate treatments were given. The stationary phase *L.major* promastigotes were opsonized with 1% human AB serum in PBS for 5 min at 37°C following which one wash was given with phenol-red free RPMI-1640 medium. The *L.major* promastigotes were added to the macrophage culture at a macrophage: parasite ratio of 1:10 or 1:50 and incubated for 6 h at 37°C following which the unbound parasites were removed by giving 3 washes with warm RPMI-1640 medium.

Fresh complete medium was added and the plates were transferred to 37°C for further incubation. The percentage infection was monitored at appropriate time intervals post-infection by staining the cells with Syto Green 11 nucleic acid dye and the parasite nuclei were visualized by fluorescence microscopy.

3.3. Animal experiments

3.3.1. Bilateral oophorectomy and sham surgery in mice

Bilateral oophorectomy, the surgical removal of both the ovaries, was performed in mice to simulate a condition of estrogen depletion. All procedures in mice were performed after obtaining approval from the Institutional Animal Ethics Committee (National Institute of Immunology, New Delhi). Female BALB/c mice were used in the study.

General anesthesia:

Hair from the skin overlying the left and right dorsal flanks were removed using electrically operated razor. The skin overlying the abdomen was sterilized by wiping with 70% ethanol. Ketamine (100 mg/kg) and xylocaine (2%) (20 mg/kg) were mixed and administered intraperitoneally. The mice were returned to the cage and the onset of anesthetic effect was monitored. The mice were considered to be in surgical anesthesia when there was loss of palpebral reflex, righting reflex, and toe pinch reflex. Respiratory rate and heart rate were monitored continuously.

Bilateral oophorectomy:

The anesthetized mice were operated under strict aseptic conditions inside a laminar flow hood. The mouse was placed over layers of sterile tissue paper and the skin overlying the dorsal flanks was sterilized by wiping with 70% ethanol. The flank was palpated gently to identify the kidney, and an incision (~ 5 mm) was made using a pair of scissors on the overlying skin which penetrated the skin, sub-cutaneous tissue and the muscle layer with the parietal peritoneum being exposed and intact. The para-ovarian pad of fat was identified through the intact peritoneum and a small incision was made on the peritoneum overlying it. The ovarian tissue along with the fallopian tube was mobilized and delivered through the incision site. The ovary was

released from the para-ovarian pad of fat as well as from the peritoneal reflections while care was taken to avoid injury to the ovarian vessels. A ligature was tied around the distal end of the fallopian tube including the ovarian vessels following which the ovary was excised. Hemostasis was secured before the stump of the tube was pushed back into the peritoneal cavity. The peritoneum was closed by continuous sutures using 2-0 silk. The same protocol was followed to perform oophorectomy on the contralateral side. The muscular layer and skin were closed together using surgical clips.

Sham surgery:

Sham surgery was performed on mice as described above except that the ovary and tubes after being delivered from the incision site were pushed back into the peritoneum in an intact state.

Post-operative care:

The mice were returned to a cage and were kept under a 100W bulb light source to prevent hypothermia. Care was taken to ensure that the eyes are kept covered. The respiratory rate and heart rate were monitored till the mice regained complete consciousness. They were fed ab-limitum post-operatively. Metronidazole (20 mg/kg) was added to the drinking water and the mice were fed this medicated water for 5 days post-operatively. On the 7^{th} post-operative day, the health of the wound was observed and the surgical clips were removed from the skin.

3.3.2. *L.major* infection in mouse footpad

$5x10^5$ *L.major* promastigotes were cultured in 5 mL modified DMEM supplemented with 10% FCS. At the end of 5 days of culture, the stationary phase promastigotes were harvested and resuspended in Hanks balanced salt solution at a cell density of $4x10^7$/mL. The cell suspension was aspirated into a 1 mL syringe and 50 µL was injected into the footpad of mice. The mice were returned to the cage and fed ab-limitum. The onset and progression of cutaneous lesion was monitored at 2 weekly intervals by observing an increase in the thickness of the footpad.

3.3.3. Histopathological examination of cutaneous leishmaniasis lesion

The cutaneous lesion developed in the *L.major* infected footpad and tissue from the corresponding region of normal footpad was harvested for histopathological examination. The tissue was fixed in 4% formaldehyde for 24 h following which the tissue was dehydrated by incubating it with ascending concentrations of alcohol (50%, 70%, and 100% ethanol for 1 h each). Subsequently, tissue clearing was performed by incubating with xylene for 1 h following which paraffin embedding was performed. The embedded tissue was cut into multiple sections of 5 μm thickness using a microtome. The paraffin sections were then coated on slides and deparaffinization was carried out by treating with xylene. Subsequently, hematoxylin-eosin staining was performed and the slides were visualized under a light microscope.

3.4. Molecular biology techniques

3.2.1. Total RNA isolation

Total RNA was isolated from cells using TRIzol reagent following the manufacturer's protocol. Briefly, $2x10^6$ cells were harvested by non-enzymatic cell dissociation buffer and washed once with PBS. The cell pellet was lysed with 1 mL ice-cold TRIzol reagent. The lysate was centrifuged at 12,000 x *g* for 10 min at 4°C to pellet down cellular debris, polysaccharides, and high molecular weight DNA. The supernatant was gently decanted into a fresh microcentrifuge tube and 200 μL of chloroform /mL of TRIzol was added and the tube was shaken vigorously for 15 s. The mixture was incubated at room temperature for 2-3 min before centrifugation at 12,000 x *g* for 15 min at 4°C. This resulted in the separation of the mixture into a lower organic phase and an upper aqueous phase. The aqueous phase containing the RNA was gently aspirated and transferred into a fresh microcentrifuge tube and 500 μL of isopropanol /mL of TRIzol reagent was added to precipitate the RNA. The mixture was centrifuged at 12,000 x *g* for 10 min at 4°C to isolate the RNA as a pellet. The supernatant was discarded and the pellet was washed once with 70% ethanol, centrifuged and the pellet was air-dried and re-dissolved in appropriate quantity of nuclease-free water. The purity ($A_{260}/A_{280} > 1.8$) and concentration (A_{260}

X dilution factor X 40) of the obtained RNA was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}).

3.4.2. First strand synthesis by reverse transcription

First strand synthesis of mRNA into cDNA was performed using First strand cDNA synthesis kit from Invitrogen following manufacturer's protocol. Briefly, 4 μg of total RNA was denatured at 65°C for 5 min in the presence of Oligo dT_{16} and dNTPs and incubated at 42°C for another 2 min with DTT, $MgCl_2$, and RNaseOUT in 10 X reverse transcription buffer. 1 μL/reaction of the Superscript Reverse Transcriptase enzyme was added to the denatured RNA and incubated at 42°C for 50 min. The enzyme was denatured by heating at 70°C for 15 min. The reaction was completed by a quick high-speed centrifugation and the complementary RNA strand degraded by incubating with RNaseH for 20 min at 37°C. The preparation was stored at -70°C.

3.4.3. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify specific nucleotide sequences from cDNA derived from human macrophages. The reaction consisted of

Gene	Forward primer	Reverse primer
ER-α	5′-GTGGGAATGATGAAAGGTGG-3′	5′-TCCAGAGACTTCAGGGTGCT-3′
ER-β	5′-TGAAAAGGAAGGTTAGTGGGAACC-3′	5′-TGGTCAGGGACATCATCATGG-3′
Bcl-2	5′-GTGGAGGAGCTCTTCAGGGA-3′	5′-AGGCACCCAGGGTGATGCCA-3′
Mcl-1	5′-CGGCAGTCGCTGGAGATTAT-3′	5′-GTGGTGGTGGTTGGTTA-3′
Bfl-1	5′-AGCTCAAGACTTTGCTCTCCACC-3′	5′-TGGAGTGTCCTTTCTGGTCAACAG-3′
iNOS	5′-GGCCTCGCTCTGGAAAGA-3′	5′-TCCATGCAGACAACCTT-3′
Actin	5′-GTGGGGCGCCCCAGGCACCA-3′	5′-CTCCTTAATGTCACGCACGATTTC-3′

Figure 3.1. The table shows the forward and reverse primers designed against specific genes used for amplifying products using PCR.

an initial denaturation at 94°C for 4 min, followed by 20-30 cycles of denaturation at 94°C for 30 s, annealing at primer specific temperature for 30 s, and extension at 68-

72°C for 45 s – 1 min. A final extension at 68-72°C for 10 min was performed. Relative expression of specific genes in cells subjected to different treatments was determined by semi-quantitative PCR. The optimal number of cycles required for achieving a linear amplification of serially diluted template was determined, which was then used with other samples to quantify the expression of specific genes. The PCR products were resolved on 1-2% agarose gel containing ethidium bromide and visualized under ultraviolet illumination. The specific primers used are shown in Figure 3.1.

3.4.4. Agarose gel electrophoresis

DNA fragments were resolved on 1-2% agarose gel containing 0.5 μg/mL ethidium bromide in Tris-Acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.1). The samples were mixed with 6X loading dye containing bromophenol blue, and the samples were resolved by applying a voltage of ~5-7 V/cm. The resolved DNA fragments were visualized under ultraviolet illumination and the relative band size was determined by comparison against a DNA ladder with bands of known sizes. When required, images were acquired using a UVP Gel Documentation system.

3.4.5. Elution of DNA from agarose gel

To elute DNA from agarose gel, the samples were loaded on a gel (1-1.8%) cast with low melting point agarose (LMP agarose). The samples were resolved and visualized under UV transilluminator, and the band of interest was excised quickly using a scalpel blade. The volume of gel slice was quantitated by weighing and the DNA eluted using MinElute Gel Extraction kit (Qiagen) as per manufacturer's protocol. Briefly, the gel was solubilized by incubating it with buffer QG at 50°C for 10 min. The solubilized gel was loaded onto binding columns and centrifuged at 12,000 x *g* for 1 min. The flow-through was discarded and the column was washed once with buffer PE containing ethanol. The DNA bound to the column was eluted using the elution buffer provided with the kit, or alternatively with nuclease-free water. The concentration of the obtained DNA was estimated by measuring the absorbance at 260 nm (A_{260}) and using the following formula: DNA concentration = A_{260} X 50 X dilution factor.

3.4.6. Sub-cloning of PCR products into pGEM-T_{Easy} vector

The DNA fragments eluted from the agarose gel were cloned into pGEM-T_{Easy} vector which allows efficient sequencing using the sequencing primers for T7 and SP6 promoters. 3 μL of eluted DNA (1 μg/μL) was ligated with 1 μL of pGEM-T_{Easy} vector in the presence of 1 μL of T4 DNA ligase in a 10 μL reaction volume. The reaction was allowed to proceed at 4°C for 16 h following which 8 μL of the ligation mix was used to transform DH5-α strain of *E.coli* following standard protocols (9). The transformation mix was spread onto LB-agar plates containing appropriate ampicillin (100 μg/mL) and the blue-white selection reagent (40 μL/plate) (Sigma chemical company). The plate was incubated at 37°C for 12 h following which the white colonies were picked up for screening for presence of the gene of interest.

3.4.7. DNA sequencing

DNA was sequenced by the di-deoxy method (10) at the DNA sequencing facility of Department of Biochemistry, University of Delhi, South Campus, New Delhi, India.

3.5. Densitometry and statistics

Densitometric measurements for quantitation of signals on immunoblots or ethidium bromide stained agarose gels were performed using a UVP Gel Documentation instrument, and the acquired data was analyzed on LabWorks image analysis and acquisition software (UVP, v.4.0.0.8). Data from at least 3 experiments were quantitated to arrive at the average value of the signal. All measurements were normalized to internal loading controls. To determine statistical significance, the data was analyzed by Student's T test and the values were expressed as mean±SEM. The values were considered to be significantly different at $p<0.05$.

3.6 Instrumentation

Optiphot fluorescence microscope, E600W fluorescence microscope and T2000E Confocal microscope C1 were from Nikon (Tokyo, Japan).

Multitemp III water bath and EPS 500/400 power supply were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Gyratory water bath shaker was purchased from New Brunswick Scientific Co., Inc (Edison, NJ).

Centrivac and Biofuge table top centrifuge were from Heraeus (Allerod, Denmark).

μ- Quant microplate reader was from Bio-tek Instruments Inc. (Winooski, VT).

Protean II polyacrylamide gel system and Mini Trans blot system were from Bio-Rad Laboratories (Hercules, CA).

Submarine DNA electrophoresis system was procured fro Bangalore Genei (Bangalore, India).

Laminar flow hoods were purchased from Kartos Ltd. (New Delhi, India).

Eppendorf 5810R centrifuge was purchased from Eppendorf (Hamburg, Germany).

LS50B flourimeter was from Perkin Elmer Biosystems (Norwalk, CT). Fluostar Optima fluorimeter was from BMG labtech (Offenburg, Germany)

BD-LSR flow-cytometer was from Bectinson Dickinson Biosciences (San Jose, CA).

Peltier Thermal Cycler- 200 was purchased from MJ research (Waltham, MA).

Doc-It Gel Documentation system was procured from UVP Bio Imaging System Incorporation (Upland, CA).

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Chapter 4

Estrogen receptor expression in human macrophages

4.1. Introduction

Macrophages respond to estrogen present in their microenvironment and hence should express functional estrogen receptors unless estrogen acts through a receptor-independent pathway (1). Also, it is known that human macrophages express mRNA transcript for estrogen receptor – α and estrogen receptor – β (2), but the expression of respective proteins have not been demonstrated. Although in many other cell types the sub-cellular localization of the ER's is known, their sub-cellular distribution in macrophages is not known. The receptor sub-types expressed and their sub-cellular localization is important to know as it is well known that the outcome of estrogen signaling depends upon the relative levels of ER-α and ER-β as well as their sub-cellular localization (3). This is evident from the distinct phenotypes obtained with ER-α and ER-β knockout mice (4), and in addition, evidence for differential receptor activity come from studies showing overlapping but exclusive sets of downstream target genes for the two sub-types (5). As information on macrophage estrogen receptors was scanty and fragmented, in the first part of our study the expression of ER-α and ER-β at the transcript and protein level as well as their sub-cellular localization was explored.

4.2. THP-1 macrophages express ER-α and ER-β mRNA

The expression of ER-α and ER-β mRNA transcript in macrophages derived from THP-1 cells was determined by reverse transcription polymerase chain reaction (RT-PCR) using specific primers designed against ER-α and ER-β. As a positive control, RNA isolated from MCF-7 cells, a human breast cancer cell line known to express both ER-α and ER-β was used for amplification of the receptors. Figure 4.1 (A and B) shows a clear amplification of both ER-α and ER-β in both THP-1 and MCF-7 cells. The identity of ER-α and ER-β amplification products obtained was further confirmed by DNA sequencing. Therefore, both ER-α and ER-β are expressed in macrophages derived from THP-1 acute monocytic leukemia cells. Macrophages derived from this cell line have been used in many other studies as a model of human macrophages (6, 7).

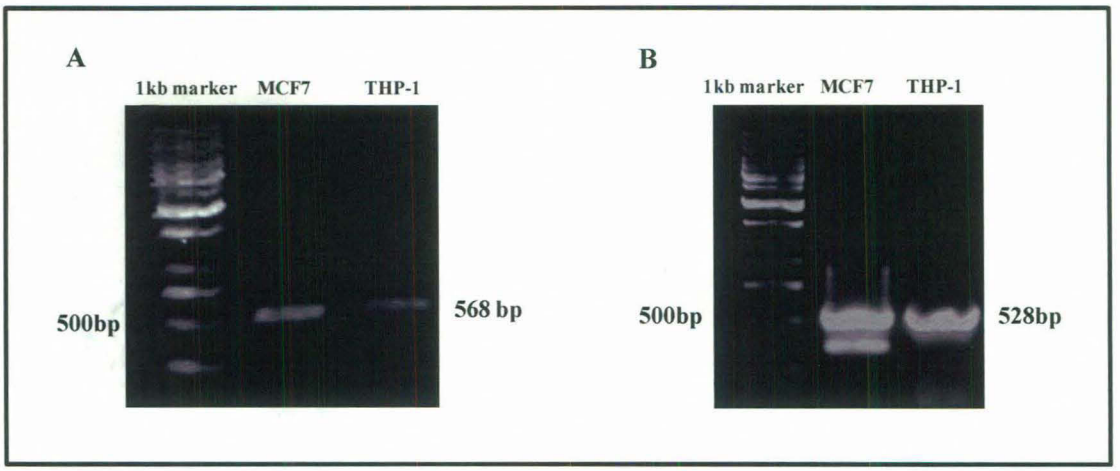

Figure 4.1. Human THP-1 macrophages express transcripts for both ER-α and ER-β. RT-PCR analysis of ER-α (A) or ER-β (B) expression using primers designed against regions specific to ER-α or ER-β. MCF-7 was used as a positive control for ER-α/β expression.

4.3. Sub-cellular localization of estrogen receptors in THP-1 macrophage

To analyze the sub-cellular distribution of estrogen receptors, the sub-cellular fractions of THP-1 macrophages were probed for ER-α and ER-β using an antibody which recognizes ER-α and ER-β as distinct bands of 66 kDa and 54 kDa respectively.

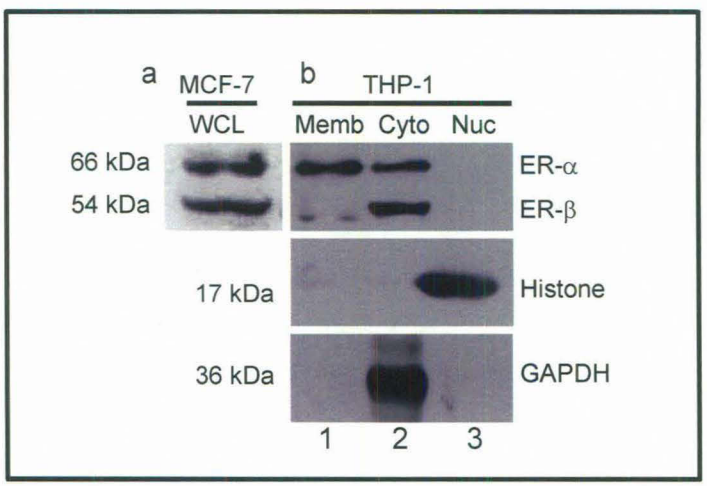

Figure 4.2: Sub-cellular localization of estrogen receptors in THP-1 macrophages. Western blot was performed on membrane, cytoplasmic, and nuclear fractions of THP-1 macrophages using an antibody that recognizes both ER-α and ER-β. Note the absence of ER-β on the plasma membrane and also the total absence of estrogen receptors in the nuclear fraction. MCF-7 whole cell lysate was used as positive control for ER-α and ER-β expression. Western blot for histone and GAPDH served as controls to determine the homogeneity of the obtained nuclear and cytosolic fractions respectively. Memb, Membrane; Cyto, Cytoplasm; Nuc, Nucleus; WCL, Whole cell lysate.

As shown in Figure 4.2, the expression of ER-α was found on both the plasma membrane and the cytosol (Figure 4.2, lanes 1 and 2), while ER-β was expressed exclusively in the cytoplasm (Figure 4.2, lane 2). Interestingly, no nuclear localization of these receptors could be detected (Figure 4.2, lane 3). To further confirm the sub-cellular localization, immunocytochemistry was performed on live and fixed THP-1 macrophages using the same antibody.

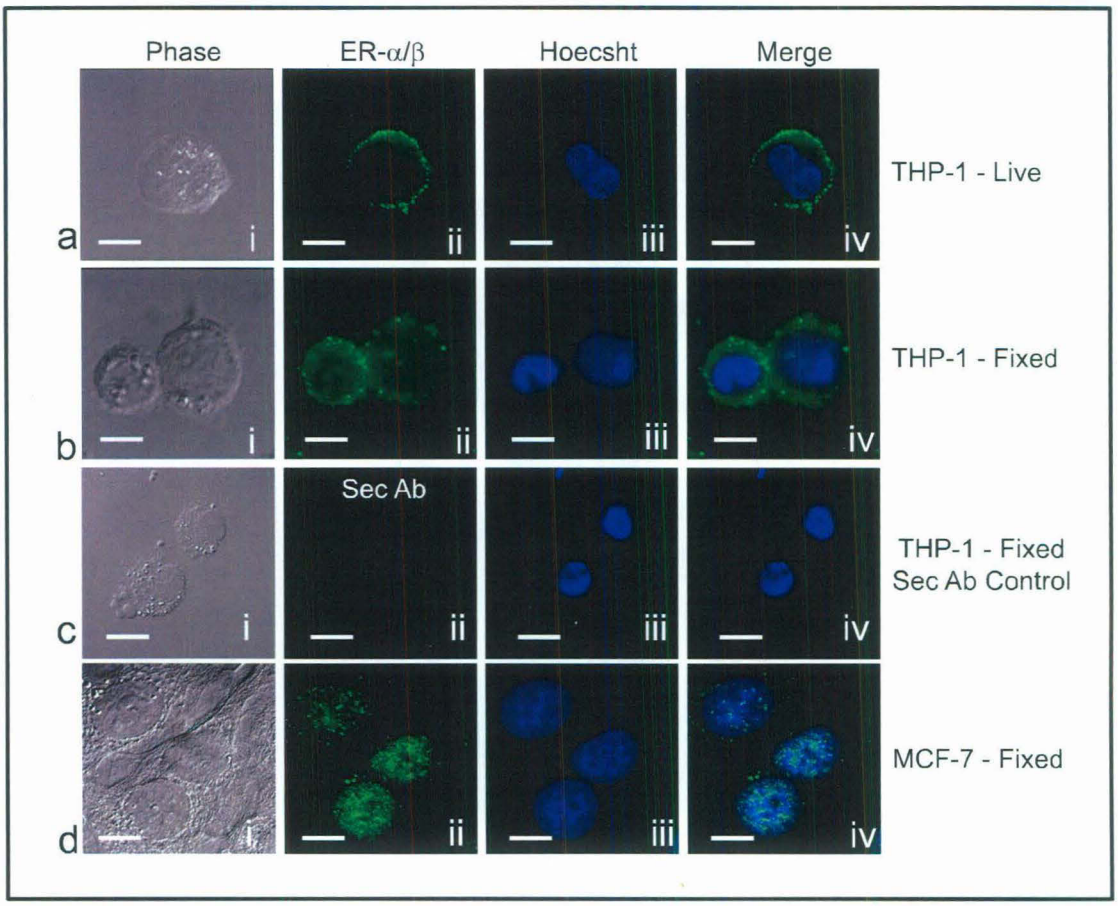

Figure 4.3. Immunocytochemistry for estrogen receptor expression. Immunocytochemistry and fluorescence microscopy was performed on live (a) and fixed (b) THP-1 macrophages using an antibody that recognized both ER-α and ER-β. Note the punctate staining of estrogen receptor on the plasma membrane (a, iv) and the cytoplasm (b, iv). Panel (c) represents the cells that were incubated with the secondary antibody alone to serve as a control to eliminate non-specific binding. Panel (d) represents immunocytochemistry performed on fixed MCF-7 cells showing distinct nuclear localization of estrogen receptors (d, iv). The staining in green (ii) represents staining for estrogen receptor, the staining in blue (iii) represents staining with the nuclear dye Hoechst 33342, (iv) represents the merged image of (ii) and (iii), while (i) represents the respective phase contrast image. Bar represents 10 μm.

Live cell staining showed distinct immunostaining on the surface (Figure 4.3, a, iv) confirming the presence of membrane localized estrogen receptors. Immunocytochemistry on fixed and permeabilized cells revealed the presence of receptors on the plasma membrane as well as the cytosol with very minimal localization noted in the nucleus (Figure 4.3, b, iv). This was in agreement with the data obtained by Western blotting shown in Figure 4.2. Also, the demonstration of nuclear localized estrogen receptors in MCF-7 cells (Figure 4.3, d, iv) using the same antibody excluded the possibility that the antibody used in the study was unable to detect the nuclear estrogen receptors.

Furthermore, to confirm that the detection of membranous receptor was not an artifact, E2-BSA-FITC (estrogen conjugated to bovine serum albumin-fluorescein isothiocyanate complex) was used which is a fluorescent membrane impermeable form of estrogen. Figure 4.4 demonstrates the binding of E2-BSA-FITC to the plasma membrane of THP-1 macrophages as shown by the clear shift in fluorescent intensity (Figure 4.4, red line) as compared to cells labeled with BSA-FITC (Figure 4.4, black line). The specificity of this binding was further confirmed by competition experiments with unlabelled E2 (Figure 4.4, blue and pink lines) where the bulk of the staining could be displaced by E2.

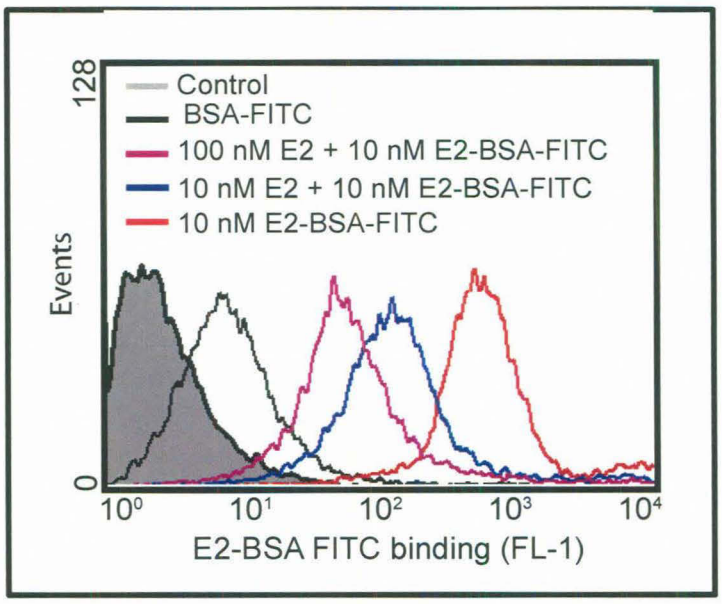

Figure 4.4: Surface binding of E2-BSA-FITC. The histogram represents flow-cytometric analysis performed on THP-1 cells treated with E2-BSA-FITC for 10 min or co-incubated with different concentrations of unlabelled E2. Note the distinct shift in fluorescent intensity in cells treated with E2-BSA-FITC as compared to BSA-FITC and the reduction in binding on competition with unlabelled E2.

4.4. Human peripheral blood monocyte derived macrophages express estrogen receptors-α and β.

As shown above, human THP-1 macrophages express ER-α and ER-β. Though THP-1 macrophages serve as a model cell line to study human macrophages (6, 7), it was imperative to demonstrate the presence of estrogen receptors in primary cells to establish parity and to exclude any artifact of the leukemic cell line. The presence of transcripts for ER-α and ER-β in human peripheral blood monocyte derived macrophages (hPBMDM) was established by reverse transcription-polymerase chain reaction using primers specific for ER-α and ER-β (Figure 4.5, A and B). The expression of ER-α and ER-β proteins in hPBMDM was demonstrated by Western blotting using an antibody that recognizes both ER-α and ER-β (Figure 4.5, C).

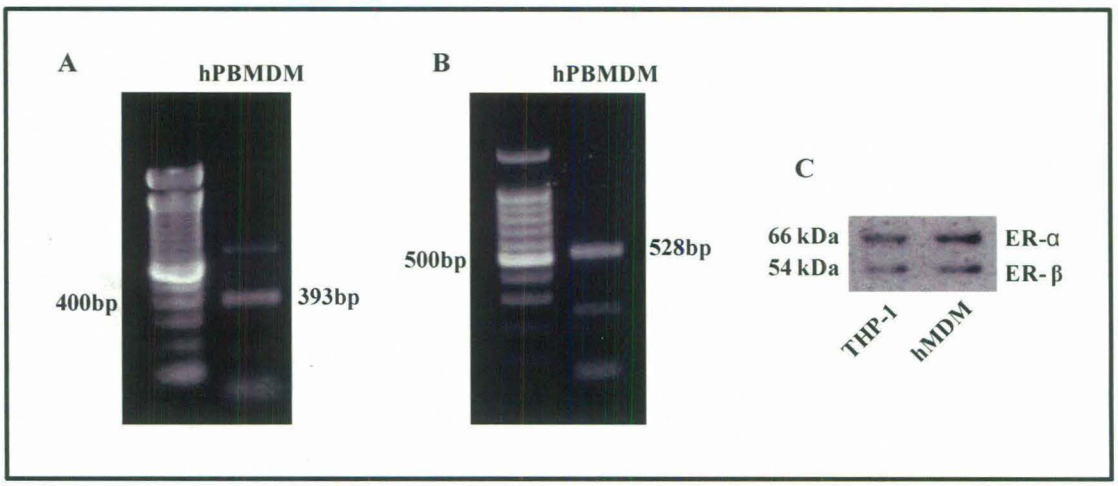

Figure 4.5. Human peripheral blood monocyte derived macrophages express both ER-α and ER-β. The transcript for ER-α (A) and ER-β (B) was amplified from RNA isolated from hPBMDM using specific primers by RT-PCR. (C) Western blot analysis for expression of ER-α and ER-β in hPBMDM using an antibody which recognizes ER-α and ER-β as 66 kDa and 54 kDa bands respectively.

Furthermore, to characterize the sub-cellular localization of these receptors in hPBMDM, immunocytochemistry and fluorescence microscopy was performed on live and fixed cells using an antibody which recognizes both ER-α and ER-β. As shown in Figure 4.6, the estrogen receptor is localized on the plasma membrane and the cytosol with very little localization to the nucleus similar to that observed in human THP-1 macrophage cell line.

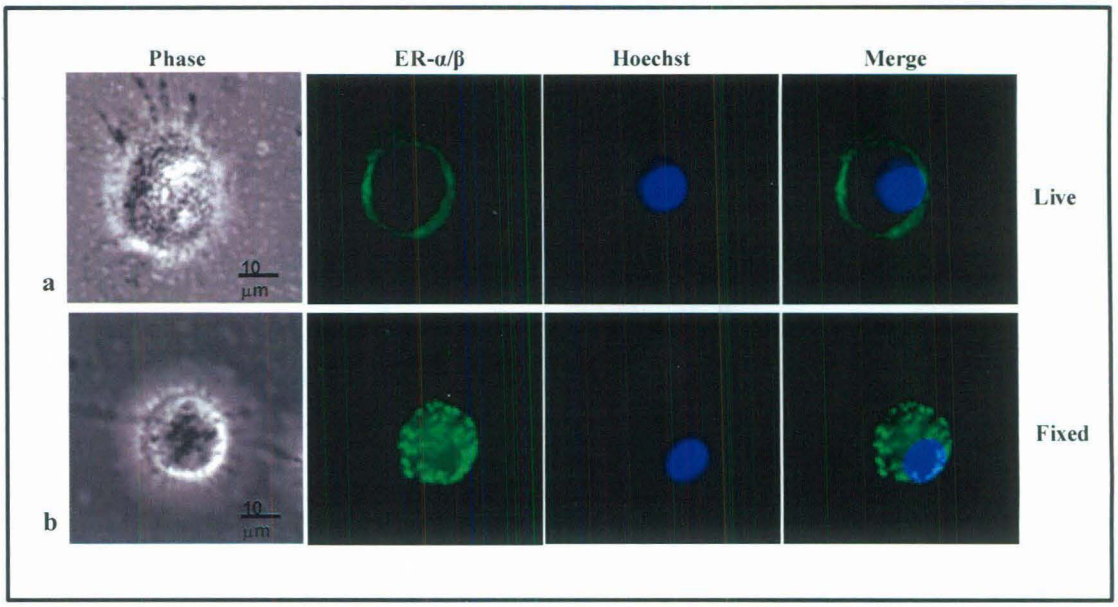

Figure 4.6. Microscopic analysis of sub-cellular localization of estrogen receptors in hPBMDM. Immunocytochemistry and fluorescence microscopy for ER-α and ER-β was performed on live (a) or fixed (b) hPBMDM. The green staining represents ER-α/β staining; the blue stain represents Hoechst 33342 nuclear dye. The bar represents 10 μm.

4.5 Summary

In this chapter, we have demonstrated that macrophages derived from human THP-1 acute monocytic leukemia cell line as well as from human peripheral blood monocytes express both ER-α and ER-β mRNA transcript and protein. The expression of ER-α and ER-β mRNA transcripts have been demonstrated by RT-PCR using ER-α and ER-β specific primers and further confirmed by DNA sequencing of the obtained amplicon. Furthermore, we have demonstrated that ER-α sub-type is distributed on the plasma membrane and cytoplasm while the ER-β receptor sub-type is distributed exclusively in the cytoplasm. Interestingly, the nuclear compartment was found to be devoid of estrogen receptors in the absence of its cognate ligand estrogen. However, in the presence of estrogen, a proportion of estrogen receptor was found to be localized within the nucleus. Also, the expression of membrane bound estrogen receptor was confirmed by multiple techniques including demonstration of binding of membrane impermeable estrogen on the cell surface, immunocytochemistry on live cells and Western blotting on sub-cellular fractions using a specific antibody against

estrogen receptor. These data taken together has revealed the presence of ER-α and ER-β in distinct sub-cellular compartments of human macrophages.

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Chapter 5

Study of the role of estrogen in human macrophage survival

5.1. Introduction

The literature on the effect of estrogen on macrophage survival is ambiguous with various studies demonstrating that estrogen induces apoptosis on cells of monocytic lineage (1, 2), whereas others suggest a protective role (3). The discrepancies observed among these various studies may be attributed to the fact that they have been performed on various cell types and species such as murine alveolar macrophages, murine peritoneal macrophages, human acute promonocytic leukemia cell line, human microglial cells, human osteoclasts and osteoclastic cell line etc., and the results have been extrapolated to human macrophages. However, it is well known that the effect of estrogen is highly species and cell-type specific and is dependent on the relative expression of ER-α *versus* ER-β, the sub-cellular localization of these receptors, as well as the availability of various co-factors and co-repressors. Moreover, the pleiotropic effect could also be due to the differential regulation of various pro- and anti-apoptotic proteins in these various cell types. Since, macrophages are critical to the regulation of a number of physiological and pathological processes, a systematic study of the effect of estrogen on human macrophage survival is imperative. In this study, we have utilized the human macrophage cell line THP-1 as a model system and at every step the results have been verified on primary cells, the human peripheral blood monocyte derived macrophages.

5.2. Effect of estrogen on human macrophage survival

In an effort to study the effect of estrogen on human macrophage survival, both THP-1 macrophage cell line and human peripheral blood monocyte derived macrophages (hPBMDM) were exposed to different concentrations of estrogen (E2) for 24 h. The concentrations of E2 used ranged from 1 nM to 1 μM, with 1 nM and 10 nM being within the physiological range (4), whereas 1 μM was supraphysiological. The viability of these E2 treated macrophages from five independent experiments is shown in Figure 5.1. The data reveals that estrogen does not affect the viability of either human THP-1 macrophages (Figure 5.1, black bars) or the hPBMDMs (Figure 5.1, grey bars) at the tested concentrations. Since, 10 nM E2 was the physiological concentration, further time kinetic analysis of viability was performed at this

concentration. The result shown in Figure 5.1, B, demonstrates that E2 treatment for upto 72 h does not affect the viability of these macrophages.

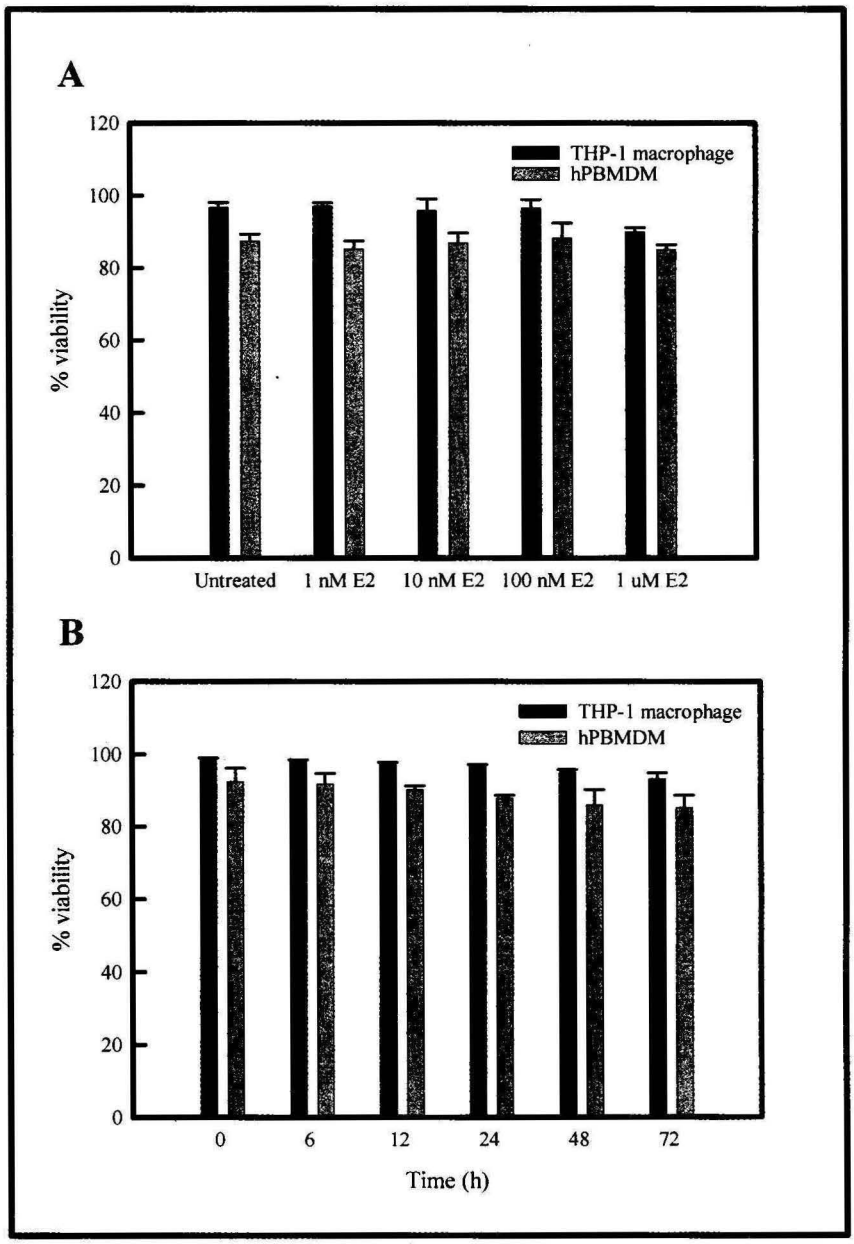


Figure 5.1. Effect of estrogen on human macrophage viability. (A) Appropriate groups of THP-1 macrophages (black bars) or hPBMDM (grey bars) were treated with indicated concentrations of E2 and viability was analyzed on flow-cytometer by propidium iodide dye exclusion method. The bars represent mean of 5 independent experiments and error bars represent ±SEM. (B) Time kinetic analysis of viability of THP-1 macrophages (black bars) and hPBMDM (grey bars) treated with 10 nM E2 for upto 72 h. Propidium iodide dye exclusion assay was performed to analyze viability. The bars represent mean of 3 independent experiments and the error bars represent ±SEM.

5.3. Effect of estrogen on viability of human macrophages exposed to stress stimuli

As shown above, estrogen does not affect the viability of human macrophages. However, we tested the possibility of estrogen affording some protection in the event of exposure to a stress stimulus. We have employed lipopolysaccharide (LPS) derived from *Salmonella typhosa* as a stress stimuli as it is one of the most common activation stimulus for macrophages *in-vivo* and is also known to mediate activation-induced cell death in the process (5). When human THP-1 macrophages were treated with LPS (4 μg/mL), a significant decrease in cell death was observed in those groups pre-incubated with E2 as compared to cells that were not exposed to E2 (Figure 5.2). This suggested that E2 affords protection against LPS-induced macrophage death.

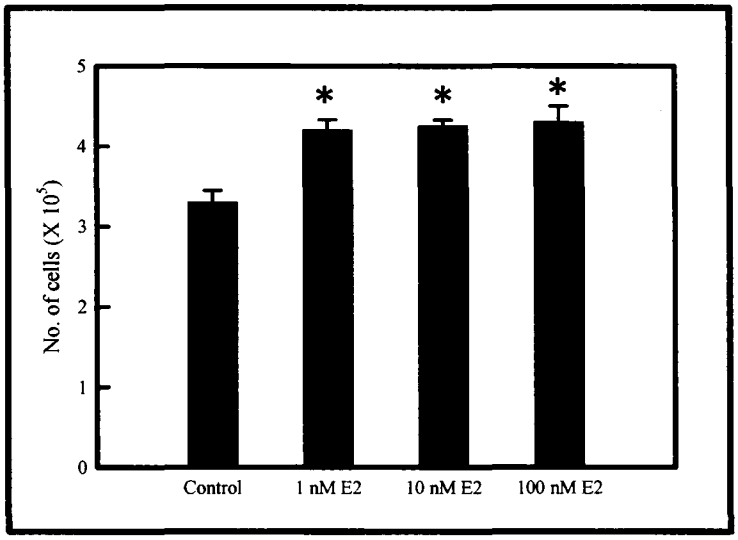


Figure 5.2. Estrogen protects human macrophages against LPS-induced cell death. THP-1 macrophages were pre-incubated with indicated concentrations of E2 for 12 h prior to addition of 4 μg/mL LPS and further incubated for 12 h following which viable cell count was made by trypan blue dye exclusion method. $5X10^5$ cells were plated in each group at the beginning of the experiment. The bars represent the mean number of cell recovered in the indicated groups. The error bars represent ±SEM. *, $p<0.05$ as compared to control. The data is representative of 3 independent experiments.

5.4. Estrogen modulates the expression of Bcl-2 family of proteins in human macrophages

The data shown above demonstrating a protective effect of E2 against cell death prompted the hypothesis that E2 may mediate this effect by modulating pro- and anti-apoptotic members of the Bcl-2 family of proteins. THP-1 macrophages upon

exposure to E2 demonstrated a dose-dependent increase in the expression of the anti-apoptotic protein Bcl-2 (Figure 5.3, A). However, other members of the Bcl-2 family such as the anti-apoptotic Mcl-1 and Bfl-1 and pro-apoptotic Bax and Bad levels remained unchanged (Figure 5.3, A, B).

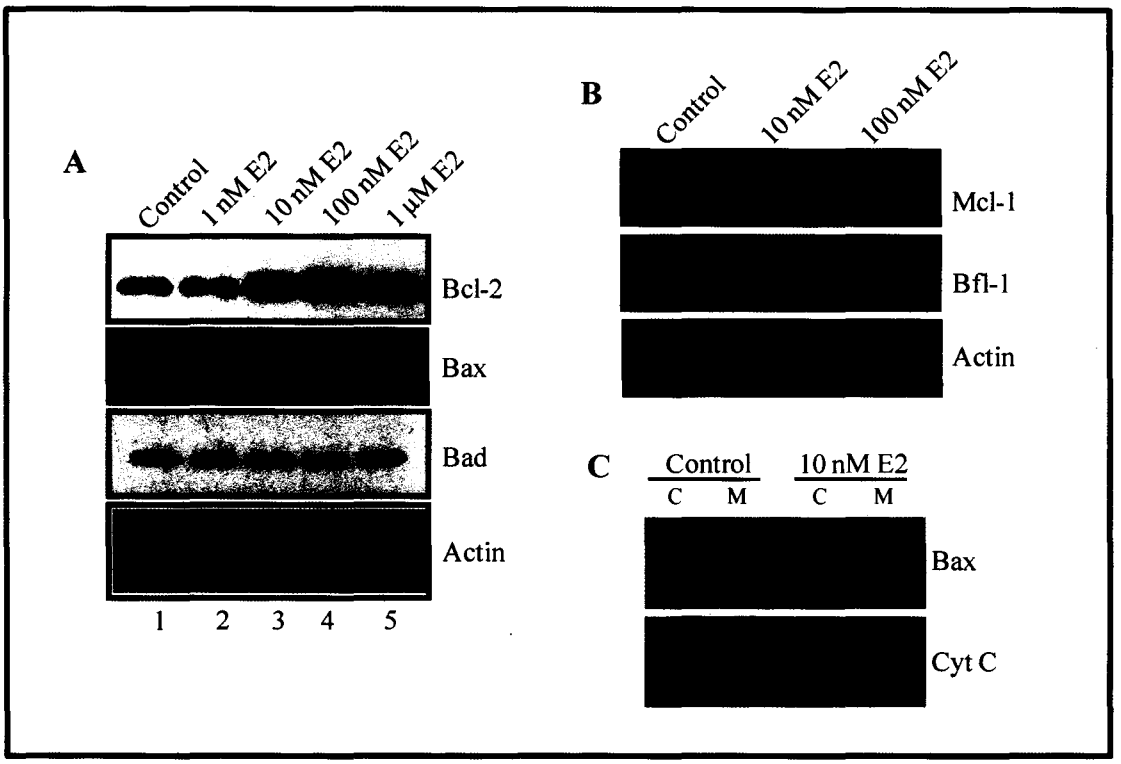


Figure 5.3. Estrogen modulates Bcl-2 family of proteins in THP-1 macrophages. (A) Western blot analysis for expression of Bcl-2, Bax, and Bad in THP-1 macrophages treated with indicated concentrations of E2 for 24 h. Note the dose-dependent increase in Bcl-2 expression. Western blot for actin was used as endogenous loading control. (B) Semi-quantitative RT-PCR analysis of expression of Mcl-1 and Bfl-1 transcripts using specific primers in THP-1 macrophages exposed to indicated concentrations of E2 for 24 h. PCR for actin was used as loading control. (C) Western blot analysis of localization of Bax in mitochondrial and cytosolic fractions of THP-1 macrophages obtained by sub-cellular fractionation of cells treated with 10 nM E2 for 4 h. Western blot for cytochrome c was performed to determine the homogeneity of the obtained fractions.

It is known that the mechanism of pro-apoptotic action of Bax involves its translocation to the mitochondria upon being activated. In this context, it is interesting to note that though the levels of total Bax remained essentially unchanged, an alteration in its sub-cellular localization was observed in the form of translocation from the cytosol to the mitochondria (Figure 5.3, C) suggesting elicitation of a pro-apoptotic signal by estrogen.

5.5. The role of estrogen receptor in estrogen-induced modulation of Bax and Bcl-2

To determine if the modulation of Bcl-2 and Bax observed upon E2 treatment was estrogen receptor mediated, THP-1 macrophages were treated with ICI 182,780, a pure estrogen receptor antagonist prior to addition of E2. ICI 182,780 acts by inhibiting the binding of estrogen to estrogen receptor and also by inducing degradation of the estrogen receptor through interference with the nucleo-cytoplasmic shuttling of these receptors (6). THP-1 macrophages exposed to E2 showed about 1.5 fold up-regulation of Bcl-2 (Figure 5.4, lane2) as compared to control which was abrogated upon pre-incubation of these cells with ICI 182,780 (Figure 5.4, lane 3) indicating that E2-induced Bcl-2 up-regulation is dependent on signaling *via* the estrogen receptor.

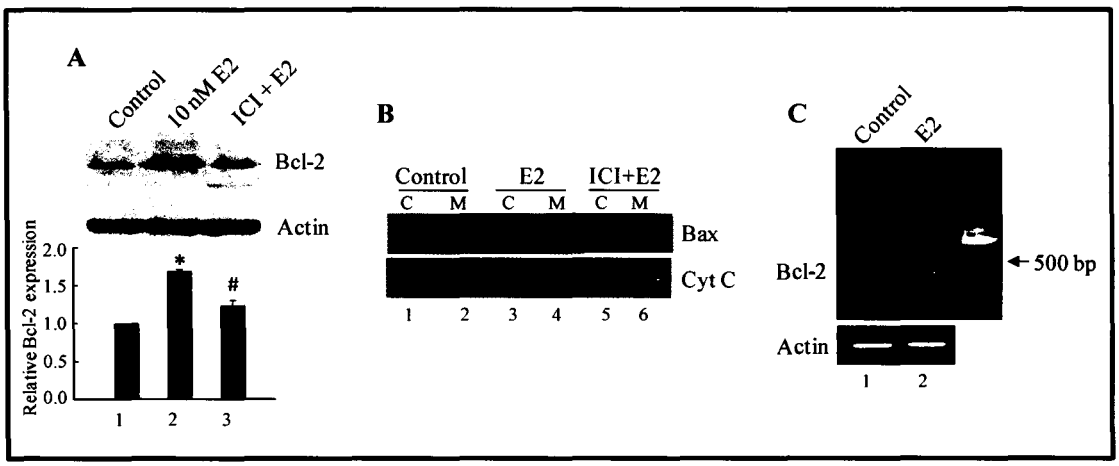


Figure 5.4. Bcl-2 and Bax are modulated via signaling through the estrogen receptor. (A) THP-1 macrophages were treated with 10 nM E2 for 2 h or were incubated with 1 μM ICI 182,780 for 1 h prior to addition of E2. Whole cell lysates were analyzed by Western blot for expression of Bcl-2. The bar graph represents relative Bcl-2 expression as compared to control and normalized to actin derived by densitometric analysis of multiple immunoblots (n=3). *, $p<0.05$ as compared to control; #, $p<0.05$ as compared to E2 treated. (B) Western blot for analysis of Bax localization performed on cytosolic and mitochondrial fractions of THP-1 macrophages treated with 10 nM E2 for 4 h or exposed to 1 μM ICI 182,780 for 1 h prior to E2 addition. Immunoblot for Bcl-2 was performed to determine the homogeneity of the obtained fractions. C, Cytosol; M, Mitochondria. (C) Semi-quantitative RT-PCR analysis for expression of Bcl-2 mRNA transcript in THP-1 macrophages treated with or without E2. PCR for Actin was used as endogenous loading control.

Moreover, the translocation of Bax from cytosol to the mitochondria observed in THP-1 macrophages treated with E2 (Figure 5.4, B, lanes 3 and 4) was completely

abrogated upon pre-incubation with the estrogen receptor antagonist ICI 182,780 (Figure 5.4, B, lanes 5 and 6) suggesting that estrogen-induced Bax translocation is an estrogen receptor dependent phenomenon. Further, to investigate whether E2-induced Bcl-2 up-regulation is mediated *via* a transcriptional mechanism or by post-transcriptional or post-translational mechanism, semi-quantitative RT-PCR was performed on RNA isolated from THP-1 macrophages treated with or without E2 using primers specific to Bcl-2. There was an actual increase in mRNA transcript of Bcl-2 (Figure 5.4, C, lane 2) in cells treated with E2 as compared to the control indicating a transcriptional mechanism for regulation of Bcl-2 by estrogen.

5.6. Role of ER-α and ER-β in modulation of Bcl-2 and Bax

As demonstrated above, estrogen modulates Bcl-2 and Bax in human macrophages by an estrogen receptor dependent mechanism. Also, we have shown that human macrophages express both ER-α and ER-β and hence it was imperative to study if signaling from the two receptor sub-types have differential roles in modulating Bcl-2 and Bax as it is known for several other signaling pathways (7). To dissect the effect of signaling exclusively through ER-α or ER-β, a siRNA mediated knockdown of these individual receptors was performed. The siRNA transfection efficiency achieved was ~ 90% as determined by transfection of Cy3-labelled negative control siRNA (Figure 5.5, A) and the viability of these transfected cells was over 95 %. THP-1 macrophages transfected with siRNA against ER-α or ER-β showed a specific knockdown of the respective mRNA transcript (Figure 5.5, B) and protein (Figure 5.5, C). The knockdown efficiency of ER-α was ~ 60% while that of ER-β was ~ 75% at the level of protein expression (Figure 5.5, C). Moreover, cells transfected with ER-α siRNA demonstrated a complete loss of membranous population of estrogen receptor – α (Figure 5.5, D, b, iii) as compared to cells transfected with negative control siRNA (Figure 5.5, D, a, iii). ER-α siRNA transfected cells when treated with E2 do not show up-regulation of Bcl-2 (Figure 5.6, A, lane 4) while ER-β siRNA (Figure 5.6, A, lane 6) and negative control siRNA transfected cells (Figure 5.6, A, lane2) demonstrate an increase in Bcl-2 levels suggesting that estrogen induces up-regulation of Bcl-2 by signaling via the ER-α receptors.

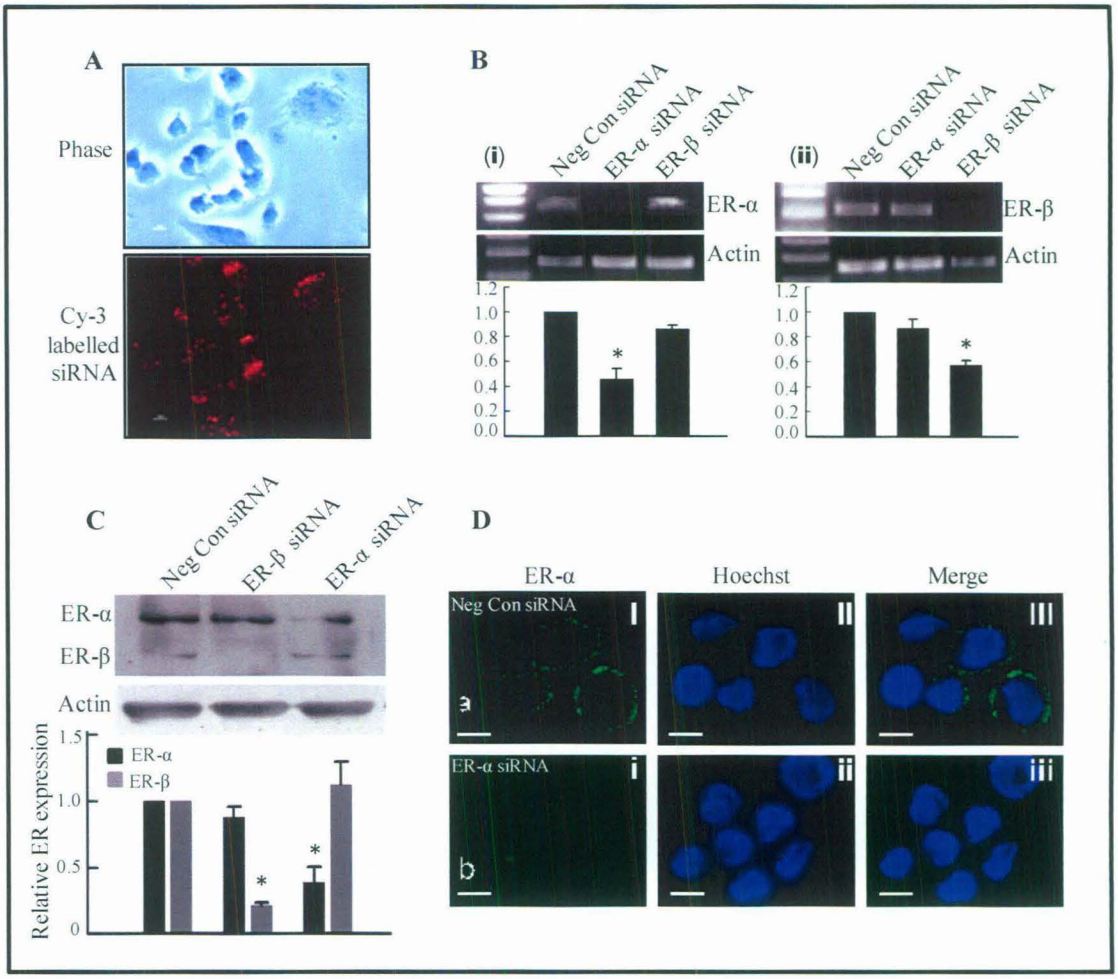


Figure 5.5. siRNA mediated knockdown of ER-α and ER-β. (A) Fluorescence microscopic analysis of Cy3-labelled siRNA to monitor transfection efficiency. Note the distinct intracellular distribution of siRNAs. (B) Semi-quantitative RT-PCR analysis of mRNA transcripts for ER-α (i) and ER-β (ii) in THP-1 macrophages transfected with the indicated siRNAs at 24 h post-transfection. The bar graph represents the densitometric analysis of relative expression of ER-α or ER-β in the various transfection groups. *, $p<0.05$ as compared to cells transfected with negative control siRNA. (C) Western blot analysis of knockdown of ER-α and ER-β in THP-1 macrophages transfected with the indicated siRNAs at 24 h post-transfection. The bar graph represents the densitometric analysis of relative expression of ER-α and ER-β revealing a specific knockdown with the respective siRNAs. *, $p<0.05$ as compared to cells transfected with negative control siRNA. (D) Immunocytochemistry and fluorescence microscopy for membranous ER-α expression on live cells transfected with ER-α siRNA (b, i-iii) or negative control siRNA (a, i-iii). Note the distinct loss of membranous ER in ER-α siRNA transfected cells. The green stain represents staining for ER-α, the blue stain represents nuclear staining with Hoechst 33342. The bar represents 10 μm.

The knockdown of either ER-α or ER-β alone did not have any effect on the Bcl-2 levels (Figure 5.6, A, lanes 1, 3, and 5). In contrast, the estrogen-induced translocation of Bax from the cytosol to the mitochondria was unaffected in ER-α knockdown cells

(Figure 5.6, B, lanes 5 and 6) while ER-β knockdown cells showed complete abrogation of Bax translocation (Figure 5.6, B, lanes 3 and 4). This indicated that the signal for estrogen-induced translocation of Bax is mediated *via* ER-β.

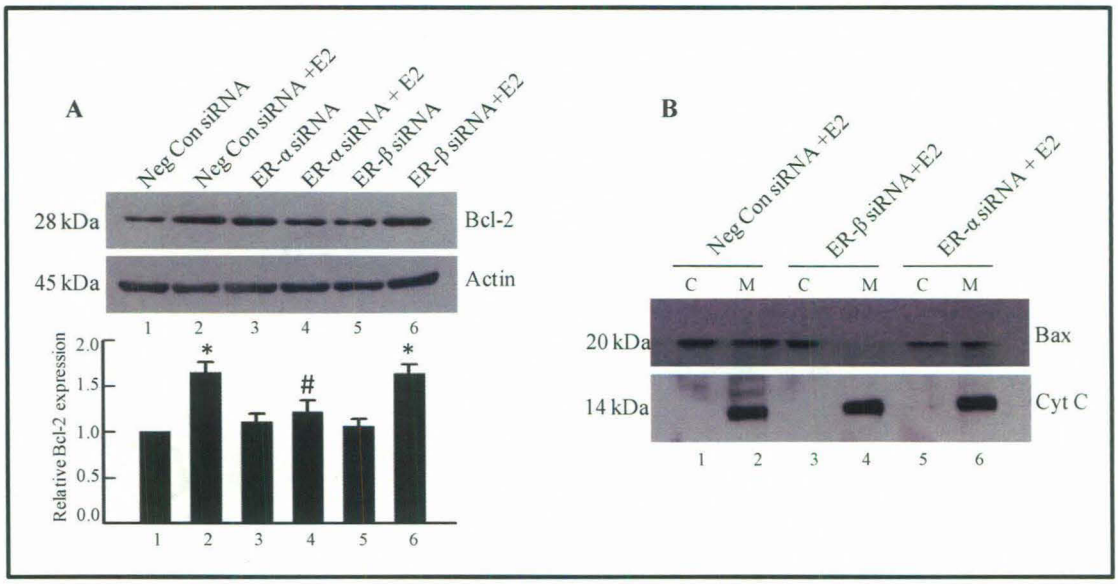


Figure 5.6. A differential role for ER-α and ER-β in modulation of Bcl-2 and Bax. (A) Western blot analysis for Bcl-2 expression in THP-1 macrophages transfected with the indicated siRNAs. After 24 h of transfection, these cells were treated with 10 nM E2 for 6 h. Note the inhibition of Bcl-2 up-regulation in ER-α siRNA transfected cells. The bar graph represents the relative Bcl-2 expression as compared to negative control siRNA transfected cells normalized to actin. *, $p<0.05$ as compared to negative control siRNA group; #, $p<0.05$ as compared to negative control siRNA group treated with E2. (B) Analysis of sub-cellular localization of Bax by Western blotting in cytosolic and mitochondrial fractions obtained from THP-1 macrophages treated with 10 nM E2 24 h post-transfection with the indicated siRNAs. A clear absence of translocation of Bax to the mitochondria is seen in cells transfected with ER-β siRNA. Western blot for cytochrome c was used to assess purity of the obtained fractions. Cyt C, cytochrome c; Neg con siRNA, Negative control siRNA; C, cytosolic fraction; M, mitochondrial fraction. The data in A and B are representative of 3 independent experiments.

5.7. The role of estrogen receptors localized in distinct sub-cellular compartments in the modulation of Bcl-2 and Bax.

As shown in Chapter 4, the estrogen receptor sub-types show distinct sub-cellular localization; the plasma membrane is populated by ER-α alone, the cytoplasm possesses both ER-α and ER-β, while the nucleus is devoid of estrogen receptors. Also, as demonstrated in Figure 5.4, C, estrogen-induced up-regulation of Bcl-2 is mediated by a transcriptional mechanism. To elucidate the mechanism of this

transcriptional up-regulation, bio-informatic analysis of the Bcl-2 promoter was performed which revealed the presence of both an estrogen response element (ERE) (8) and a cyclic-AMP response element (CRE) (9). This raised the possibility that though the estrogen receptors are not localized in the nucleus, but upon ligand binding may translocate into the nucleus and bind to ERE thereby initiating transcription. Analysis of sub-cellular localization of estrogen receptor in E2 treated THP-1 macrophages showed a marked co-localization of estrogen receptor within the nuclear compartment (Figure 5.7, b, iv and v) as compared to cells that were in an estrogen deprived environment (Figure 5.7, a, iv and v).

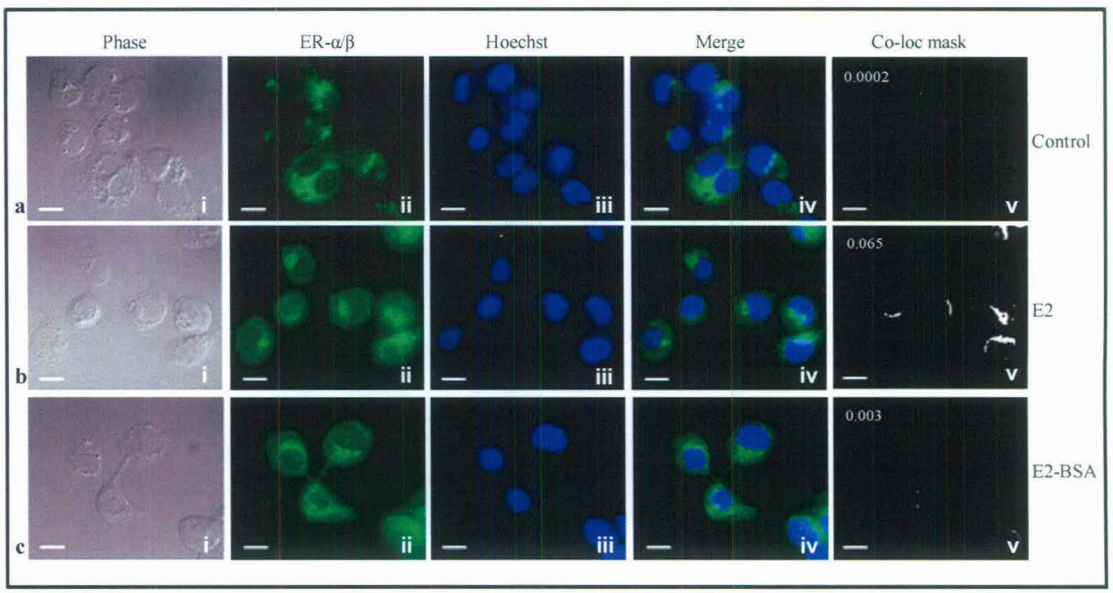


Figure 5.7. Nuclear translocation of estrogen receptor upon ligand binding. Immunocytochemistry was performed using an antibody which recognizes both ER-α and ER-β on fixed THP-1 macrophages which were either left untreated (a) or treated with 10 nM E2 (b) or 10 nM E2-BSA (c) for 30 min. The sub-panel (ii) represents ER-α/β staining, (iii) represents staining with the Hoechst 33342 nuclear staining dye, (iv) represents the merge of panels (ii) and (iii) and panel (v) represents the colocalization mask. The value within panel (v) represents the co-efficient of co-localization between ER-α/β staining and nuclear staining. Note the significant co-localization of estrogen receptor within the nuclear compartment in cells treated with E2. The bar represents 10 μm.

Moreover, cells treated with estradiol-bovine serum albumin conjugate (E2-BSA), which is a membrane impermeable form of estrogen, did not induce the nuclear translocation (Figure 5.7, c, iv and v). This confirmed that there is indeed a translocation of the estrogen receptor from the cytoplasm into the nucleus upon binding to estrogen.

Arguably, if E2-ER complex translocates into the nucleus and binds ERE to initiate transcription, then E2-BSA should be unable to induce an up-regulation of Bcl-2 as it neither crosses the plasma membrane nor does it induce nuclear translocation of estrogen receptors. However, interestingly, E2-BSA induced an up-regulation of Bcl-2 (Figure 5.8, A, lane 4) comparable to that induced by E2 (Figure 5.8, A, lane 2). Also, the E2-BSA induced Bcl-2 increase was abrogated by pre-incubation with the estrogen receptor antagonist ICI 182,780 (Figure 5.8, A, lane 5) suggesting that the signal for Bcl-2 up-regulation is mediated by plasma membrane localized estrogen receptors. Given the fact that Bcl-2 up-regulation is mediated by membranous estrogen receptor and that the membrane component of estrogen receptor is ER-α (Figure 4.2) and that the knockdown of ER-α abrogates Bcl-2 expression (Figure 5.6, A), it can be inferred that the signal for Bcl-2 up-regulation is mediated *via* the

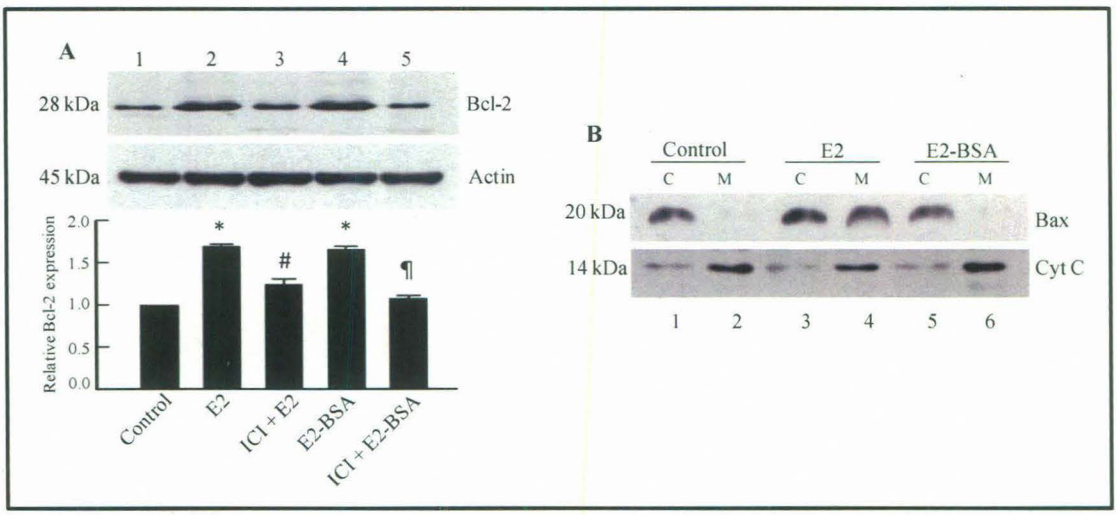


Figure 5.8. Membranous estrogen receptor signals for Bcl-2 up-regulation but not Bax translocation. (A) Bcl-2 expression analysis by Western blot in THP-1 macrophages treated with 10 nM E2 or 10 nM E2-BSA for 6 h. ICI 182,780 was added 1 h prior to addition of E2 or E2-BSA. The bar graph represents the relative Bcl-2 expression derived by densitometric analysis of immunoreactive bands after normalization with actin loading control. *, $p<0.05$ as compared to control, #; $p<0.05$ as compared to E2 treated group; ¶, $p<0.05$ as compared to E2-BSA group. (B) Western blotting for Bax localization in mitochondrial and cytosolic fractions of THP-1 macrophages treated with 10 nM E2 or 10 nM E2-BSA for 4 h. cytochrome c blot was performed to determine the purity of the obtained fractions. Cyt C, cytochrome c; C, cytosolic fraction; M, mitochondrial fraction.

membrane localized ER-α. In contrast, E2-BSA was unable to induce translocation of Bax from the cytosol to the mitochondria (Figure 5.8, B, lanes 5 and 6), while the translocation was complete in E2 treated THP-1 macrophages (Figure 5.8, B, lanes 3

and 4). This suggested that the signal for Bax translocation is independent of membranous receptor signaling and is dependent on signaling *via* the intracellular pool of estrogen receptors. This was in corroboration with our previous data that Bax translocation is mediated *via* ER-β which is localized intracellularly in the cytoplasm.

5.8. Estrogen-induced up-regulation of Bcl-2 is mediated by ERK phosphorylation

The data presented above clearly demonstrated that E2-induced transcriptional up-regulation of Bcl-2 was not mediated by binding of E2-ER complex to the ERE. Hence, we tested the alternate hypothesis that it may be mediated by cyclic-AMP response element binding protein (CREB) binding to CRE on Bcl-2 promoter. When THP-1 macrophages were exposed to E2, there was a time-dependent phosphorylation of CREB (Figure 5.9, A) indicating a role for it in modulation of Bcl-2.

One of the most common upstream kinase which phosphorylates CREB is extracellular signal regulated kinase (ERK). Both E2 and E2-BSA were able to induce rapid phosphorylation of ERK (Figure 5.9, B, lanes 2 and 4 respectively) within 5 min of addition that was inhibited by pre-incubation with ICI 182, 780 (Figure 5.9, B, lanes 3 and 5) suggesting that the process was estrogen receptor mediated. If the E2-induced phosphorylation of ERK was linked to up-regulation of Bcl-2, then inhibition

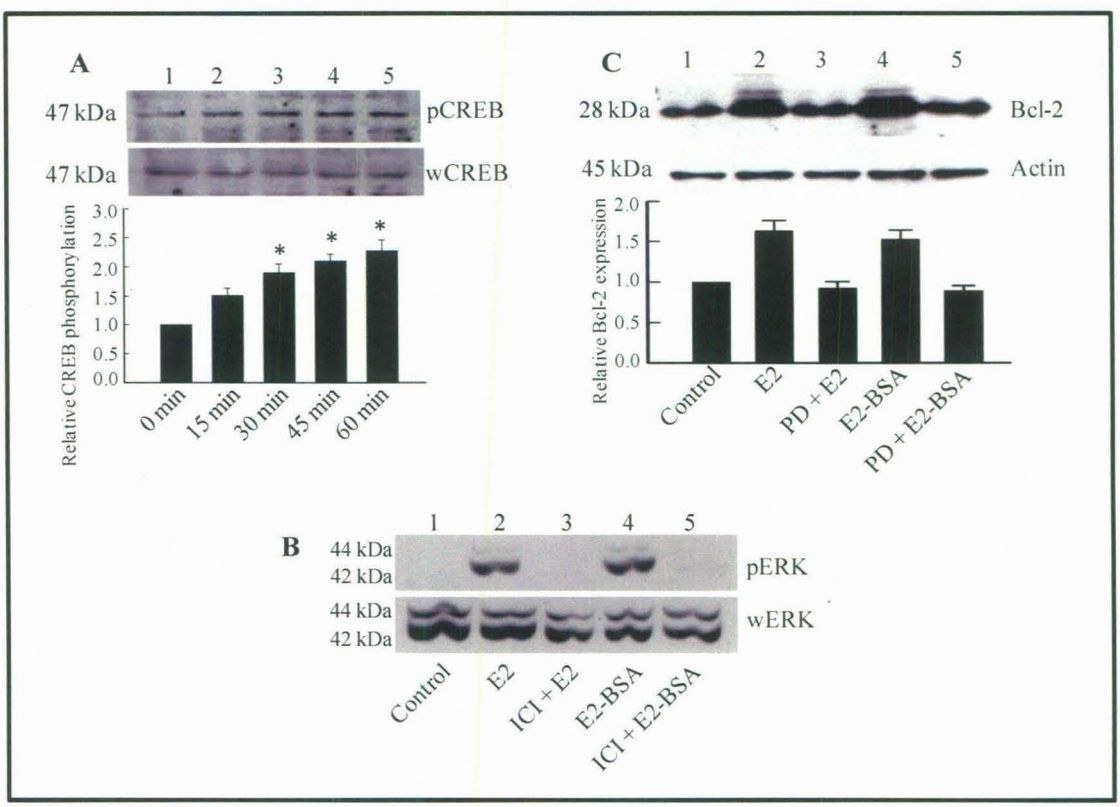


Figure 5.9. Role of ERK phosphorylation in Bcl-2 up-regulation. (A) Analysis of phosphorylated CREB (pCREB) by Western blotting in THP-1 macrophages treated with 10 nM E2 for 0-60 min. The bar graph represents relative CREB phosphorylation normalized to total CREB (wCREB). *, $p<0.05$ as compared to 0 min. (B) Western blotting to detect phosphorylated ERK1/2 (pERK) in THP-1 macrophages treated with 10 nM E2 or 10 nM E2-BSA for 5 min. In the inhibitor treated groups, the cells were pre-incubated with 1 μM ICI 182,780 for 10 min prior to E2 or E2-BSA addition. Western blot for total ERK (wERK) was used as loading control. (C) Analysis of Bcl-2 expression in THP-1 macrophages treated with 10 nM E2 or 10 nM E2-BSA with or without pre-incubation with the MEK inhibitor PD 98,059 (25 μM) for 10 min prior to addition of E2 or E2-BSA. The bar graph represents relative Bcl-2 expression as compared to control and normalized to actin which served as endogenous loading control. *, $p<0.05$ as compared to control; #, $p<0.05$ as compared to E2 or E2-BSA treated group.

of ERK phosphorylation should abrogate the increase in Bcl-2. When phosphorylation of ERK was inhibited using the MEK inhibitor PD 98,059, there was complete abrogation of E2 and E2-BSA-induced increase in Bcl-2 (Figure 5.9, C, lanes 3 and 5 respectively) thereby verifying the hypothesis and confirming the link between ERK phosphorylation and Bcl-2 up-regulation.

5.9. Ca^{2+} dependent regulation of ERK phosphorylation

Phosphorylation of ERK is mediated by upstream kinases belonging to the MAPK pathway which may be activated by proximal kinases such as phosphatidylinositol-3-kinase (PI3K) or protein kinase C (PKC) in a Ca^{2+} dependent or independent manner. When the intracellular Ca^{2+} concentration was measured using the fluorescent Ca^{2+} indicator Fluo3-AM, a distinct Ca^{2+} flux was observed in THP-1 macrophages treated with E2 (Figure 5.10, A, red line, and Figure 5.10, B, iii and iv). The rise in Ca^{2+} occurred within seconds of E2 addition and then showed a gradual decrease at around 10 min of treatment to be followed by a second rise which led to stabilization of intracellular Ca^{2+} at around 90 nM as compared to the basal level of 45 nM. This increase in Ca^{2+} was estrogen receptor mediated as it was inhibited by pre-incubation with the estrogen receptor antagonist ICI 182,780 (Figure 5.10, A, green line, and Figure 5.10, B, v and vi). Moreover, the source of Ca^{2+} for mediating the increase was extracellular as it was completely abolished by addition of EGTA, the extracellular Ca^{2+} chelator (data not shown). Further analyzing the mechanism of Ca^{2+} entry, it was observed that the Ca^{2+} influx was inhibited by the L-type Ca^{2+} channel inhibitor verapamil (Figure 5.10, A, blue line, and Figure 5.10, B, vii and viii), but not by

pimozide (data not shown) which is a T-type Ca^{2+} channel inhibitor. This increase in intracellular Ca^{2+} is essential for mediating phosphorylation of ERK as demonstrated by inhibition of ERK phosphorylation upon treatment of cells with the L-type Ca^{2+} channel inhibitor verapamil (Figure 5.11, A, lane 3). Furthermore, the role of Ca^{2+} dependent proximal kinase such as PKC in ERK phosphorylation was studied using a specific inhibitor to PKC, and as seen in Figure 5.11, B, lane 4 , there was an inhibition of phosphorylation of ERK upon use of the inhibitor. Hence, it can be inferred that E2 treatment results in activation of L-type Ca^{2+} channel leading to Ca^{2+}

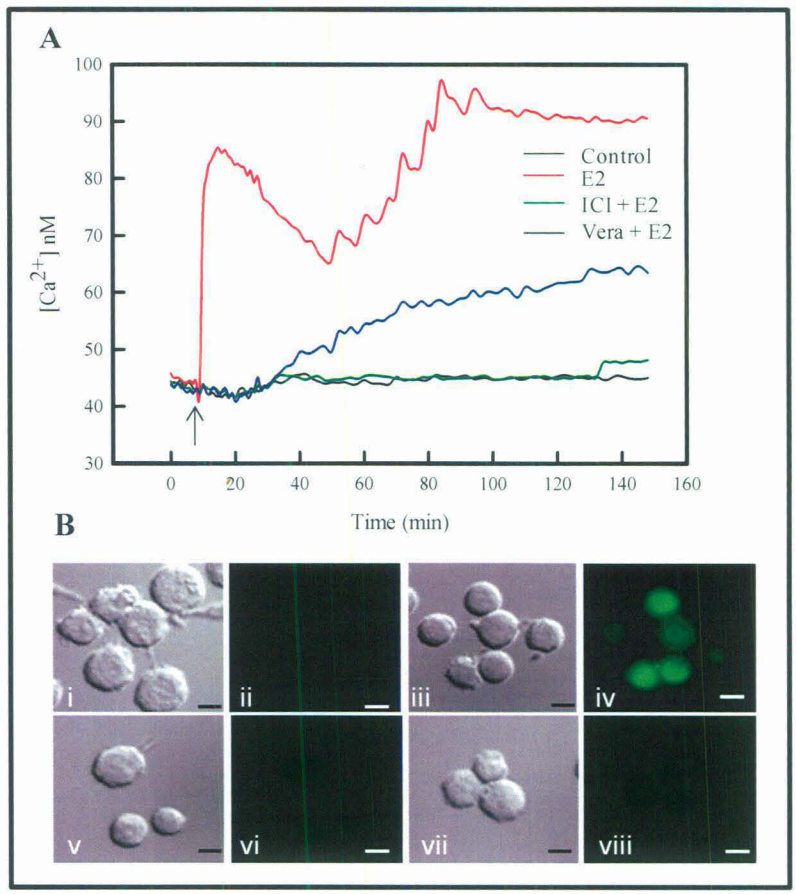


Figure 5.10. E2 induces a rapid intracellular Ca^{2+} flux. (A) Fluorimetry based time-kinetic analysis of intracellular Ca^{2+} using the Ca^{2+} indicator Fluo3-AM was performed on THP-1 macrophages treated with 10 nM E2 or pre-incubated with ICI 182,780 (1 μM) or verapamil (25 μM) for 10 min prior to initiation of measurements. The arrow indicates the point of addition of E2. (B) Analysis of Ca^{2+} influx by fluorescence microscopy using Fluo3-AM in THP-1 macrophages treated with 10 nM E2 for 5 min (iii and iv), or pre-incubated with ICI 182,780 (v and vi) or verapamil (vii and viii). Panel (i and ii) represents control cells which were not exposed to E2.

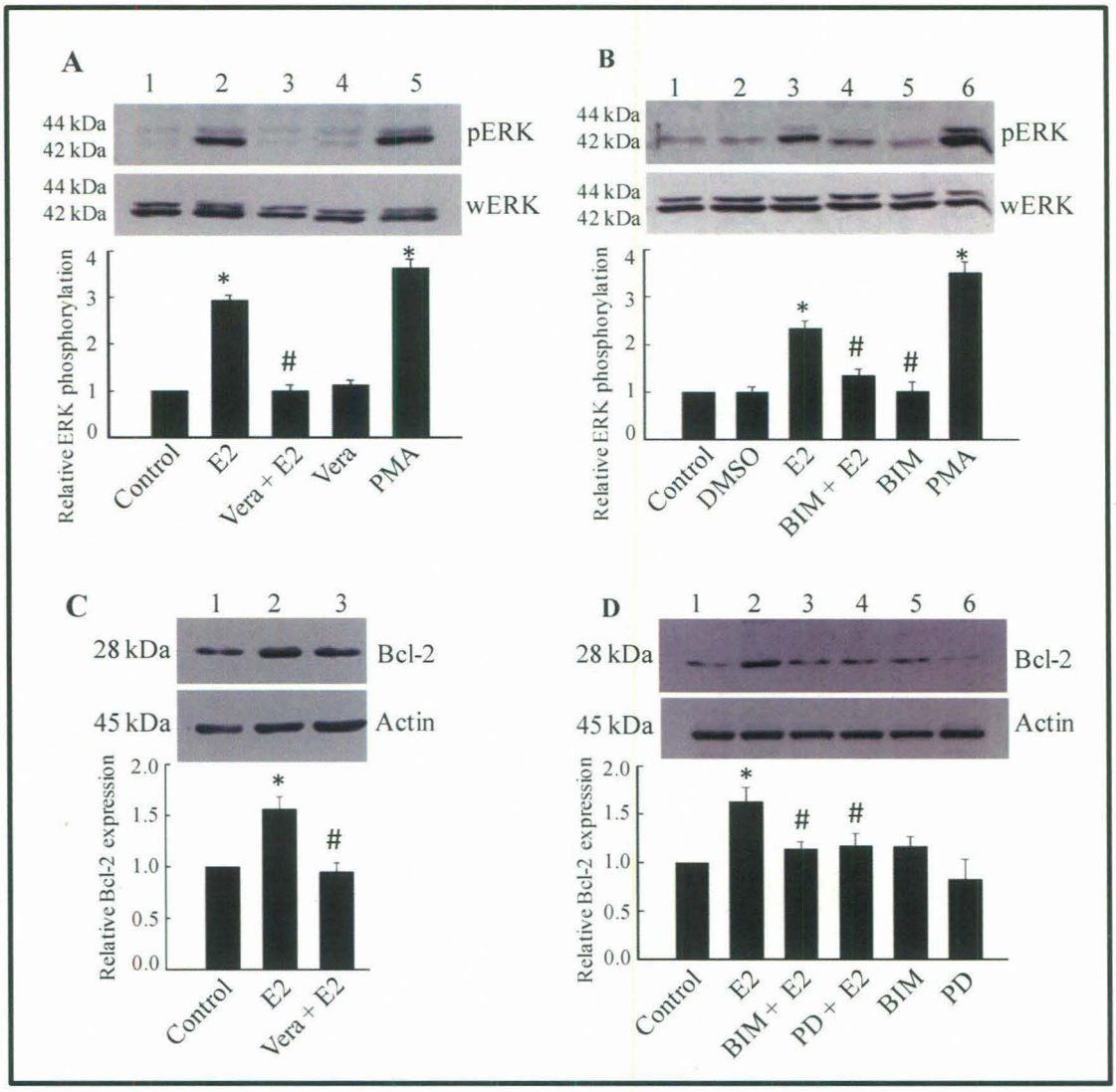


Figure 5.11. Bcl-2 up-regulation is mediated by ERK phosphorylation. (A and B) THP-1 macrophages were treated with 10 nM E2 for 5 min in the presence of verapamil (25 μM), or BIM VIII (25 μM) and levels of phosphorylated ERK was detected by Western blotting. DMSO was used as a solvent control as inhibitors were reconstituted in DMSO. PMA (100 ng/mL) was used as a positive control for induction of ERK phosphorylation. The bar graph represents the relative levels of phosphorylated ERK as compared to control. *, $p<0.05$ as compared to control; #, $p<0.05$ as compared to E2 treated group. (C and D) Western blot analysis of Bcl-2 expression in THP-1 macrophages treated with 10 nM E2 for 6 h in the absence or presence of verapamil (25 μM) or BIM VIII (25 μM), or PD 98,059 (25 μM). The bar graph represents the relative Bcl-2 expression as compared to control and normalized to actin. *, $p<0.05$ as compared to control; #, $p<0.05$ as compared to E2 treated group.

influx which activates PKC resulting in phosphorylation of ERK *via* the MAPK pathway. However, as to whether this Ca^{2+} dependent pathway of phosphorylation of ERK is actually involved in regulating Bcl-2 up-regulation needed to be investigated. Figure 5.11 clearly demonstrates that inhibition of either Ca^{2+} influx *via* the L-type

channel (Figure 5.11, C, lane 3), or inhibition PKC (Figure 5.11, D, lane 3) inhibits up-regulation of Bcl-2 mediated by E2 thereby establishing the pathway of Bcl-2 up-regulation as involving a Ca^{2+} dependent activation of PKC with subsequent ERK phosphorylation and CREB phosphorylation resulting in transcription of Bcl-2.

5.10. Role of estrogen-induced Ca^{2+} influx and ERK phosphorylation on translocation of Bax

As evidenced above, estrogen-induced Ca^{2+} influx followed by ERK phosphorylation modulates Bcl-2 expression. Hence, it was interesting to study if they also cross-talk in the signaling pathway responsible for translocation of Bax. However, inhibition of Ca^{2+} influx using the L-typed Ca^{2+} channel inhibitor verapamil (Figure 5.12, lanes 5 and 6) or inhibition of phosphorylation of ERK using PD 98,059, an inhibitor of the upstream kinase MEK (Figure 5.12, lanes 7 and 8), did not influence the estrogen-induced translocation of Bax. This suggested a pathway for Bax translocation independent of Ca^{2+} influx or phosphorylation of ERK.

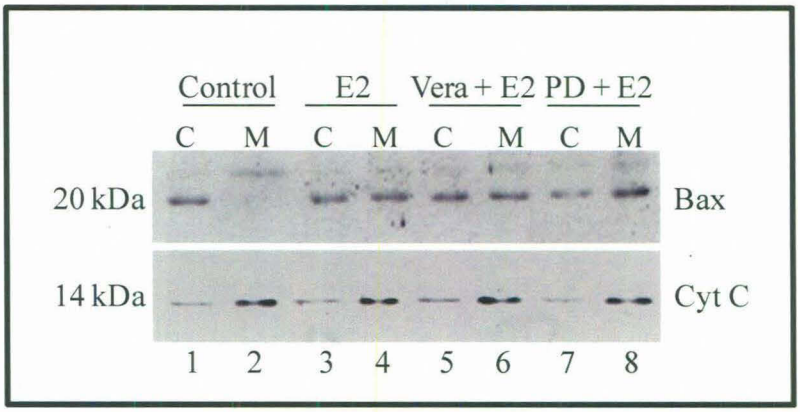


Figure 5.12. Estrogen-induced Bax translocation is independent of Ca^{2+} influx and ERK phosphorylation. Western blot analysis of sub-cellular localization of Bax in mitochondrial and cytosolic fractions obtained from THP-1 macrophages treated with 10 nM E2 for 2 h with or without pre-incubation with 25 μM verapamil or 25 μM PD 98,059 for 10 min prior to addition of E2. Western blot for cytochrome c was performed to determine the homogeneity of the obtained fractions. Cyt C, cytochrome c; Vera, verapamil; PD, PD 98,059; C, cytosolic fraction; M, mitochondrial fraction.

5.11. Analysis of the mechanism of estrogen-induced translocation of Bax

The mechanisms leading to activation of Bax and its subsequent translocation to the mitochondria is not well understood, though several hypothesis have been proposed, including, release of Bax from scaffold proteins (10), phosphorylation (11), intracellular acidification (12), and intracellular alkalinization (13). We analyzed the

role of one of the scaffold proteins clusterin, which holds Bax in its inactive conformation (10). Estrogen treatment of THP-1 macrophages did not affect the levels of either the secretory form of clusterin which is the anti-apoptotic form, or the nuclear clusterin, which is the pro-apoptotic form (Figure 5.13, A, lane 2), thereby ruling out its involvement in mediating Bax translocation. However, when changes in intracellular pH was monitored using the pH sensitive dye SNARF-1-AM, an increase in intracellular pH was observed from the basal level of 7.5 to 7.7 in E2 treated cells (Figure 5.13, B, red line). This increase was inhibited by estrogen receptor antagonist ICI 182,780 (Figure 5.13, B, blue line) suggesting pH change to be an estrogen receptor dependent process. Also, the observation that E2-BSA, the membrane impermeable form of estrogen did not alter the intracellular pH (Figure 5.13, B, green line) indicated that the alkalinization was mediated *via* signaling through the intracellular pool of estrogen receptors.

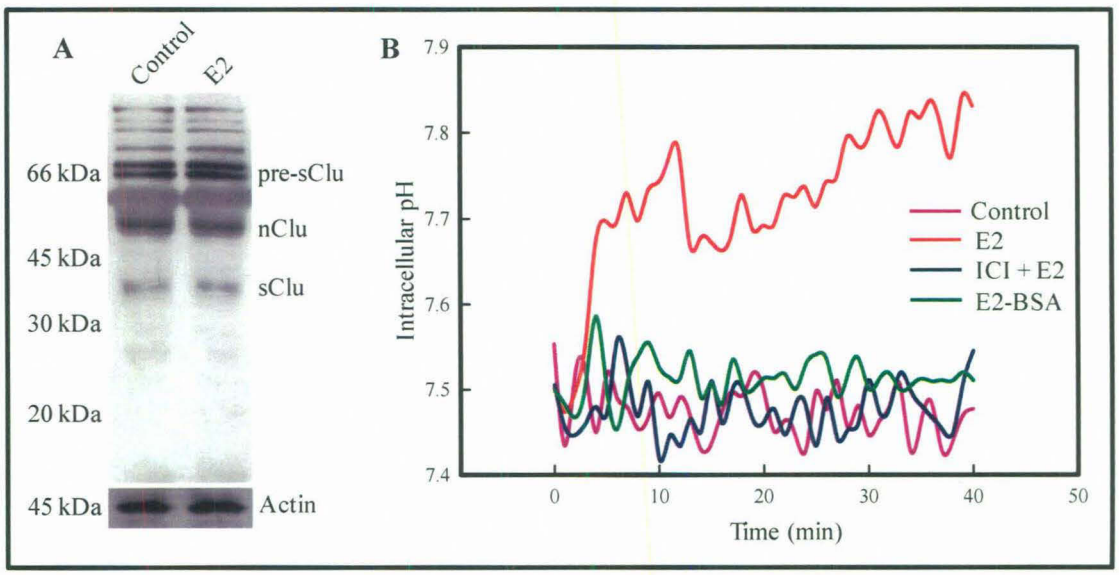


Figure 5.13. Estrogen induces intracellular alkalinization. (A) Western blot analysis of expression of clusterin in THP-1 macrophages treated with or without 10 nM E2 for 4 h. Actin was used as endogenous loading control. sClu, secretory clusterin; nClu, nuclear clusterin; pre-sClu, precursor of secretory clusterin. (B) Time-kinetic analysis of intracellular pH changes by fluorimetry in THP-1 macrophages treated with 10 nM E2 or 10 nM E2-BSA, or pre-incubated for 10 min with ICI 182,780 (1 μM). ICI, ICI 82,780.

Based on the above observations, we hypothesized that Bax translocation was mediated by estrogen-induced intracellular alkalinization. To test this hypothesis, THP-1 macrophages were exposed to E2 with the intracellular pH being maintained at

the basal level of 7.5 or at the elevated level of pH 7.7. This was achieved by treating cells with Nigericin, a K^+-H^+ antiporter which maintains the intracellular pH the same as that of the extracellular medium in a buffer containing high K^+. When THP-1 macrophages were exposed to E2 with the intracellular pH being maintained at the basal level, no translocation of Bax was observed (Figure 5.14, A, lanes 3 and 4), however, the translocation was complete in cells placed in a buffer of pH 7.7 (Figure 5.14, A, lanes 5 and 6) indicating that the process of intracellular alkalinization was linked to Bax translocation. Interestingly, inhibition of intracellular alkalinization did not affect the E2-induced Bcl-2 up-regulation (Figure 5.14, A) indicating that modulation of Bcl-2 is independent of changes in pH.

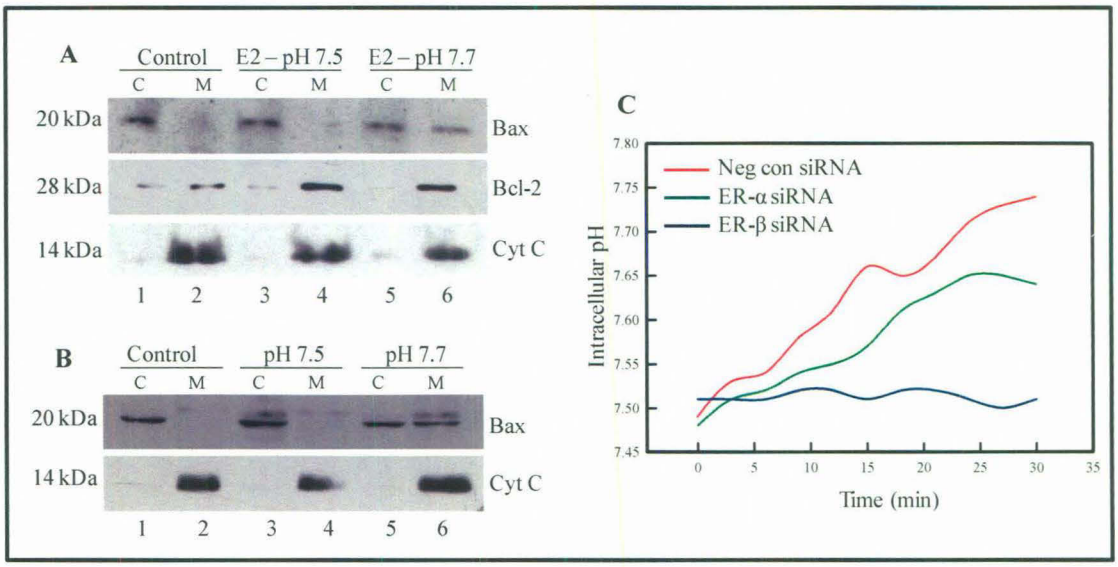


Figure 5.14. Estrogen-induced intracellular alkalinization mediates Bax translocation. Appropriate groups of THP-1 macrophages were resuspended in high-K^+ buffer of pH 7.5 or 7.7. The control group was left untreated whereas the other two groups were incubated with 1 μM nigericin and treated with (A) or without (B) 10 nM E2 for 6 h. Western blotting was performed for Bax, Bcl-2 and cytochrome c on the mitochondrial and cytosolic fractions obtained from these treated macrophages. Cyt C, cytochrome c; C, cytosolic fraction; M, mitochondrial fraction. (C) Fluorimetry based intracellular pH measurement using SNARF-1-AM in THP-1 macrophages transfected with negative control siRNA, ER-α siRNA, or ER-β siRNA following treatment with 10 nM E2. The graph shows the absence of increase in intracellular pH in cells transfected with ER-β siRNA.

To ascertain if translocation of Bax could occur whenever there was a pH change independent of other stimuli, an increase in intracellular pH was induced in the absence of E2, and a change of pH to 7.7 resulted in Bax translocation (Figure 5.14, B, lanes 5 and 6) but not when pH was maintained at 7.5 (Figure 5.14, B, lanes 3

and 4). This data suggested that an increase in intracellular pH was sufficient to induce translocation of Bax independent of other pathways that may be activated by E2. Moreover, ER-β knockdown cells treated with E2 did not shown any alteration in intracellular pH (Figure 5.14, C, blue line) while an increase in intracellular pH was observed in ER-α knockdown cells (Figure 5.14, C, green line) corroborating with the previous evidence that Bax translocation is signaled through ER-β, which we now demonstrate to be dependent on an intracellular alkalinization mediated mechanism.

In several cellular systems the intracellular pH is maintained by the co-ordinated activity of a number of ion channels and their respective ions, the most important of which are the sodium-hydrogen (Na^+/H^+) exchangers (NHE) and the bicarbonate (HCO_3^-) transporters. Hence, the involvement of each of these transporters in estrogen-induced pH alteration was investigated.

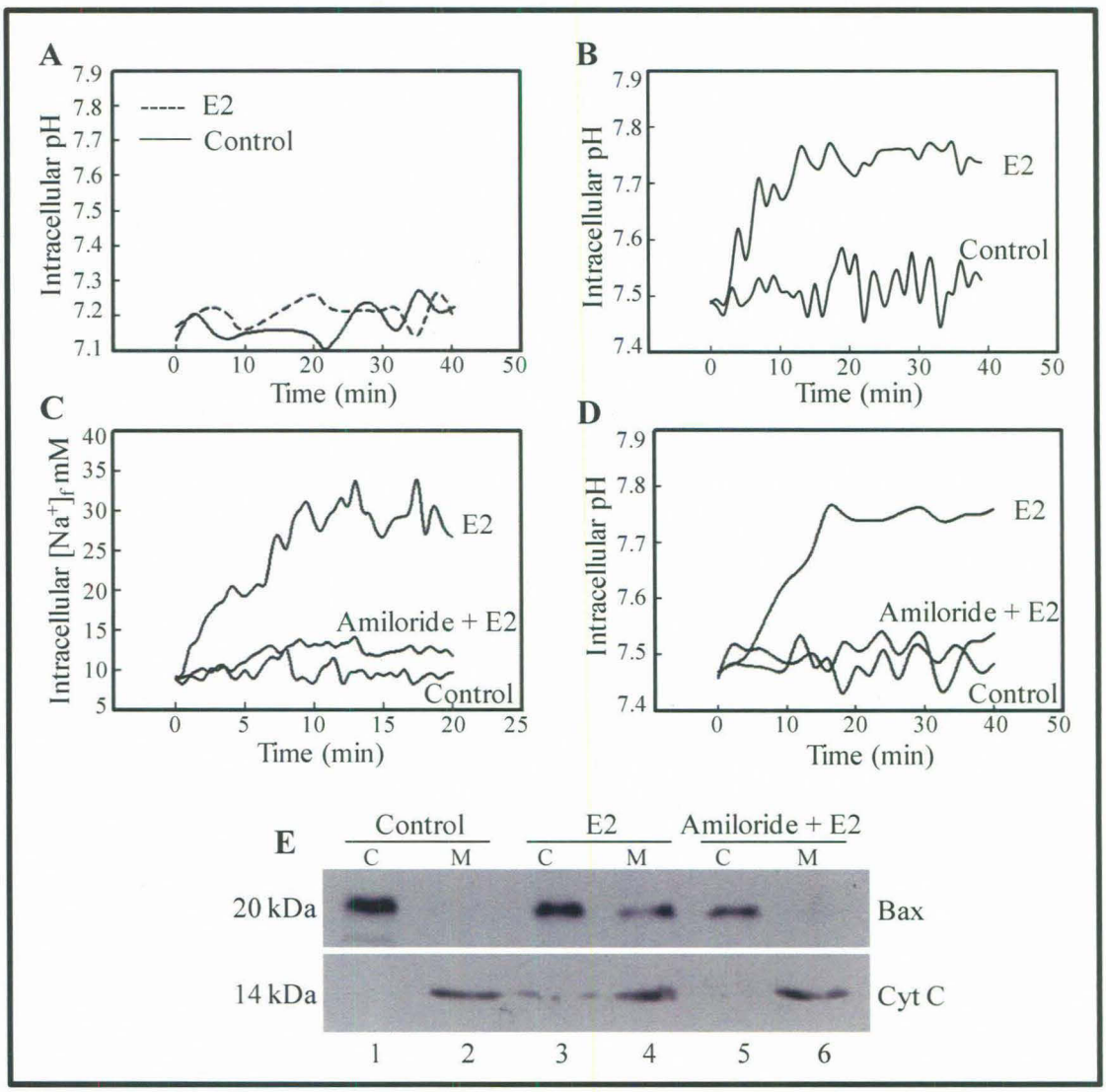


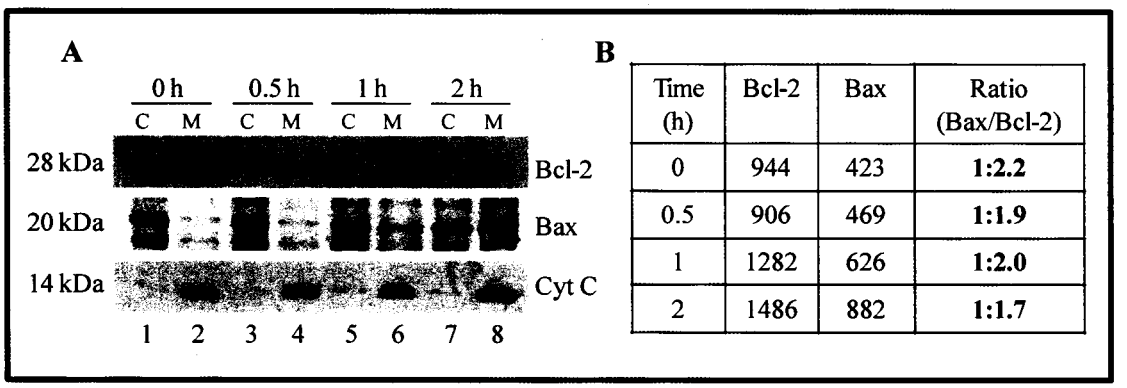
Figure 5.15. Estrogen activates NHE to mediate intracellular alkalinization. THP-1 macrophages were resuspended in Na^+-free buffer (A) or HCO_3^- free buffer (B), and intracellular pH was measured using SNARF-1-AM dye after treatment with 10 nM E2 over a time period of 40 min. (C) Intracellular Na^+ measurement using Sodium Green™ in THP-1 macrophages treated with 10 nM E2 with or without 10 min pre-incubation with amiloride (2 μM). (D) Intracellular pH measurement in THP-1 macrophages exposed to 10 nM E2 and pre-incubated with or without the Na^+/H^+ exchanger inhibitor amiloride (2 μM). Note the abrogation of E2-induced increase in pH in the presence of amiloride.

NHE functions in the maintenance of intracellular pH by pumping out intracellular H^+ for extracellular Na^+, and hence its activity is indicated by both an increase in intracellular Na^+ and alkalinization of the cytoplasm due to expulsion of H^+ ions. When THP-1 macrophages were suspended in a Na^+-free media, E2 was unable to induce a pH change (Figure 5.15, A) indicating that influx of Na^+ was essential for alkalinization. However, HCO_3^- was not required as E2 was able to induce alkalinization in a HCO_3^- free buffer (Figure 5.15, B). A possible role for NHE in mediating increase in intracellular pH was indicated by an increase in intracellular Na^+ in response to E2 as observed by an increase in Sodium Green fluorescence (Figure 5.15, C). This was further confirmed when amiloride, an NHE inhibitor lowered Na^+ levels (Figure 5.15, C) and also prevented alkalinization of the cytoplasm (Figure 5.15, D). If NHE activity regulates estrogen-induced alkalinization which mediates translocation of Bax, then inhibition of alkalinization by amiloride should prevent Bax translocation. This was confirmed by Western blotting of cytosolic and mitochondrial fractions obtained from THP-1 macrophages treated E2 in the presence of amiloride, which showed complete abrogation of Bax translocation (Figure 5.15, E, lanes 5 and 6).

5.12. The role of Bcl-2 up-regulation in survival of macrophages exposed to estrogen

The data presented so far indicate that estrogen provokes a death response by initiating translocation of Bax from the cytosol to the mitochondria, however, it also initiates a survival response in the form of up-regulation of Bcl-2. Hence, we hypothesized that a balance between the levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 in the mitochondria helps maintain macrophage survival upon exposure to E2. When the levels of Bax and Bcl-2 on the mitochondria of THP-1 macrophages treated with E2 was analyzed over time (Figure 5.16, A), it was noticed

that despite the translocation of Bax to the mitochondria, the ratio of Bax:Bcl-2 on the mitochondria remained constant (Figure 5.16, B). Arguably, if up-regulation of Bcl-2 protects human macrophages from the pro-apoptotic effect of Bax translocation induced by estrogen treatment, then inhibition of Bcl-2 increase should induce cell death. When estrogen induced Bcl-2 up-regulation was inhibited by siRNA mediated knock down of ER-α (Figure 5.17, A, (iii)), or by inhibiting Ca2+ influx by verapamil (Figure 5.17, B), or by inhibiting PKC activation by BIM VIII (Figure 5.17, B), or by inhibiting ERK phosphorylation by the MEK inhibitor PD 98,059 (Figure 5.17, B), a significant increase in cell death was observed. Since these inhibitors target kinases of



Time (h)	Bcl-2	Bax	Ratio (Bax/Bcl-2)
0	944	423	**1:2.2**
0.5	906	469	**1:1.9**
1	1282	626	**1:2.0**
2	1486	882	**1:1.7**

Figure 5.16. Estrogen maintains the ratio of Bax and Bcl-2 on the mitochondria. (A) Western blot for analysis Bax and Bcl-2 levels in the mitochondrial fractions of THP-1 macrophages treated with 10 nM E2 for 0, 0.5, 1, or 2 h. Immunoblot for cytochrome c was performed to assess the purity of the obtained fraction. Cyt C, cytochrome c; C, cytosolic fraction; M, mitochondrial fraction. (B) The table represents absolute values of Bcl-2 and Bax on the mitochondria and their ratio at various time intervals after E2 treatment as determined by densitometric analysis of specific immunoreactive bands on blots, a representative of which is shown in (A).

critical signaling pathways such as PKC and ERK, it may introduce inadvertent artifacts in viability analysis. To overcome this problem, an siRNA mediated specific knockdown of Bcl-2 was performed. Upon transfection with Bcl-2 siRNA about 60% knockdown of endogenous Bcl-2 was obtained (Figure 5.18, A, lane 2) and also, the estrogen-induced Bcl-2 increase was significantly reduced (Figure 5.18, A, lane 4), the levels being comparable to that of control (Figure 5.18, lane 1). When these Bcl-2 knockdown THP-1 macrophages were treated with E2, a significant increase in cell death was observed, which was inhibited when pH changes were annulled by maintaining the cells at basal pH (Figure 5.18, B). Collectively, these data indicated

that estrogen-induced Bcl-2 up-regulation is the major anti-apoptotic signal, while Bax translocation acts as the major pro-apoptotic signal.

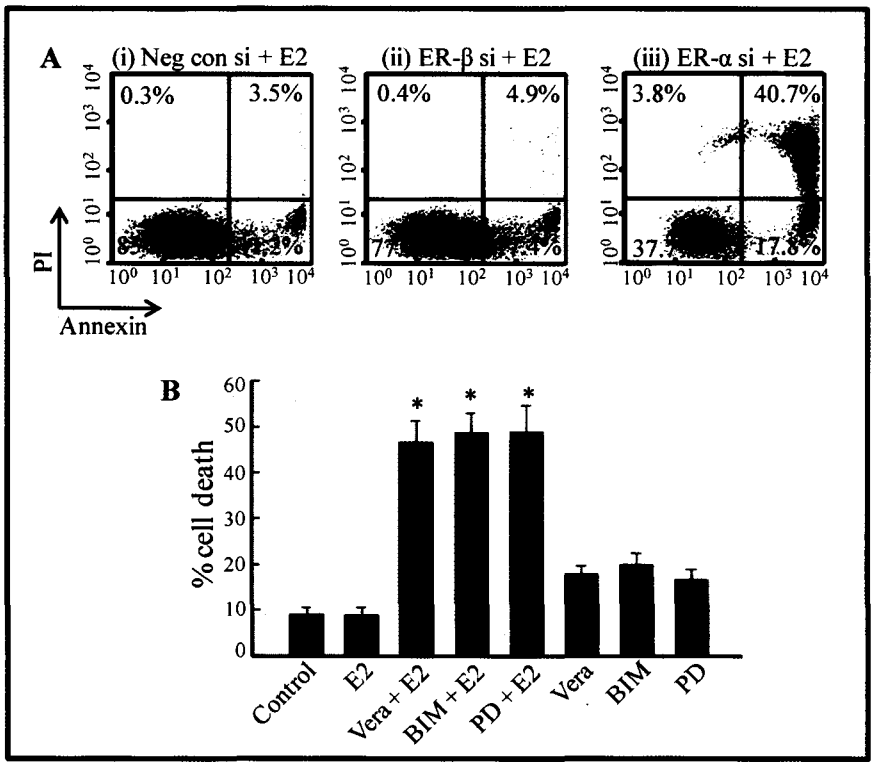


Figure 5.17. Inhibition of Bcl-2 up-regulation results in macrophage death upon exposure to E2. (A) Flow-cytometric analysis of Annexin-V-PI staining in cells transfected with negative control siRNA (i), or ER-β siRNA (ii), or ER-α siRNA (iii) following treatment with 10 nM E2 for 6 h. Note that down-regulation of ER-α in the presence of E2 shows high number of apoptotic cells. Cells in the lower left quadrant represent viable cells, lower right quadrant represent apoptotic cells, upper right quadrant represents late apoptotic cells, and cells in upper left quadrant represent necrotic cells. Neg con si, negative control siRNA; ER-β si, ER-β siRNA; ER-α si, ER-α siRNA. (B) THP-1 macrophages were pre-incubated with 20 μM verapamil or 25 μM PD 98,059 for 10 min or 1 μM BIM VIII for 30 min before treatment with 10 nM E2 for 12 h. Cell death was analyzed by fluorescence microscopy by counting propidium iodide (1 μg/mL) positive cells. *, $p<0.05$ as compared to control (n=3).

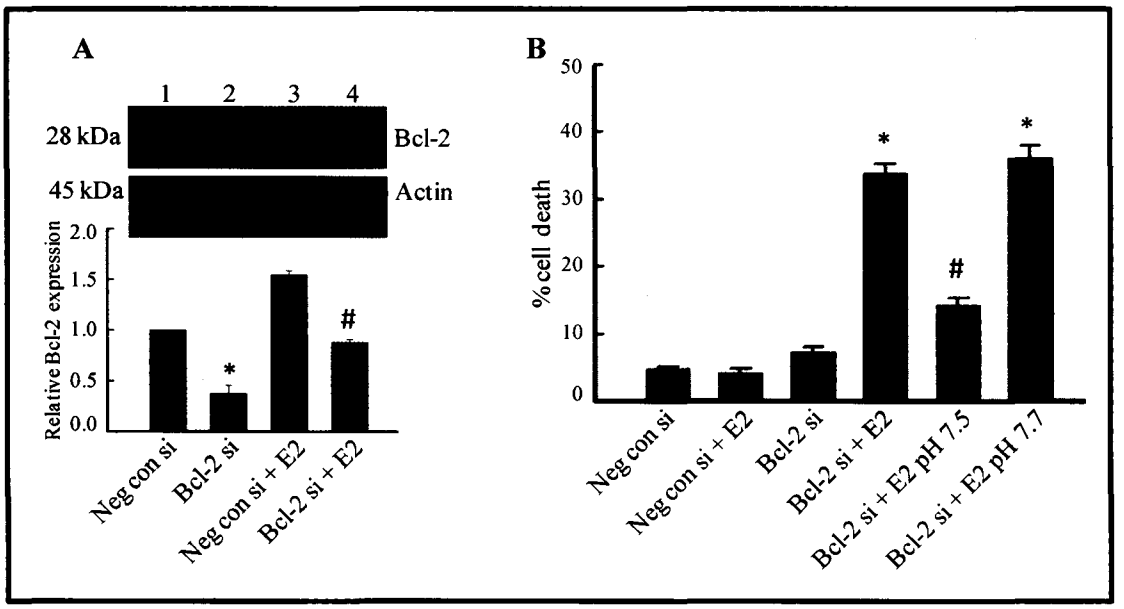


Figure 5.18. Estrogen treatment of Bcl-2 knockdown macrophages leads to cell death. Western blot analysis of Bcl-2 knockdown efficiency in THP-1 macrophages transfected with Bcl-2 siRNA and 24 h post-transfection treated with or without 10 nM E2 for 6 h. Negative control siRNA was used as a target gene specificity control. The bar graph represents relative Bcl-2 expression as compared to cells transfected with negative control siRNA and normalized to actin. *, $p<0.05$ as compared to negative control siRNA group; #, $p<0.05$ as compared to Bcl-2 siRNA transfected group. (B) THP-1 macrophages were transfected with negative control siRNA or Bcl-2 siRNA and 24 h post-transfection the cells were subjected to appropriate treatments with 10 nM E2 as indicated. All cells were resuspended in high-K^+ buffer and appropriate groups where intracellular pH was to be maintained at 7.5 or 7.7 were treated with 1 μM nigericin. E2 treatment was given for 6 h and viability was analyzed by propidium iodide dye exclusion method performed with fluorescence microscopy. The bar graph represents the percentage cell death in the various treatment groups. *, $p<0.05$ as compared to cells transfected with negative control siRNA and treated with E2; #, p,0.05 as compared to Bcl-2 siRNA transfected cells treated with E2.

5.13. Estrogen induces Bcl-2 up-regulation which is critical for survival of human peripheral blood monocyte derived macrophages

The data so far clearly demonstrated that estrogen treatment of THP-1 macrophage cell line results in up-regulation of Bcl-2 with a concomitant translocation of Bax and that the increased Bcl-2 levels play a major role in maintaining macrophage survival upon exposure to E2. Though THP-1 is described as a model human macrophage cell line, it was essential to establish that the changes induced by estrogen as detailed above are relevant in human macrophages *in vivo*. Hence, macrophages were derived by differentiation from human peripheral blood monocytes and subjected to treatment with E2. Upon E2 treatment, a distinct increase

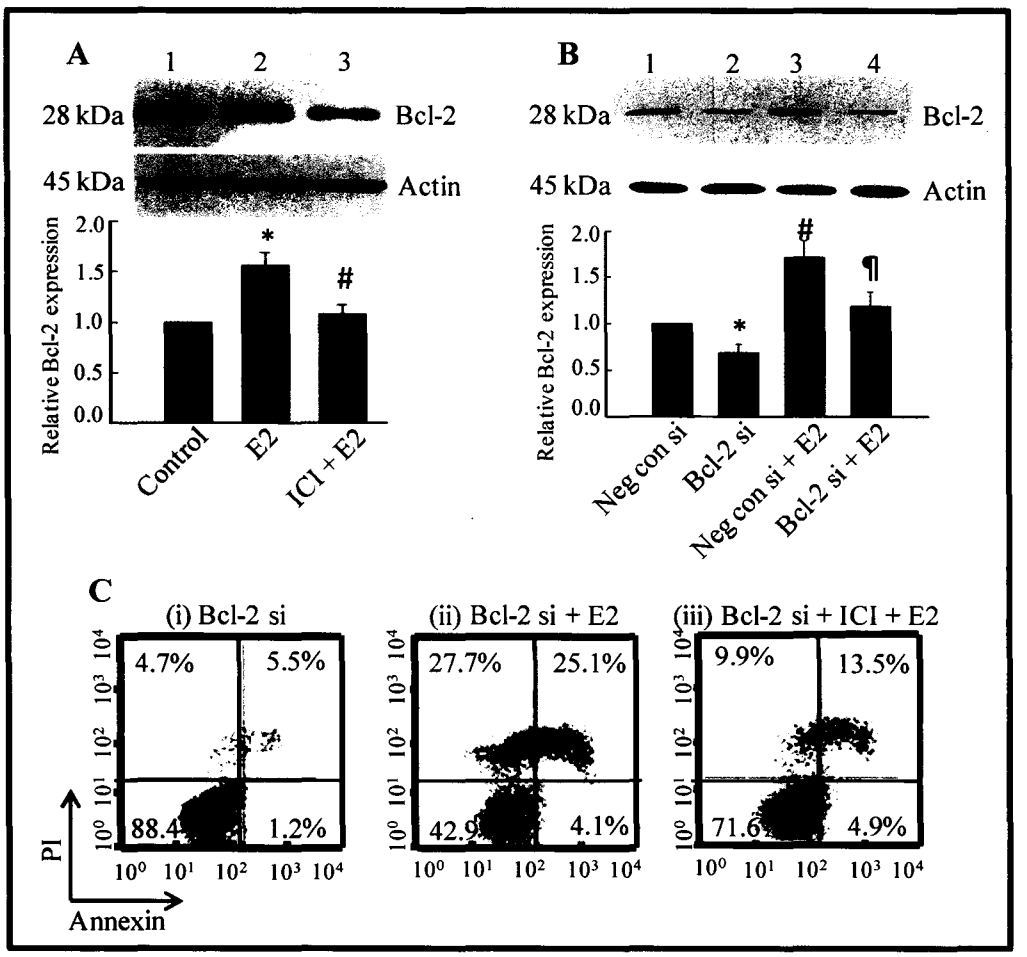


Figure 5.19. Estrogen induces Bcl-2 up-regulation in hPBMDM. (A) Human peripheral blood monocyte derived macrophages were treated with 10 nM E2 for 6 h with or without pre-incubation with ICI 182,780 (1 μM) following which the expression of Bcl-2 was assessed by Western blotting. The bar graph represents the relative Bcl-2 expression as compared to control and normalized to actin. *, $p<0.05$ as compared to control; #, $p<0.05$ as compared to E2 treated. (B) The Bcl-2 knockdown efficiency was assessed by Western blotting in hPBMDM transfected with Bcl-2 siRNA and treated with or without 10 nM E2 for 6 h. Negative control siRNA served as target gene specificity control. The bar graph represents relative Bcl-2 expression as compared to control. *, #, $p<0.05$ as compared to negative control siRNA group; ¶, $p<0.05$ as compared to negative control siRNA group treated with E2. (C) Flow-cytometric analysis of viability in hPBMDM by simultaneous annexin-V and propidium iodide staining of cells transfected with Bcl-2 siRNA and treated with 10 nM E2 for 6 h with or without pre-incubation with 1μM ICI 182,780. The percentages shown represents cells analyzed that lie within each quadrant. Cells in the lower left quadrant are viable, those in the lower right are early-apoptotic, those in the upper right are late-apoptotic and those in the upper left quadrant are necrotic cells.

in Bcl-2 was observed (Figure 5.19, A, lane 2) similar to that obtained with THP-1 macrophages. Also, this increase in Bcl-2 was inhibited by pre-incubation with the estrogen receptor antagonist ICI 182,780 (Figure 5.19, A, lane 3) suggesting it to be

mediated by an estrogen receptor dependent mechanism. To further study if the up-regulation of Bcl-2 plays a critical role in hPBMDM survival, specific knockdown of Bcl-2 was performed by an siRNA mediated mechanism. In hPBMDM, a transfection efficiency of over 95% was achieved as detected by observing fluorescence of Cy3-labeled negative control siRNA. About 30% specific knockdown of endogenous Bcl-2 was observed in cells transfected with the Bcl-2 siRNA, while a significant knockdown was achieved of E2-induced Bcl-2 up-regulation, the levels being comparable to that of control cells (Figure 5.19, B). When these Bcl-2 knockdown hPBMDM were treated with E2, a significant increase in apoptotic cells was observed (55% in E2 treated group as compared to ~ 10% in the untreated group) (Figure 5.19, C) as detected by Annexin-V-PI staining of these cells. Also, this E2-induced cell death in Bcl-2 knockdown hPBMDM was abrogated by pre-incubation with the estrogen receptor antagonist ICI 182,780 (Figure 5.19, C, (iii)). All these data taken together indicate that the phenomenon studied was similar between human THP-1 macrophages and human peripheral blood monocyte derived macrophages (hPBMDM).

5.14 Summary

In this chapter, we have demonstrated that estrogen does not affect the viability of either THP-1 macrophages or human peripheral blood monocyte derived macrophages. However, E2 treatment of human macrophages results in elicitation of both a death signal in the form of Bax translocation to the mitochondria and a survival signal in the form of Bcl-2 up-regulation.

The up-regulation of Bcl-2 upon E2 treatment is mediated by signaling via the membrane localized ER-α as demonstrated by the ability of membrane-impermeable form of E2, E2-BSA to up-regulate Bcl-2 as well as by the abrogation of this effect by siRNA mediated knockdown of ER-α but not ER-β. Furthermore, we have revealed the signaling pathway downstream of ER-α as involving a rapid Ca^{2+} influx via the L-type calcium channel, protein kinase c activation, phosphorylation of ERK and CRE mediated transcriptional up-regulation of Bcl-2. In contrast, the activation and mitochondrial translocation of Bax could not be mediated by E2-BSA, suggesting that

Bax translocation requires signaling via the intracellular pool of estrogen receptors. We further demonstrated by siRNA mediated knockdown experiments that the ER-β sub-type is responsible for mediating translocation of Bax. The signaling downstream of ER-β did not involve Ca^{2+} influx, or ERK phosphorylation as demonstrated by the inability of verapamil, the L-type Ca^{2+} channel inhibitor, or the MEK inhibitor to abrogate the translocation of Bax. However, we established that E2 treatment results in a rise of intracellular pH by about 0.2 units and this intracellular alkalinization mediates activation and mitochondrial translocation of Bax. Also, we demonstrated that this increase in intracellular pH was a Na+-dependent process involving the activation of sodium-hydrogen exchangers (NHE).

This chapter also established that E2-induced increase in Bcl-2 is essential for macrophage survival as interference with Ca^{2+} influx or PKC activation or ERK phosphorylation which inhibits the up-regulation of Bcl-2 resulted in a dramatic increase in cell death in these cells. Similar results were obtained upon siRNA mediated knockdown of Bcl-2 or ER-α wherein cell death ensued upon E2 treatment due to the unopposed action of Bax. However, the complete abrogation of cell death upon inhibition of Bax translocation by disallowing the intracellular pH changes established the crucial role of Bax in mediating the pro-apoptotic effect of E2 in human macrophages. Hence, the results in this chapter demonstrate that the ratio of Bax and Bcl-2 play a critical role in the maintenance of human macrophage survival upon exposure to estrogen.

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Chapter 6

Effect of estrogen on infection-inflammatory functions of human macrophages

6.1. Introduction

Macrophages play a crucial role in combating infection and are as well responsible for elicitation of a number of inflammatory reactions which is responsible for maintaining health and disease. The gender bias observed in a number of infectious diseases such as leishmaniasis, trypanosomiasis, listeriosis, chlamydiasis, etc., (1) are hypothesized to be mediated by the presence of the female sex hormone estrogen, though it has been demonstrated in a few studies that testosterone and progesterone too can lead to significant immunomodulation (2). A recent study has demonstrated the use of Tamoxifen, a partial estrogen agonist in the treatment of visceral and cutaneous leishmaniasis in rodent models (Miquel et al, 2009), though no studies have been performed to deduce its mechanism of action (3). To understand the role of estrogen in modulating macrophage functions and to determine if estrogen was involved in retention of *Leishmania* in macrophages, we used both THP-1 cells as well as mice to explore the function of *Leishmania* infected macrophages under estrogen influence.

6.2. Effect of estrogen on macrophage phagocytosis

Phagocytosis is an essential function of macrophages necessary for uptake and clearance of parasites as well as for clearance of dead and dying cells in various tissues (4). Upon treatment with E2, about 10% more macrophages showed uptake of at least one Alexa fluor 488-labeled *E.coli* (Figure 6.1, A, (iii)) as compared to cells that were in an estrogen-deprived environment (Figure 6.1, A, (ii)). Moreover, as observed from the histogram plot, E2 treatment of human THP-1 macrophages results in an enhancement of the number of *E.*coli particles taken up per macrophage (Figure 6.1, B) demonstrating that E2 enhances the phagocytic ability of human macrophages in a small but significant manner. Moreover, the pure estrogen receptor antagonist ICI 182,780 was able to antagonize the E2-induced enhanced uptake of *E.coli* (Figure 6.1, A and B) demonstrating the effect to be mediated *via* signaling through the estrogen receptor.

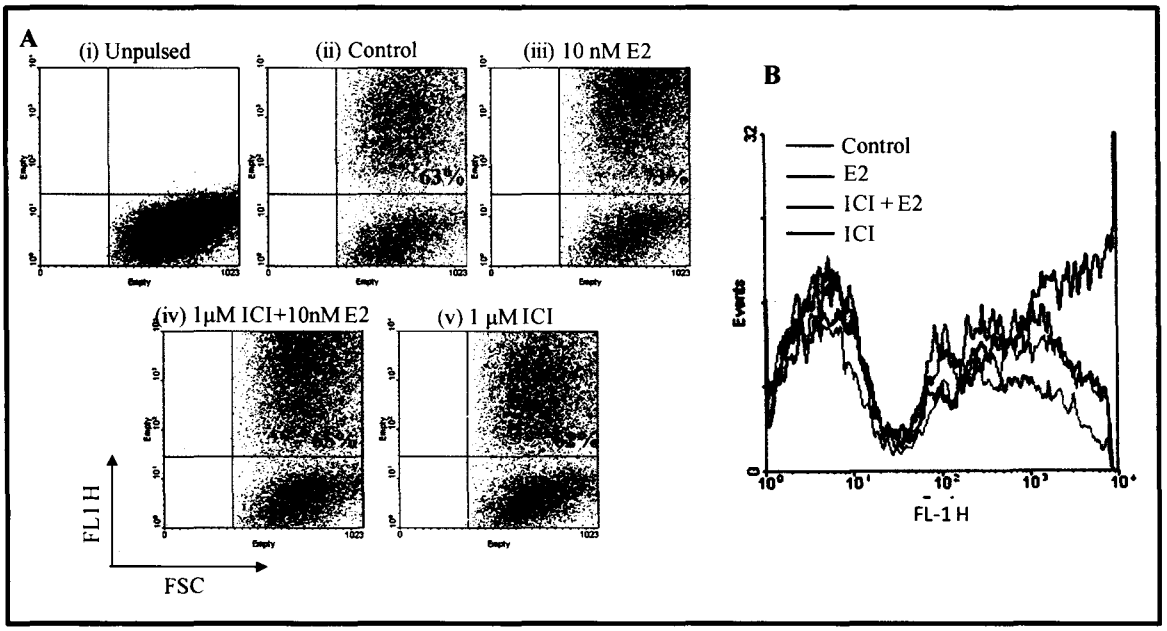

Figure 6.1. Estrogen enhances macrophage phagocytosis. THP-1 macrophages were treated with 10 nM E2 for 12 h and pre-incubated with or without 1μM ICI 182,780. These macrophages were incubated with opsonized Alexa fluor 488-labeled dead *E.coli* for 1 h and the uptake of bacteria was monitored by assessing Alexa fluor 488 fluorescence by flow cytometry. The dot plot (A) represents the bacterial uptake of macrophages. The percentages within the right upper quadrant represent the percent macrophages that have taken up at least one bacteria. The same data has been represented as a histogram plot (B) which demonstrates a higher number of *E.*coli particles taken up per responding macrophage as compared to control.

6.3. Estrogen and macrophage reactive oxygen species generation

As demonstrated above, E2 enhances the phagocytic ability of human macrophages. It is well known that macrophage phagocytosis of bacteria and parasites results in the elicitation of a respiratory burst through the NADPH oxidase system leading to generation of reactive oxygen species such as hydrogen peroxide, superoxide etc., (5). Hence, we analyzed if the enhancement in phagocytic ability was coupled to an increased reactive oxygen species (ROS) generation. Upon treatment with several concentrations of E2 (Figure 6.2, A,), no significant change in basal ROS levels were detected over a time period of 6 h as compared to control cells (Figure 6.2, A, blue line). However, several studies have demonstrated that estrogen serves as an anti-oxidant by decreasing ROS levels through an increase in the levels of glutathione, or by altering the activity of superoxide dismutatses (6). Hence, we tested the possibility that estrogen may alter the ROS generation mediated by common inflammatory stimuli such as bacterial lipopolysaccharide (LPS). However, exposure

of human macrophages to E2 did not affect the LPS-induced generation of superoxide (Figure 6.2, B) suggesting that E2 does not affect the basal ROS or the LPS-induced generation of ROS in THP-1 macrophages.

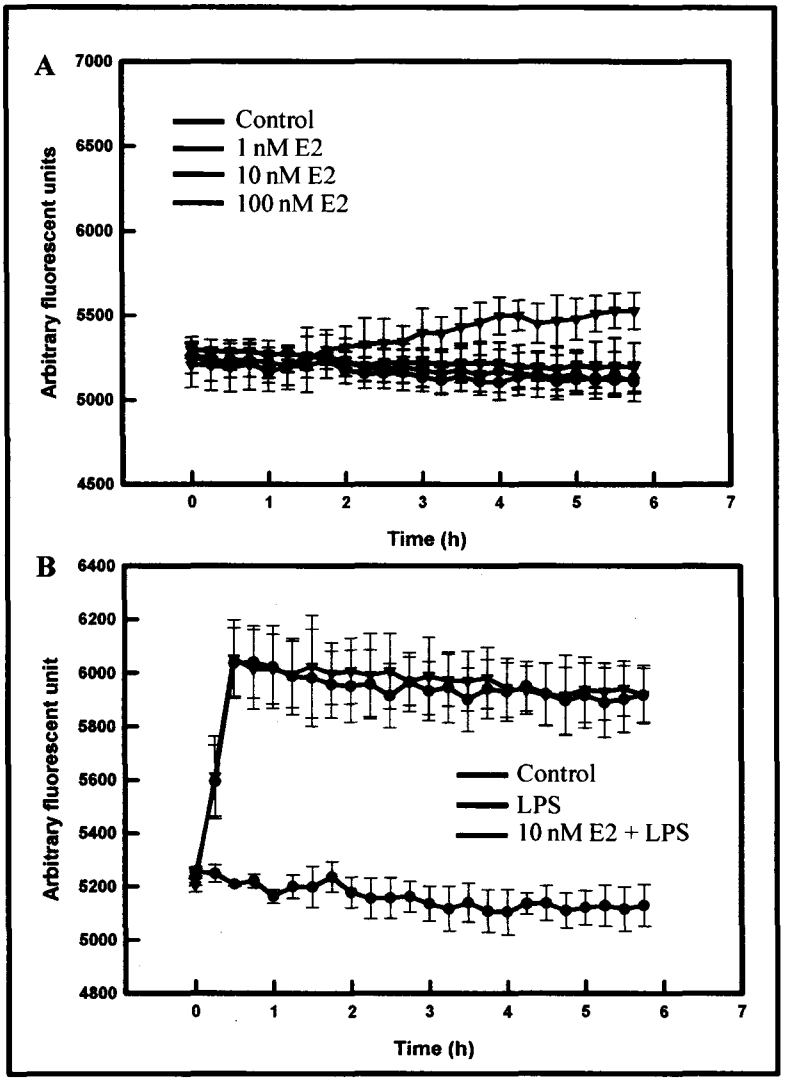

Figure 6.2. Estrogen does not affect ROS generation. (A) Time-kinetic measurement of intracellular reactive oxygen species (ROS) by fluorimetry using CMH_2DCFDA in THP-1 macrophages treated with the indicated concentrations of E2. (B) THP-1 macrophages were pre-incubated with 10 nM E2 for 12 h following which they were exposed to LPS (30 μg/mL) and intracellular ROS was measured using CMH_2DCFDA.

6.4. Role of estrogen in nitric oxide generation in human macrophages

The generation of nitric oxide (NO) plays a crucial role in the clearance of several pathogenic species by macrophages including *Leishmania* as demonstrated by non-remitting infection in $iNOS^{-/-}$ mice (7). Interestingly, E2 at tested concentrations of 10 nM and 100 nM enhanced the generation of NO in human THP-1 macrophages

(Figure 6.3, A) as demonstrated by measurement of intracellular NO using the fluorescent NO indicator DAF-FM. Previous studies have indicated that E2 may enhance NO levels by either activating the inducible nitric oxide synthase (iNOS) (8) or by acutely stimulating constitutive NOS (cNOS) (9). Aminoguanidine, a specific inhibitor of iNOS was able to abrogate the E2-induced generation of NO (Figure 6.3, A), suggesting that the process was probably mediated by activation of iNOS. Further evidence for the involvement of iNOS was derived by semi-quantitative RT-PCR analysis of mRNA transcript for iNOS and as shown in Figure 6.3, B, treatment of THP-1 macrophages with E2 resulted in an increase in the levels of iNOS transcript. Hence, it can be inferred that E2 enhances the generation of NO in human macrophages through the activation of iNOS.

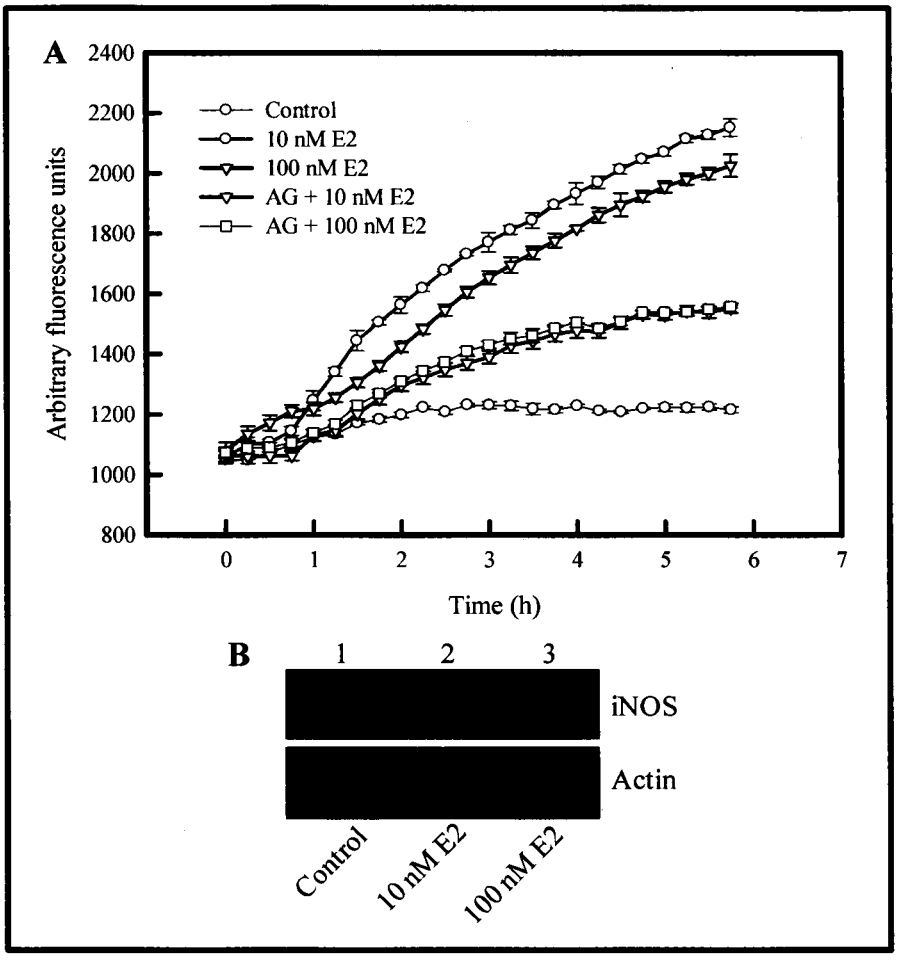

Figure 6.3. Estrogen increases NO generation *via* an iNOS dependent mechanism. (A) Intracellular nitric oxide (NO) measurement by fluorimetry using DAF-FM dye in THP-1 macrophages treated with 10 nM or 100 nM E2 with or without pre-incubation with aminoguanidine (100 μM) for 1 h prior to E2 addition. (B) Semi-quantitative RT-PCR analysis of iNOS expression using specific primers in THP-1

macrophages treated with either 10 nM or 100 nM E2 for 3 h. PCR for actin was used as an endogenous loading control.

6.5. Effect of estrogen on LPS-induced cytokine secretion in THP-1 macrophages

One of the major mechanisms involved in mediating clearance of infection as well as the progression of various pathological disorders is regulated by the secretion of a number of cytokines by macrophages (10). The ability of E2 to modulate LPS-induced secretion of cytokines was studied by using cytokine specific enzyme linked immunosorbent assay (ELISA). E2 was able to inhibit the LPS-induced secretion of IL-1β (Figure 6.4, A), IL-8 (Figure 6.4, B) and IL-12 (Figure 6.4, C). Also, ICI 182,780, the pure estrogen receptor antagonist was able to antagonize this inhibitory effect of estrogen indicating it to be an estrogen receptor mediated phenomenon (Figure 6.4, A-C).

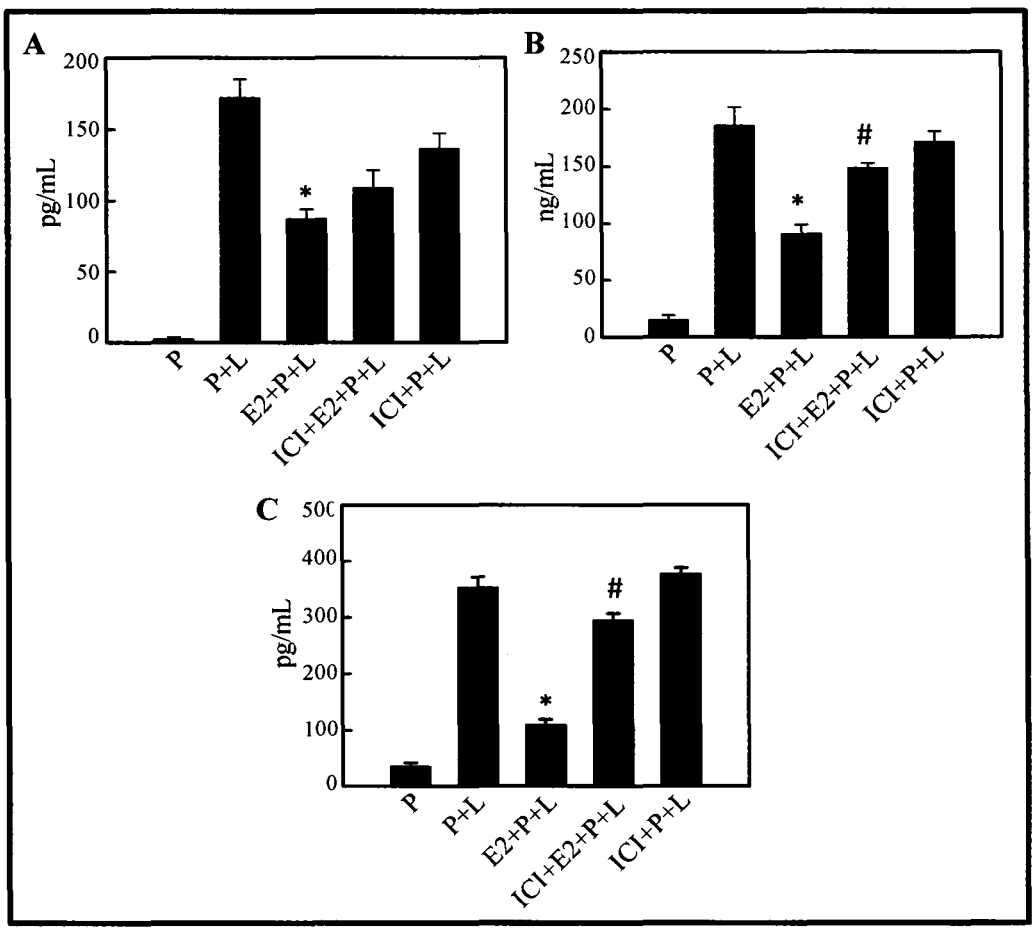

Figure 6.4. Estrogen inhibits secretion of IL-1, IL-8 and IL-12. THP-1 macrophages were pre-incubated with 10 nM E2 for 12 h in the presence or absence of 1 μM ICI 182,780 following which the

cells were exposed to LPS (100 ng/mL) for 12 h. The cell culture supernatants were used for cytokine ELISA for IL-1β (A), IL-8 (B), and IL-12 (C).

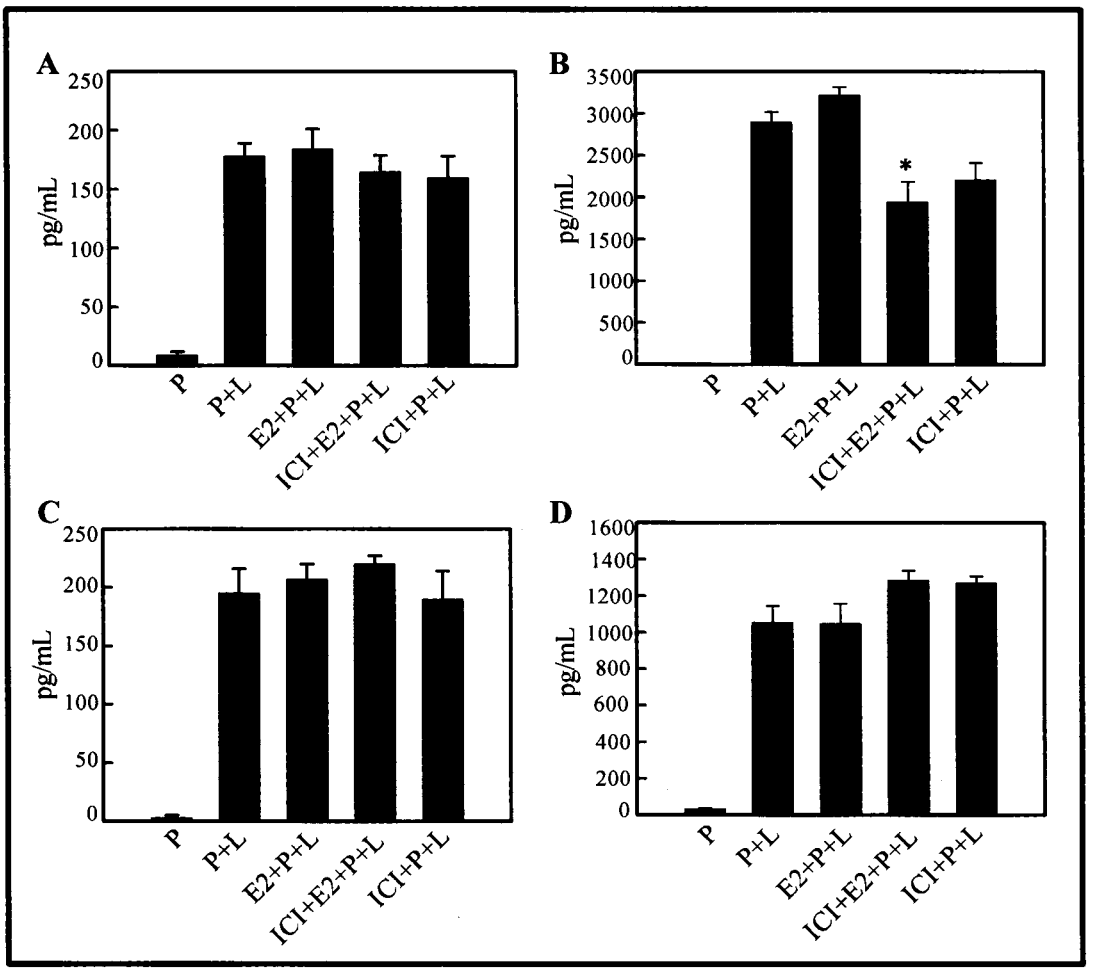

Figure 6.5. Estrogen does not affect the secretion of IL-4, IL-6, IFN-γ and TNF. THP-1 macrophages were pre-incubated with 10 nM E2 for 12 h in the presence or absence of 1 μM ICI 182,780 following which the cells were exposed to LPS (100 ng/mL) for 12 h. The cell culture supernatants were used for cytokine ELISA for IL-4 (A), IL-6 (B), IFN-γ, and TNF (C).

However, E2 was unable to modulate the LPS-induced secretion of IL-4, IL-6, IFN-γ, and TNF (Figure 6.5, A-D). ICI 182,780, the estrogen receptor antagonist by itself inhibited the LPS-induced secretion of IL-6 (Figure 6.5, B), the reason for which is not known.

6.6. Effect of estrogen on leishmanial infection *in vitro*

From the data presented above, it is evident that estrogen modulates several crucial functions performed by macrophages, for e.g., it enhances phagocytic ability, enhances NO generation, and modulates the secretion of a number of pro-inflammatory cytokines. Many of these functions are critical to the clearance of leishmanial infection *in vitro* and *in vivo*. Hence, we investigated the role of estrogen in modulating leishmanial infection of human macrophages *in vitro* using *L.major*-THP-1 macrophages as a host-pathogen model system. THP-1 macrophages were readily infected with opsonized stationary phase promastigotes of *L.major*, a representative infection is shown in Figure 6.6. Infection rates of ~ 60% is obtained routinely with around 3-4 parasites per infected macrophage. Further, the infection is retained within the macrophages for upto 72 h, but shows a gradual decline over the time period (~55% at 6 h to ~35% at 72 h).

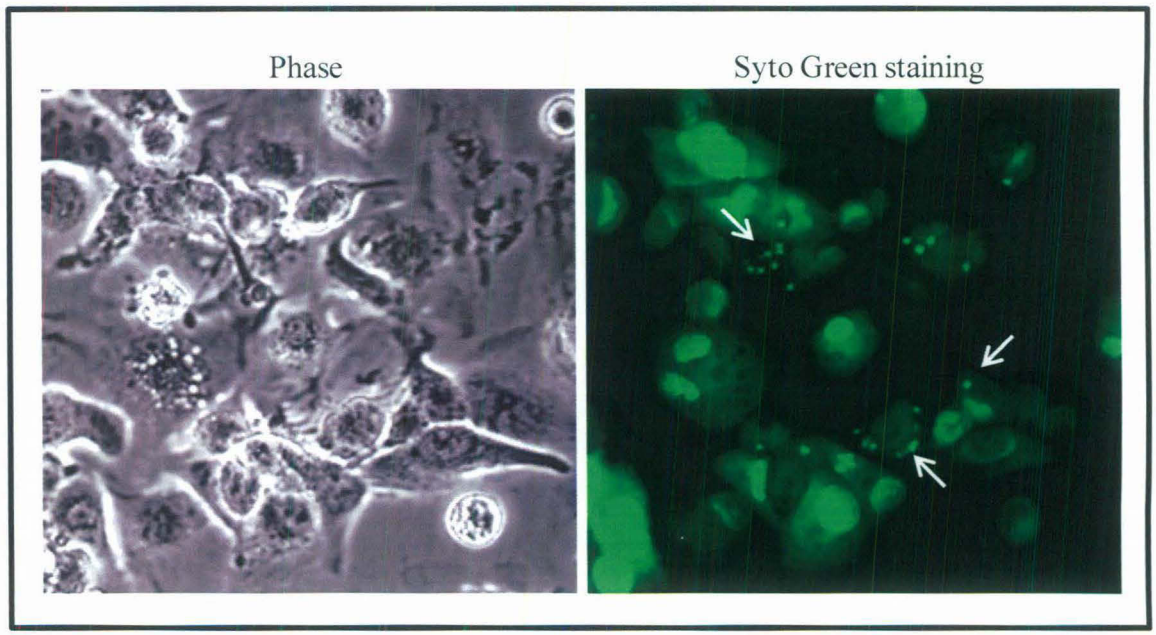

Figure 6.6. *L.major* infection in THP-1 macrophages. THP-1 macrophages were infected with opsonized stationary phase promastigotes of *L.major* at an multiplicity of infection (MOI) of 1:10 for 6 h. Infection was monitored by staining cells with Syto Green nuclear staining dye and observed under fluorescence microscope. The arrows point to intracellular *L.major* parasites.

When the effect of E2 on *in vitro L.major* infection was investigated, it was observed that at both an multiplicity of infection (MOI) of 1:10 and 1:50 there was enhanced uptake of parasites at 3 h post-infection (Figure 6.7). However, when infection was monitored beyond 6 h and upto 48 h, no significant differences were observed

between E2 treated macrophages and control macrophages (Figure 6.7, A and B). This suggested that E2 does not significantly alter the kinetics of infection or progression and clearance of *L.major* infection *in vitro*, except for the finding of

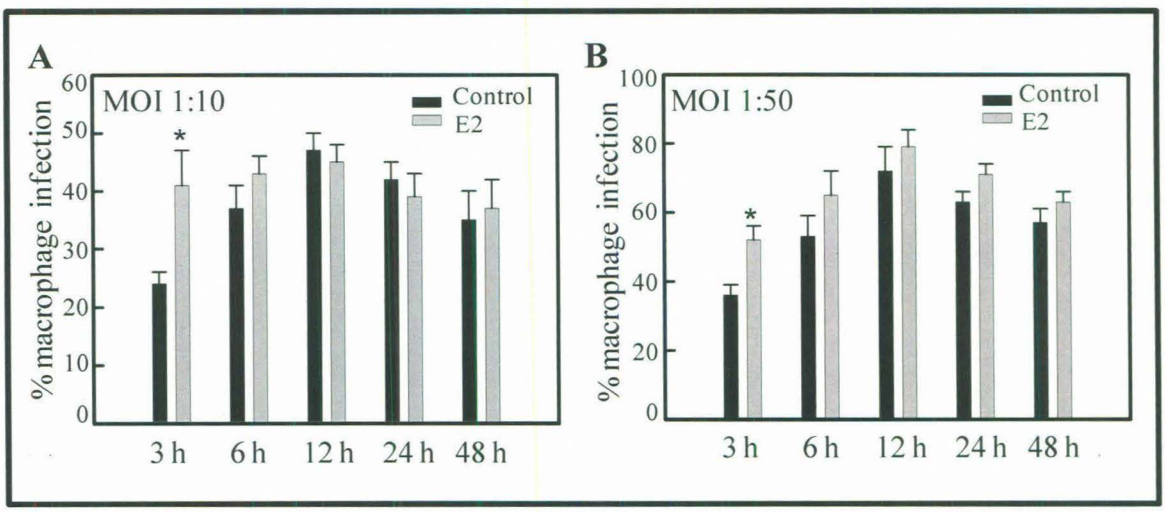

Figure 6.7. Effect of estrogen on *L.major* infection in macrophages. THP-1 macrophages treated with or without 10 nM E2 for 12 h were incubated with opsonized stationary phase promastigotes of *L.major* at an MOI of 1:10 (A) or 1:50 (B). The percentage macrophage infection was monitored under fluorescence microscope at indicated time intervals after staining the cells with the fluorescent nuclear dye Syto Green. The bar represents the percent macrophage infected with at least one parasite. The error bars represent ±SEM (n=3).

enhanced uptake of parasites in E2 treated macrophages at 3 h post-infection which could be attributed to the E2-induced enhancement in macrophage phagocytic ability as has been demonstrated earlier in our study with *E.coli* (Figure 6.1, A).

6.7. Effect of estrogen on *L.major* infection in mouse footpad

As demonstrated above, estrogen did not affect *L.major* infection of human macrophages *in vitro*. It is known that clearance of *Leishmania* infection *in vivo* is mediated by the co-ordinated activity of macrophages, T-cells and B-cells including the profile of cytokines secreted (Th1 *vs.* Th2). Hence, to determine whether estrogen could have effects on the onset and progression of *Leishmania* infection *in vivo*, we employed a cutaneous infection model of *L.major* in the mouse footpad. Estrogen

depletion in mice was achieved by performing bilateral oophorectomy while female mice subjected to sham surgery served as an estrogen replete model. The onset and progression of cutaneous *L.major* infection was monitored by measuring the size of the footpad lesion.

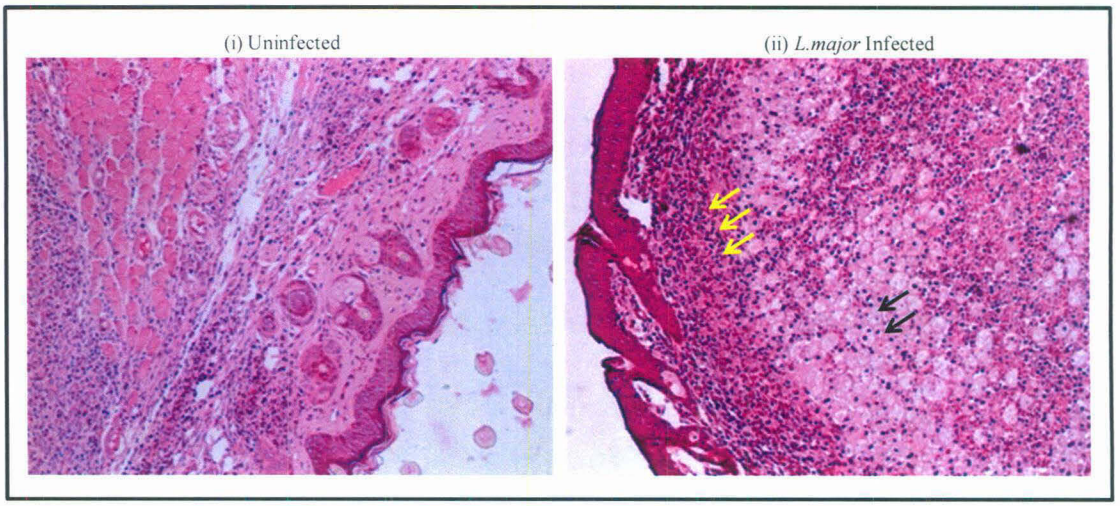

Figure 6.8. Histopathology of footpad of BALB/c mice infected with *Leishmania major*. Footpads were sectioned at 6 weeks post-infection with *Leishmania major* and stained with hematoxylin-eosin. Note the intense leukocytic infiltrate (yellow arrows) and the vacuolated macrophages containing intracellular amastigotes (black arrows) in *L.major* infected footpad (ii) as compared to the well defined cutaneous morphology of normal footpad (i).

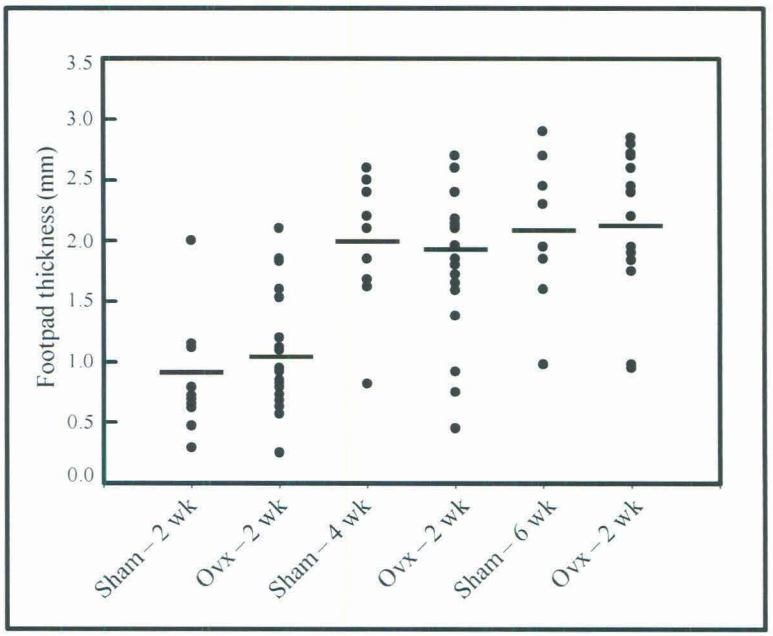

Figure 6.9. Progression of leishmanial infection in oophorectomized mice. Female BALB/c mice were either subjected to bilateral oophorectomy or sham surgery and 6 weeks post-surgery *Leishmania major* were injected into the footpad. The increase in thickness of footpad was monitored by

measurement at 2 weekly intervals. The spots represent individual animals (n=10 in sham group and n=20 for oophorectomized group). The horizontal bar represents the average for each group. Ovx, Oophorectomized mice.

Several cross-sections of normal and infected footpad were made and stained with eosin and hematoxylin to assess the gross morphology upon infection. As shown in Figure 6.8, there was an intense leukocytic infiltration in the dermal and sub-dermal tissue of the infected footpad as compared to the normal footpad (Figure 6.8, ii). Also, the pathognomonic vacuolated macrophages containing intracellular amastigotes were seen in the infected footpad. These findings established successful generation of cutaneous leishmaniasis in mice footpad. When onset and progression of cutaneous leishmaniasis was compared between oophorectomized mice and mice subjected to sham surgery, there were no significant differences between the groups (Figure 6.9).

6.8. Summary

In this chapter we have demonstrated that estrogen modulates a number of macrophage functions, for e.g. estrogen (i) enhances the phagocytic ability, (ii) enhances nitric oxide generation via activation of iNOS as revealed by an increase in iNOS transcript as well as by absence of NO generation in the presence of aminoguanidine, a specific iNOS inhibitor, and (iii) suppresses inflammatory stimuli induced release of IL-1β, IL-8 and IL-12 in human macrophages. However, estrogen did not affect the endogenous or inflammatory stimuli induced generation of ROS. Furthermore, the ability of the pure estrogen receptor antagonist, ICI 182,780 to abrogate these effects demonstrated that estrogen modulates macrophage function by signaling via the estrogen receptor. The exposure of human macrophages to estrogen does not affect the clearance of *L.major* parasites in THP-1 cells as demonstrated by the absence of any significant difference in the number of infected macrophages or the infective load per macrophage at 48 h post-infection. Interestingly, estrogen treated macrophages show higher percentage of infection at an early time interval of 3 h post-infection which might be mediated by the ability of estrogen to enhance phagocytosis. To confirm the *in vitro* data, *in vivo* infection studies were carried out in a mouse model of cutaneous leishmaniasis following bilateral oophorectomy. The

data revealed no significant difference in the size of the footpad lesion following infection between the oophorectomized mice and the sham mice demonstrating that estrogen does not affect either the onset or progression of leishmaniasis.

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Chapter 7

Discussion

This thesis presents evidence of involvement of estrogen in modulating the death pathway in human macrophages and delineates the involvement of distinct subset of estrogen receptors in executing estrogen induced survival or death signals. In addition, the downstream signaling cascade induced by estrogen is also discussed. The data presented in this thesis provides a comprehensive study of relationship of estrogen to human macrophage survival.

Estrogen exerts its effect on cells by binding to estrogen receptors localized in different sub-cellular compartments and signals arising from these distinct receptor pools could have different functional outcomes. Hence, it was important to understand the sub-cellular distribution of these receptors in human macrophages. This is the first study to clearly demonstrate the expression of the two sub-types of estrogen receptor ER-α and ER-β as well as their sub-cellular localization in human macrophages. Estrogen receptors are classically described as nuclear-localized transcription factors (1), however, our data in human macrophages demonstrates these receptors to be distributed in the cytoplasm and plasma membrane with no localization observed within the nucleus. The distribution of receptors on the plasma membrane is in corroboration with a growing body of evidence showing the presence of membrane associated ERs in various cell types which are shown to be involved in rapid signaling events and in mediating non-genomic effects of estrogen (2-4). Furthermore, we demonstrate a differential expression of estrogen receptor sub-types in different sub-cellular locations, for eg., ER-α was localized predominantly on the plasma membrane and the cytosol whereas ER-β was expressed exclusively in the cytoplasm suggesting the possibility of differential involvement of the receptors in mediating diverse estrogen actions. The localization of ER-α on the plasma membrane was confirmed by several lines of evidence including (a) the binding of E2-BSA to the cell surface receptors, (b) recognition of ER-α binding sites on live cells by specific anti-ER-α antibody and (c) knockdown of surface ER-α receptors by siRNA for ER-α but not ER-β. Furthermore, the estrogen receptors expressed on THP-1 macrophages and human peripheral blood monocyte derived macrophages were functional as demonstrated by the ability of ICI 182,780, the pure estrogen receptor antagonist to inhibit both the estrogen-induced increase in Bcl-2 expression as well as mitochondrial translocation of Bax. In this context, the presence of functional membranous estrogen receptor is of particular interest because surface receptors are

amenable to selective manipulation by cell-impermeable estrogen agonists. Also, the development of ER-α and ER-β selective agonists and antagonists (5-7) could be used to explore the role of signaling from these receptors in distinct locations to gain insights into the differential activities of these receptor sub-types.

Signals transmitted through the estrogen receptors can affect several signaling pathways in the cell. Our interest was to establish the relationship between estrogen action and cell survival or death in human macrophages. Estrogens are known to modulate macrophage survival *via* signaling through an extrinsic death receptor pathway (8) or by modulation of pro- and anti-apoptotic proteins of the intrinsic pathway of apoptosis (9). In this study, we demonstrate that estrogen does not alter the survival of human macrophages; however, it modulates the expression levels of both pro- and anti-apoptotic members of the Bcl-2 family of proteins through differential involvement of estrogen receptor sub-types localized in distinct sub-cellular compartments thereby establishing the mechanism by which estrogen regulates the macrophage mitochondrial death pathway. Our report illustrates that exposure of human macrophages to estrogen elicits both an anti-apoptotic and pro-apoptotic signal in the form of up-regulation of Bcl-2 and mitochondrial translocation of Bax respectively. The signal for estrogen-induced Bcl-2 upregulation originated from the membrane bound ER-α as demonstrated by the ability of membrane impermeable form of estrogen, E2-BSA to upregulate Bcl-2 as well as by abrogation of Bcl-2 upregulation following siRNA mediated selective knockdown of ER-α but not ER-β. Similar instances of estrogen-induced Bcl-2 upregulation have been demonstrated in other cell types such as B-cells (10), neuronal cells (11) and MCF-7 breast cancer cells (12). In contrast to upregulation of Bcl-2, the observed translocation of Bax was independent of membrane bound estrogen receptors because E2-BSA was unable to induce Bax migration. Furthermore, the translocation of Bax to mitochondria was dependent on intracellular ER-β receptors as demonstrated by abrogation of Bax translocation upon knockdown of ER-β but not ER-α. This data indicated the importance of ER-β in death inducing arm of the mitochondrial apoptotic pathway. This function has not been demonstrated in cells of monocytic origin but mediation of pro-apoptotic events by ER-β is known in cells of non-myeloid lineage like breast and colon cancer cells (13, 14).Clearly, estrogen was able

to influence both pro- and anti-apoptotic pathways by signaling through distinct estrogen receptor sub-types.

Having established that estrogen modulates both pro- and anti-apoptotic proteins, it was important to understand the signaling cascade involved in mediating these changes. To explore the downstream signaling responsible for the up-regulation of Bcl-2 and Bax translocation, we evaluated the possible role of Ca^{2+} signaling as in many cellular systems changes in intracellular Ca^{2+} is an important event determining survival (15). Also, there is a close relationship between Bcl-2 and Ca^{2+} because while Ca^{2+} can mediate Bcl-2 increase, Bcl-2 can also regulate cellular Ca^{2+} through manipulation of endoplasmic reticulum Ca^{2+} stores (16). We observed that estrogen induced a rapid increase in intracellular Ca^{2+} in human macrophages which was inhibited by the L-type Ca^{2+} channel blocker verapamil with consequent inhibition of Bcl-2 increase demonstrating that the process of Bcl-2 upregulation was Ca^{2+} dependent. This data suggested a situation similar to hippocampal neurons where estrogen activates rapid Ca^{2+} influx (17) to mediate Bcl-2 upregulation. The transcriptional upregulation of Bcl-2 was unlikely to be mediated via direct binding of estrogen-ER complex to estrogen response elements (ERE) on Bcl-2 promoter (12) as inhibition of events such as Ca^{2+} influx and ERK phosphorylation could inhibit the increase in Bcl-2. In contrast to Bcl-2 upregulation, Bax translocation was independent of Ca^{2+} influx and ERK phosphorylation as demonstrated by the inability of verapamil and PD 98,059 to inhibit Bax migration. The translocation of Bax to the mitochondria upon receipt of an apoptotic stimulus is dependent upon alteration in its conformation resulting in the exposure of its N-terminal or BH-3 domain (18, 19) and is under the control of various physiological factors (20). Prior knowledge that a change in pH could trigger Bax movement (21-23) prompted us to focus on the possibility of estrogen inducing a pH change in THP-1 macrophages. Our findings show that estrogen induces intracellular alkalinization which acts as a cue for Bax migration because inhibition of alkalinization prevented Bax movement. Interestingly, Bax translocation could be initiated upon intracellular alkalinization even in the absence of estrogen, suggesting that movement of Bax was likely to be facilitated by any stimulus capable of altering cellular pH. Estrogen-induced intracellular alkalinization was mediated by activation of sodium-hydrogen exchangers (NHE) since the process was Na^{+}-dependent and could be inhibited by amiloride, a selective

NHE inhibitor (24). A number of studies show that estrogen can alter NHE functions (25) through a NHE regulatory factor (NHE-RF) which is a primary response gene under ER control (26). However, in our system, this was unlikely because the change in pH was very rapid, making transcriptional regulation an unlikely event. However, the mechanistic basis of estrogen-mediated activation of NHE is unclear and requires further investigation.

In several systems, cellular survival is dependent on the ratio of Bax and Bcl-2. In the mitochondria, Bax could either be overwhelmed by the amount of Bcl-2 present and the equilibrium will shift to Bcl-2 favoring survival or in low Bcl-2 conditions Bax will prevail and facilitate release of cytochrome c ensuring cell death (27). Since estrogen provoked both a pro- and anti-apoptotic signal in human macrophages, we hypothesized that the upregulation of Bcl-2 could serve as a homeostatic survival mechanism to counteract the effects of activated Bax. As Bax translocation was independent of Ca^{2+} increase and activation of the PKC pathway, this provided us with an opportunity to investigate Bcl-2 function without interfering with Bax translocation. The crucial role of estrogen induced Bcl-2 increase was apparent from experiments where L-type Ca^{2+} channel blocker verapamil restricted Ca^{2+} influx and the consequent inhibition of the PKC pathway prevented ERK phosphorylation resulting in Bcl-2 decrease but did not inhibit Bax translocation as a result of which cell death ensued. Although the above data confirmed the importance of the altered Bax/Bcl-2 ratio for survival, inhibition of both PKC and ERK activity could lead to alteration in other pathways as well introducing artifacts. Therefore, to unequivocally prove the involvement of Bcl-2 in macrophage survival at the time of estrogen exposure, we used either Bcl-2 siRNA or ER-α siRNA to knockdown Bcl-2 expression, both of which resulted in significant increase in macrophage death. The above observation established that Bcl-2 upregulation is an absolute requirement for macrophage survival in the presence of estrogen. Moreover, it was not surprising when a substantial increase in cell survival was observed after estrogen-induced pH change was blocked leading to prevention of Bax translocation at the time of Bcl-2 knockdown, a situation that normally precipitates cell death. This clearly validates the observation that Bax migration to mitochondria due to pH change in the absence of concomitant Bcl-2 upregulation is responsible for the increased apoptosis. Clearly, estrogen shows two distinct set of effects; while increase in Ca^{2+} leads to ERK

phosphorylation resulting in an increase in Bcl-2 which is an anti-apoptotic signal, a distinct pro-apoptotic signal in the form of translocation of Bax from cytosol to mitochondria was generated by a Ca^{2+} signaling independent but estrogen receptor dependent pathway involving intracellular alkalinization. Therefore, in macrophages, estrogen shows a dichotomous effect and depending on other factors that could influence Bax or Bcl-2 proteins in a given circumstance, a survival or a death pathway would be chosen (Figure 7.1.).

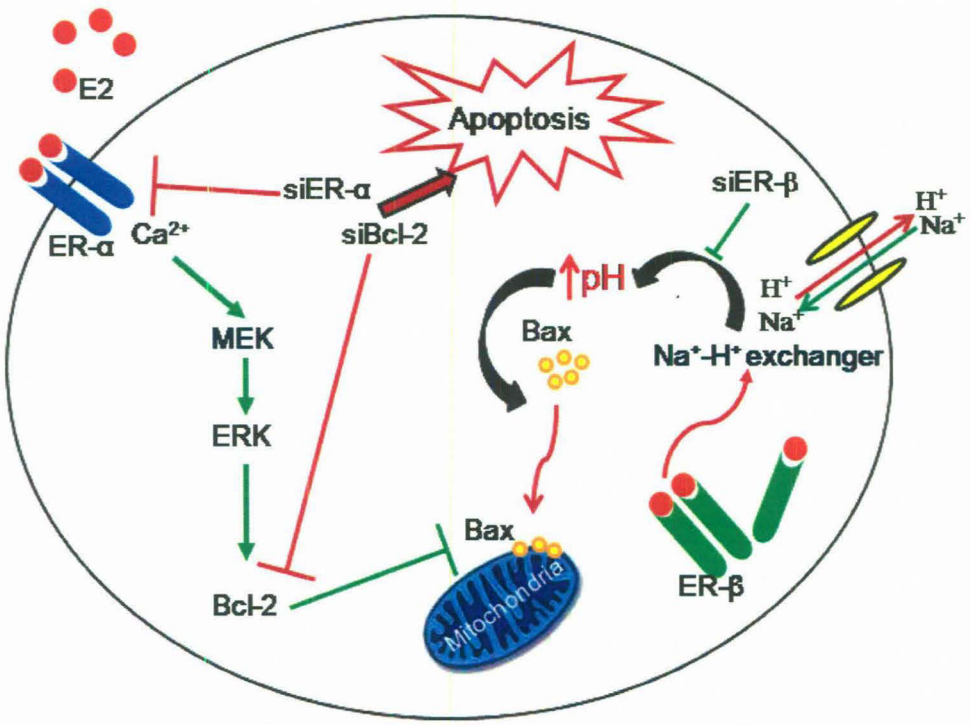

Figure 7.1. Schematic showing intracellular signaling pathways involved in estrogen action on human macrophages. The model diagram illustrates the dichotomous effect of estrogen on human macrophage survival. The pathway highlighted in green represents the anti-apoptotic signals and those highlighted in red represent the pro-apoptotic signals activated by estrogen. Estrogen signaling via ER-α induces an increase in Ca^{2+} leading to ERK phosphorylation and consequently CREB phosphorylation, resulting in an increase in Bcl-2. Translocation of Bax from cytosol to mitochondria is dependent on intracellular alkalinization mediated by signaling through ER-β via activation of Na^{+}-H^{+} exchangers. Interference with Bcl-2 increase through inhibition of Ca^{2+} (verapamil), PKC (BIM), phosphorylation of ERK (PD 98,059), ER-α knockdown or Bcl-2 knockdown leads to cell death in the presence of estrogen due to elevated levels of activated mitochondria-localized Bax.

Estrogen induced immunomodulation can be mediated by its effect on altering the cellular survival of macrophages or by a direct influence on various macrophage functions. Estrogen mediated modulation of macrophage function has been implicated in the gender bias observed in several autoimmune and infectious diseases (28). Our study has demonstrated that estrogen enhances the phagocytic ability of human macrophages, which is in concurrence with other studies performed in murine macrophages (29). This increase in ability to phagocytose may have important consequences in terms of infectious diseases as most pathogenic organisms are cleared by macrophages in the acidic lysosomal compartment following phagocytosis. On the contrary several pathogens utilize the receptor mediated phagocytic pathway to infect macrophages and in these situations, the enhancement of phagocytic ability by estrogen may be counter-productive. Also, this phenomenon may be relevant in the pathogenesis of auto-immune disorders, as elicitation of inflammatory reaction following phagocytosis of apoptotic cells is widely reported to mediate several pathologic features of these disorders (30). Though several studies have reported anti-oxidant effect of estrogen in murine macrophages (31), our study performed with human macrophages established that estrogen does not play a role in regulating either the basal or inflammatory stimuli induced ROS generation. However, estrogen induced the release of nitric oxide (NO) from human macrophages *via* stimulation of iNOS which is in concurrence with other studies performed on murine and human macrophages (32, 33). Also, estrogen inhibited the LPS-induced secretion of several pro-inflammatory cytokines such as IL-1β, IL-8, and IL-12. It is interesting to note that estrogen inhibits IL-1β and IL-8, both of which are known to mediate acute phase reaction in response to injury and inflammation (34), thereby partly explaining the protective effect of estrogen noted in several trials studying the effect of estrogen supplementation in the treatment of endotoxic shock (35, 36). Also, IL-8 is known to mediate chemotaxis of neutrophils and monocytes to inflammatory sites and thereby estrogen by inhibiting IL-8 may mediate anti-inflammatory effects (37). The evidence that estrogen inhibits the secretion of IL-12, which is one of the cytokines influencing the generation of Th1-type response (38), partly explains the mechanism of skewed Th2-type response observed in females in general and more so in pregnant women (39). This skewing to Th2-type response may have consequences in several auto-immune and infectious diseases, for eg., clearance of leishmanial infection requires a robust Th1-type response, while elicitation of Th2-type response leads to unremitting

disease (40). Given the fact that estrogen modulates several factors responsible for macrophage mediated clearance of leishmanial infection such as phagocytosis, nitric oxide generation, inhibition of IL-12 secretion etc., it was interesting to note that in our study, there was no significant effect of estrogen on onset or progression of leishmanial infection *in vitro* or *in vivo*. This is in corroboration with other epidemiological studies where no significant difference was observed in the incidence of infection between males and females (41) and possibly the gender bias observed in certain other studies may be mediated by differential rates of exposure to an infected sandfly bite due to occupational reasons (42) as well as sex-based factors independent of hormonal effects. Hence, further investigations are necessary to determine the mechanistic basis of gender bias observed in human leishmaniasis.

How might our findings be integrated into a model of macrophage behavior in high estrogen microenvironments? From a physiological point of view, estrogen induced Bcl-2 upregulation is obligate for cellular survival and hence necessary for the manifestation of the immunomodulatory effects of estrogen on macrophages. From a pathological point of view, there are two possible scenarios in which this estrogen mediated cell survival could be of relevance. Optimally, macrophage survival at tumor or inflammatory sites during chemotherapy is essential during the orderly process of cell death to restrict harm to host tissues (43). Our report provides a new insight into macrophage function in response to estrogen where the hormone is able to generate a cyto-protective response in macrophages through manipulation of the Bcl-2 proteins. This will be crucial under conditions of Bcl-2 downregulation (44, 45) when macrophage population will be depleted if Bcl-2 knockdown is attempted in tumors of estrogen targets. On the contrary, in many tumors, tumor associated macrophages play a pro-tumorogenic role by secreting growth factors and angiogenic factors and current anti-tumor strategy includes targeted destruction of these cells in which case macrophage killing through Bcl-2 downregulation would be beneficial for tumor therapy.

In summary, this study highlights the importance of estrogen signaling via distinct receptor subtypes localized in different sub-cellular compartments in modulating the mitochondrial death pathway of human monocyte derived macrophages. The observations raise interesting possibilities of exploring the use of

selective estrogen receptor modulators specific for ER-α or ER-β or those which could signal exclusively through the membranous or cytoplasmic pool of receptors. For example, estren which is an agonist signaling selectively on the membranous ER with no known transcriptional effects via the classical ER mechanism (46) could be used for generating anti-apoptotic effects. The development and use of such agents will further enhance our understanding of the role of estrogen receptors localized in distinct sub-cellular compartments and could also be used to target specific receptor population to achieve desired therapeutic effects like manipulation of death pathways in favour or against cell survival.

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Chapter 8

Summary

The modulation of macrophage survival and function by estrogen (E2) is of immense importance in the context of regulation of all functions pertaining to immune responses. Previous studies have described the effect of E2 on survival and function of murine and rat macrophages and these studies have been used to extrapolate the findings to human macrophages. However, the effect of E2 is highly species and cell-type specific and therefore to understand E2 action on human cells it is essential to directly investigate the effect of the hormone on macrophages derived from human sources. This thesis is the first description of the effect of E2 on human macrophage survival and the signaling cascades involved in the macrophage apoptotic pathway. For this study, human THP-1 monocytic cells, an acute monocytic leukemia cell line was differentiated to macrophages and used as a model system to study estrogen action. In addition, ex-vivo human peripheral blood monocyte derived macrophages were used to verify findings derived from the model cell line.

Our data clearly demonstrates expression of the two sub-types of E2 receptors ER-α and ER-β in the THP-1 macrophage cell line as well as in human peripheral blood monocyte-derived macrophages. These receptors are differentially distributed in the various sub-cellular compartments, for e.g., ER-α sub-type is expressed on the plasma membrane and the cytoplasm, while the ER-β sub-type is expressed exclusively in the cytoplasm. This distribution pattern is unlike in several other cells where the E2 receptors are localized within the nucleus. The knowledge of the distribution pattern of receptors is important in the context of studying functional effects of E2 and this study demonstrates the importance of such differential distribution in executing E2 function as described below.

E2 induces both a pro-survival and a pro-apoptotic signal in the form of upregulation of Bcl-2 and mitochondrial translocation of Bax respectively in both THP-1 derived macrophages as well as in human peripheral blood monocyte derived macrophages. The E2-induced upregulation of Bcl-2 was mediated through membrane bound ER-α receptors as was evident from the ability of membrane impermeable E2 to stimulate Bcl-2 increase and downregulation of this increase through ER-α siRNA mediated intervention. This ER-α knockdown mediated Bcl-2 inhibition was specific because siRNA against ER-β could not inhibit Bcl-2 increase. The critical role of Bcl-2 upregulation was evident from the dramatic increase in cell death when Bcl-2 elevation was inhibited by using either Bcl-2 or ER-α siRNA thereby allowing the

translocated Bax to carry out the pro-apoptotic function. This demonstrated that though there was an increase in the level of pro-apoptotic Bax in the mitochondria upon exposure to E2, a concomitant upregulation of mitochondria localized Bcl-2 ensured that the net ratio of Bcl-2:Bax remained the same thereby favoring cell survival. The Bax translocation signals were mediated through ER-β because ER-β siRNA interfered with the translocation. The stimulus for change in Bax location coming from E2 was independent of surface receptors because membrane impermeable E2 and ER-α siRNA were ineffective in inducing or abrogating translocation respectively. Therefore, dichotomous effect of E2 mediated through the two ER subtypes, ER-α mediating Bcl-2 increase and ER-β arbitrating Bax translocation was evident. These data taken together demonstrate that there is a fine balance between the levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax upon exposure to E2, and the ratio of the two determines cell fate.

The signaling pathways for E2 receptor induced effects on the Bcl-2 and Bax were primarily worked out on THP-1 cells. The E2-induced upregulation of Bcl-2 was transcriptionally regulated and could be inhibited by (a) siRNA for Bcl-2 (b) the L-type Ca^{2+} channel inhibitor verapamil, (c) PKC inhibitor bisindoleylmaleimide VIII and (d) MEK inhibitor PD 98,059 demonstrating that the upregulation of Bcl-2 was signaled by a process which was dependent upon Ca^{2+} influx via the L-type Ca^{2+} channel, with subsequent activation of PKC, phosphorylation of ERK, and activation of CREB. On the contrary, E2-induced activation and mitochondrial translocation of Bax occurred *via* signaling through intracellular alkalinization mediated by activation of sodium-hydrogen exchangers as demonstrated by the inhibition of Bax translocation by specific sodium-hydrogen exchanger inhibitor amiloride that lowered Na^{+} levels and prevented alkalinization of the cytoplasm. Inhibition of Bax translocation by preventing intracellular alkalinization rescued the Bcl-2 knockdown cells from death upon exposure to E2. This confirmed that Bax acts as the major pro-apoptotic signal upon exposure to E2.

This thesis also establishes that E2 modulates a number of macrophage functions via an estrogen receptor dependent pathway, for e.g., it enhances the macrophage phagocytic ability, increases nitric oxide generation by activation of iNOS, and inhibits LPS induced secretion of inflammatory cytokines such as IL-1β,

IL-8 and IL-12. Despite the ability of E2 to modulate a number of factors that may affect the outcome of macrophage infection by intracellular pathogens, no significant effect of E2 was observed on the initial infection or clearance of *Leishmania major* parasites *in vitro* or the onset or progression of *L.major* infection *in vivo* in a mouse model of cutaneous leishmaniasis.

Taken together, this study highlights the importance of E2 signaling through distinct ER subtypes in modulating the mitochondrial death pathway of human monocyte derived macrophages and underscores the importance of integrative signaling modality from multiple estrogen receptor pools in modulating E2 responses in human macrophages. The observations raise interesting possibilities of exploring the use of selective estrogen receptor modulators specific for ER-α or ER-β or those which could signal exclusively through the membranous or cytoplasmic pool of receptors to manipulate death pathway in human macrophages. The development and use of such agonists and antagonists could be used to target specific receptor population in target cells to achieve desired therapeutic effects like manipulation of death pathways in favor or against cell survival.

Up-Regulation of Bcl-2 through ERK Phosphorylation Is Associated with Human Macrophage Survival in an Estrogen Microenvironment[1]

Manikandan Subramanian and Chandrima Shaha[2]

Estrogen is a known immunomodulator with pleiotropic effects on macrophage function that partly accounts for the gender bias observed in numerous autoimmune, cardiovascular, and neurodegenerative disorders. The effect of estrogen on the survival of human macrophages is largely unknown, and in this study we demonstrate that 17β-estradiol (E2) provokes a death response in human THP-1 macrophages by initiating Bax translocation from cytosol to the mitochondria; however, a concomitant up-regulation of Bcl-2 creates a Bax to Bcl-2 ratio favorable for Bcl-2, thus ensuring cell survival. Both Bcl-2 up-regulation and Bax translocation are estrogen receptor-dependent events; however, Bcl-2 augmentation but not Bax translocation is dependent on Ca^{2+} increase, activation of protein kinase C, and ERK phosphorylation. This estrogen-induced Bcl-2 increase is crucial for the survival of THP-1 macrophages as well as that of human peripheral blood monocyte-derived macrophages, which is evident from E2-induced cell death under small interfering RNA-mediated Bcl-2 knockdown conditions. Hence, this study demonstrates that E2-induced Bcl-2 up-regulation is a homeostatic survival mechanism necessary for the manifestation of immunomodulatory effect of estrogen on human macrophages. *The Journal of Immunology,* **2007, 179: 2330–2338.**

Macrophages express estrogen receptor subtypes α and β (1–3) and are therefore capable of responding to increase in estrogen during the follicular phase of menstrual cycle (4), at the time of exposure from exogenous sources such as phytoestrogens (5), following administration for prophylactic and therapeutic purposes (6), or during accidental exposure to estrogenic chemicals (7, 8). Estrogen affects a variety of macrophage functions; for example, it can reduce accumulation of cholesteryl esters in macrophages (2), stimulate production of NO (9, 10), increase arachidonic acid release (11), regulate activation-related events (2, 12), decrease monocyte adhesion to vasculature (13), enhance macrophage phagocytosis (14), and facilitate Ca^{2+} influx (10). Some functions are implicated in mediating the gender bias observed in numerous autoimmune (15), cardiovascular (16), and neurodegenerative disorders (17). In addition, estrogen is able to modulate macrophage death, which is of great relevance because macrophage survival or death is crucial for disease pathogenesis (18). However, data available on the influence of the hormone on the macrophage cell death process are contradictory. Existing literature show that macrophage-like U937 cells undergo apoptosis when exposed to estrogen (19), but the same cell type is protected from TNF-α-induced apoptosis by the hormone (20). According to other reports, estrogen is able to induce apoptosis in undifferentiated U937 monocytes, but macrophages differentiated from these cells are refractory to such effects of estrogen (21). Further examples of cell types in which estrogen is reported to induce cell death include bone macrophages like murine osteoclasts, preosteoclastic FLG 29.1 cell line, and mouse peritoneal macrophages (22–25).

The apparently paradoxical effect of estrogen on apoptosis in cells of the monocytic lineage could be interpreted to be the result of its ability to differentially modulate antiapoptotic and proapoptotic proteins like the members of the Bcl-2 family that share sequence-homology domains within the group (26). The various proapoptotic and antiapoptotic Bcl-2 family members are able to heterodimerize (26), and their relative concentrations function as a rheostat for the apoptotic program (26). Certain apoptotic stimuli like exposure to NO (27), oxysterol (28), and activation-inducing agents increase macrophage Bcl-2 or other members of the Bcl-2 family of proteins like Bfl-1 (29), but the involvement of Bcl-2 family members in regulating the macrophage death pathway is not completely understood. In the context of tumor development, mechanisms regulating macrophage death are important because these cells constitute a large proportion of the tumor cells and are evidently important for either progression or regression of tumors (30). Survival of macrophages in estrogen microenvironment is relevant especially in the cells populating estrogen target tissues like uteri, breast, brain, and cervix. Also, understanding of the mechanism of macrophage survival under altered Bcl-2 level becomes important in the backdrop of development of Bcl-2 small molecule inhibitors, antisense oligonucleotides, and RNA interference against Bcl-2, which were intended to be used for treatment of resistant carcinoma and some of which are currently in phase I and phase II clinical trials (31–35). This study was designed to identify the key players that are vital for modulating human macrophage survival under estrogen exposure.

We demonstrate that 17β-estradiol (E2)[3] treatment not only provokes a death response via Bax translocation in macrophages derived from THP-1 human acute monocytic leukemia cells, but also

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[3] Abbreviations used in this paper: E2, 17β-estradiol; siRNA, small interfering RNA; MFI, mean fluorescence intensity; fluo-3-AM, fluo-3 acetoxymethyl ester; BIM, bisindoleylmaleimide; MDM, monocyte-derived macrophage; PKC, protein kinase C.

initiates an antiapoptotic response through the up-regulation of Bcl-2 via a Ca^{2+}-dependent ERK-mediated pathway. The importance of E2-induced Bcl-2 up-regulation in macrophage physiology is demonstrated by increased cell death when Bcl-2 is down-regulated through interference with Ca^{2+} influx, ERK phosphorylation, or small interfering RNA (siRNA)-mediated Bcl-2 mRNA degradation. E2 also induces cell death in human peripheral blood monocyte-derived macrophages (MDM) when Bcl-2 is knocked down with anti-Bcl-2 siRNA.

Materials and Methods

Materials

E2 (cyclodextrin encapsulated) was obtained from Sigma-Aldrich. ICI 182780, PPT (4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol), and DPN (2,3-bis(4-hydroxyphenyl)-propionitrile) were procured from Tocris Cookson. Primary Ab against Bcl-2 was obtained from Santa Cruz Biotechnology. Abs against phospho-ERK, whole ERK, and phospho-CREB were purchased from StressGen Biotechnologies. Anti-Bax, anti-cytochrome *c*, and anti-CD14 FITC Abs were purchased from BD Biosciences. Secondary Abs raised in either mice or rabbits conjugated to HRP were obtained from Jackson ImmunoResearch Laboratories. Fluo-3-acetoxymethyl ester (fluo-3-AM) and secondary anti-mouse IgG conjugated to Alexa Fluor 488 were obtained from Molecular Probes. Anti-actin and anti-estrogen receptor C-terminal Abs were from Calbiochem. The Vybrant apoptosis detection system was purchased from Promega. All reagents for Western blotting and ECL development were obtained from Amersham Biosciences. The Bcl-2 siRNA was purchased from Upstate Biotechnology, whereas Cy3-labeled negative control siRNA was procured from Ambion. Transpass R2 transfection reagent was from New England Biolabs. Phenol-red free RPMI 1640 and dextran-coated charcoal stripped FCS was obtained from Biological Industries. Verapamil, PMA, Ca^{2+} ionophore A 23187, EGTA, PD98059, bisindoleylmaleimide (BIM), Histopaque 1077, and any other chemical used were obtained from Sigma-Aldrich, unless otherwise mentioned.

Peripheral blood monocyte isolation, cell lines, and cell culture

Peripheral blood was collected from healthy male volunteers after obtaining informed consent as per regulations of the Institutional Human Ethics Committee (National Institute of Immunology, New Delhi, India). The PBMC were isolated by density gradient centrifugation using Histopaque 1077 where human whole blood was layered on Histopaque 1077 and centrifuged at 400 × *g* for 35 min at 25°C. The mononuclear cell population was isolated from the plasma-histopaque interface, and the monocytes were further purified by washing off the nonadherent cells after incubating the total PBMC for 1 h at 37°C. The homogeneity of the obtained population was determined by analyzing immunostaining of the cells with an Ab against monocyte-specific marker CD14-conjugated to FITC. The monocytes were further cultured for 7 days to allow macrophage differentiation. THP-1 cells, a human acute monocytic leukemia cell line obtained from American Type Culture Collection were maintained in RPMI 1640 medium supplemented with 10% FCS. Macrophage differentiation was induced by treatment of THP-1 cells with 10 ng/ml PMA for 36 h, following which cells were maintained in PMA-free medium for 12 h before experimentation. Forty-eight hours before experiment, both cell types were transferred to phenol red-free RPMI 1640 supplemented with 10% dextran-coated charcoal-stripped FCS to remove any extraneous source of estrogen.

Intracellular free Ca^{2+} assay

Changes in intracellular free Ca^{2+} concentration were monitored using the Ca^{2+} binding fluorescent probe fluo-3-AM as previously described (36). Briefly, 10^6 cells/ml loaded with 0.5 μM fluo-3-AM containing 0.5 μM pluronic acid F-127 were used for different experiments, and free Ca^{2+} was monitored at an excitation of 480 nm and emission of 520 nm with a Fluostar Optima spectrofluorometer (BMG Technologies). Fluorescence values were converted into absolute intracellular free Ca^{2+} concentration, using procedures and calculations as previously described (36).

Cell viability assay

For cell viability assays, propidium iodide exclusion by cells and identifying phosphatidylserine exposure by Annexin V-labeling was conducted as previously described (37) using Vybrant apoptosis assay kit. Labeling was analyzed by flow cytometry in which data acquisition was done with a BD LSR flow cytometer equipped with a 488 nm air-cooled argon ion laser. Analysis was done using WinMDI software (Microsoft v.2.8).

RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies), and cDNA was synthesized as previously described (36). The specific primers used were the following: Bcl-2 (sense) 5′-GTGGAGGAGC TCTTCAGGGA-3′, (antisense) 5′-AGGCACCCAGGGTGATGCCA-3′; and actin (sense) 5′-GTGGGGCGCCCCAGGCACCA-3′, (antisense) 5′-CTCCTTAATGTCACGCACGATTTC-3′.

PCR was performed after determining the cycle number in which a linear amplification of serially diluted template could be achieved. The PCR products were then resolved on 1.5% agarose gel and visualized by ethidium bromide staining and quantitated by densitometry.

siRNA transfection

THP-1 macrophages and human peripheral blood MDM were transfected with 15 pmol SMARTpool Bcl-2 siRNA or negative control siRNA using Transpass R2 transfection reagent as per the manufacturer's instructions. Briefly, Bcl-2 siRNA or negative control siRNA were added to transfection reagent diluted in serum-free medium and incubated for 20 min to allow the formation of transfection complexes. The siRNA transfection complexes were added at a final concentration of 15 pmol to 10^5 cells/well grown in 24-well plates and incubated for 6 h following which fresh complete medium was added. Transfection efficiency was estimated by observing Cy3 fluorescence of the negative control siRNA with a Nikon TE2000-E fluorescence microscope using a tetramethyl rhodamine filter (530–580 nm). Target protein knockdown was assessed 24 h posttransfection by probing extracts of transfected cells on Western blots with anti-Bcl-2 Ab.

Subcellular fractionation

THP-1 macrophages were harvested and suspended in homogenization buffer (0.5 M sucrose, 10 mM Tris, pH 7.4) containing 1 mM EDTA and protease inhibitor cocktail from Roche Diagnostics with a mixture of various protease inhibitors. Cell lysis was performed by sonication (Sonifier 450; Branson) on ice at 30% duty cycle for a total of 9 cycles. The sonicate was centrifuged at 4000 × *g* for 10 min to obtain the nuclear pellet. From the resulting supernatant, the mitochondrial fraction was extracted by further centrifugation at 10,000 × *g* for 10 min (38) in an ultracentrifuge (SW55Ti rotor, optima XL-110K; Beckman Coulter). The postmitochondrial supernatant was further centrifuged at 100,000 × *g* for 30 min to isolate the microsomal fraction as a pellet and the supernatant obtained was the cytosolic fraction.

SDS-PAGE and Western blot

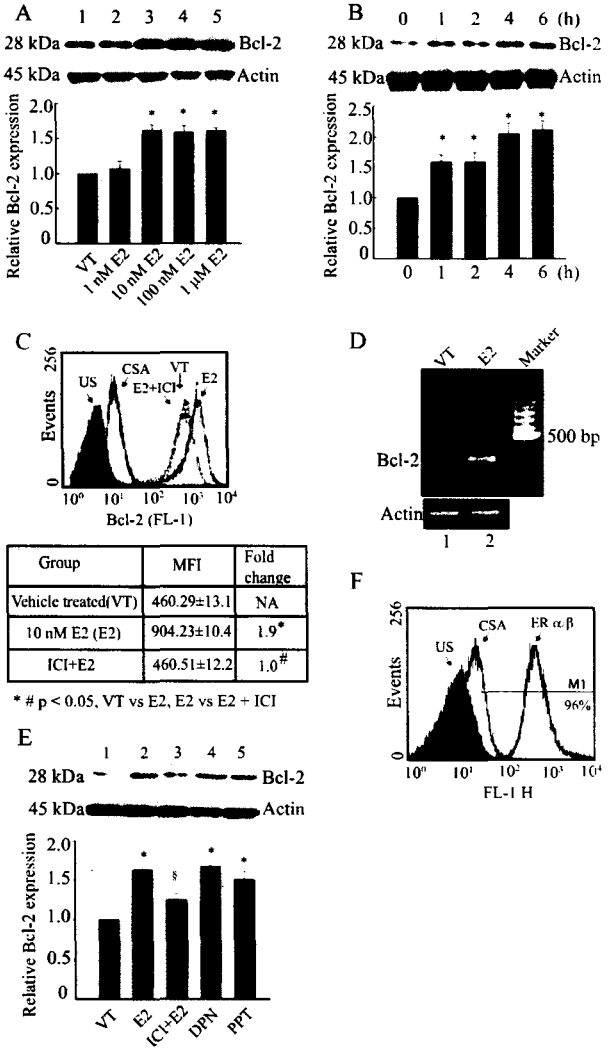
Whole cell lysates were prepared by mixing cells with lysis buffer (0.125 M Tris, 4% SDS, 20% glycerol, and 10% 2-ME), and the lysates were resolved on 12% SDS-PAGE gel following which they were transferred onto nitrocellulose membrane as previously described (36). The quantitation of protein in cell lysates was conducted with a CBX protein assay kit (G-Biosciences). Western blots were incubated with 5% ECL blocking reagent in 0.05% PBS-Tween 20 for 1 h to block nonspecific binding sites. Primary and secondary Abs were used at appropriate dilutions and reactivity was visualized by ECL using an ECL Western blotting detection system in which membranes were exposed to x-rays for appropriate time periods and subsequently developed before densitometry.

Immunocytochemistry

Formaldehyde (4%) fixed cells were blocked with 3% normal goat serum containing 0.1% saponin at room temperature for 30 min and subsequently incubated with primary Ab at 1/50 dilution for 1 h at 37°C followed by secondary Ab conjugated to Alexa Fluor 488 at 1/100 dilution for 1 h at the same temperature. Subsequently, the cells were washed and resuspended in PBS and analyzed on a BD LSR flow cytometer equipped with an air-cooled 488 nm argon ion laser.

Densitometry

Densitometric measurements for quantitation of signals on immunoblots or ethidium bromide stained agarose gels were performed using a UVP gel documentation instrument, and the acquired data were analyzed with the LabWorks image analysis and acquisition software (v.4.0.0.8.; UVP). At least three Western blots per experiment were quantitated to arrive at the average value of the signal. All measurements were normalized to internal loading controls.



Group	MFI	Fold change
Vehicle treated(VT)	460.29±13.1	NA
10 nM E2 (E2)	904.23±10.4	1.9*
ICI+E2	460.51±12.2	1.0#

* # $p < 0.05$, VT vs E2, E2 vs E2 + ICI

FIGURE 1. Estrogen increases Bcl-2 expression in THP-1 macrophages through an estrogen receptor-dependent mechanism. *A*, Increase in Bcl-2 levels after 24 h treatment with various doses of E2 is shown on representative immunoblots of THP-1 macrophage lysates probed with anti-Bcl-2 Ab (80 ng/ml). Actin was used as a loading control. *B*, Time-kinetic analysis of Bcl-2 expression with THP-1 macrophage lysates collected at different time points after 10 nM E2 exposure (0–6 h) showing a time-dependent increase in Bcl-2 levels. Actin was used as a loading control. *C*, Flow cytometric analysis of Bcl-2 expression as determined by immunostaining of THP-1 macrophages with anti-Bcl-2 mAb (600 ng/ml) after 24 h of treatment of cells with E2 and ICI 182780. Note the distinct shift obtained with E2 as compared with vehicle treated control (VT) and E2 with ICI 182780 (E2+ICI). CSA, control secondary Ab; US, unstained cells. Data shown at bottom are the MFI of the indicated treatment groups and the fold change in Bcl-2 expression ($n = 3$). *, $p < 0.05$ for vehicle treated vs E2 treated; #, $p < 0.05$ for E2 vs E2+ICI. *D*, RT-PCR analysis for the expression of Bcl-2 at 12 h after treatment with E2 (10 nM) (E2) showing increase in Bcl-2 amplicon (304 bp) with cDNA isolated from THP-1 macrophages (*lane 2*) as compared with vehicle treated (VT) control (*lane 1*). Actin RT-PCR served as loading control. *E*, Immunoblots for Bcl-2 on THP-1 extracts at 24 h after the following treatments: *lane 1*, vehicle treated (VT); *lane 2*, 10 nM E2 treatment; *lane 3*, pretreatment with 1 μM ICI 182780 before E2 treatment; *lanes 4* and *5*, treatment with 100 nM DPN, an estrogen receptor-β-specific agonist; and 100 nM PPT, an estrogen receptor-α-specific agonist. Data shown below immunoblots represent the relative Bcl-2 expression levels normalized to actin as compared with vehicle treated control, calculated by densitometric analysis of multiple immunoblots as detailed in *Materials and Methods*. Error bars in *A*, *B*, and *E* are ± SEM with $n = 3$ experiments in duplicate. *, $p < 0.05$ as compared with vehicle treated; §, $p < 0.05$ as compared with E2 treated. *F*, Estrogen receptor expression was analyzed by flow cytometry with THP-1 macrophages immunostained with anti-estrogen receptor Ab reactive to both estrogen receptors α and β. The marker represents the percentage population that stain positive for the receptors. CSA, control secondary Ab.

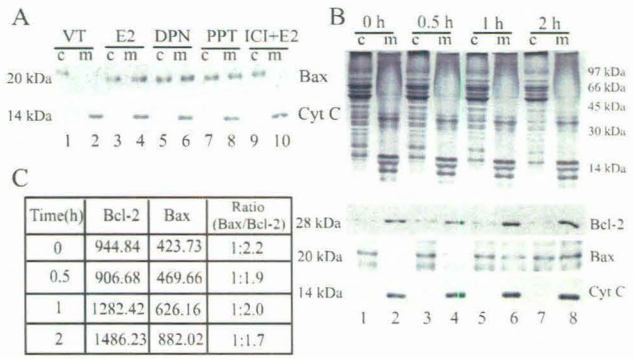
Statistical analysis

Data were analyzed by Student's *t* test and values are expressed as mean ± SEM. The values were considered significantly different at $p < 0.05$.

Results

E2 does not affect THP-1 macrophage viability while it increases Bcl-2 levels

To examine whether E2 has any effect on cell survival, THP-1 macrophages were exposed to several doses of the hormone ranging from 1 nM to 1 μM and cell death was estimated at 24 h by propidium iodide (1 μg/ml) exclusion method. No significant cell death could be recorded with any of the doses as compared with vehicle treated (cyclodextrin dissolved in water) controls. Percentage of survival was at the following: vehicle treated, 94 ± 1; 1 nM E2 treatment, 95 ± 2; 10 nM E2 treatment, 96 ± 1; 100 nM E2 treatment, 96 ± 1; and 1 μM E2 treatment, 96 ± 2. Contrary to our findings, some studies show that estrogen causes death in cells of monocytic lineage (19, 21–25). Arguably, if estrogen is able to induce death in cells of similar lineage, the failure of the hormone to do so in THP-1 macrophages could mean differential regulation of the proapoptotic and antiapoptotic proteins leading to maintenance of viability. A distinct increase in THP-1 macrophage Bcl-2 above the constitutive levels was observed with 10 nM, 100 nM, and 1 μM E2 treatment (Fig. 1*A*). Because beyond 10 nM there was no appreciable increase in Bcl-2 (Fig. 1*A*) and 10 nM being within the physiological range (39), all other studies used this dose of E2. With the same dose, a time-dependent augmentation in Bcl-2 protein levels occurred from 1 h onward doubling at 4 h from the constitutive levels at 0 h (Fig. 1*B*) and was sustained till 72 h (data not shown). Flow cytometric quantitation of Bcl-2 expression in cells of different treatment groups labeled sequentially with anti-Bcl-2 Ab followed by secondary Ab conjugated to Alexa Fluor 488 showed a distinct shift in the mean fluorescence intensity (MFI), which doubled after treatment with E2 as compared with constitutive Bcl-2 levels (vehicle treated) (Fig. 1*C*). Presence of estrogen receptor antagonist ICI 182780 prevented a shift in fluorophore labeling (Fig. 1*C*, E2+ICI), and the MFI was similar to vehicle treated controls (Fig. 1*C*). Therefore, both densitometric quantification of Western blots and flow cytometric analysis demonstrated a 1.5- to 1.9-fold increase in Bcl-2 expression. An increase in Bcl-2 mRNA transcript level after E2 treatment (Fig. 1*D*) suggested Bcl-2 up-regulation through the genomic route. E2 action on cells could possibly occur through either a receptor-mediated or a receptor-independent pathway (40), therefore to gain insights into which receptor involvement, estrogen receptor antagonists, and agonists were used. Presence of a specific estrogen receptor antagonist ICI 182780 during E2 exposure prevented Bcl-2 up-regulation (Fig. 1*E*, *lane 3*). Subtype-specific estrogen receptor agonists, namely DPN, a specific agonist for estrogen receptor-β (41) and PPT, a specific agonist for estrogen receptor-α (42) were able to up-regulate Bcl-2 (Fig. 1*E*, *lanes 4* and *5*) in the absence of E2. Therefore, data with both agonists and antagonists implicated estrogen receptor involvement. The presence of estrogen receptors was confirmed by flow cytometric analysis of cells stained with anti-estrogen receptor Ab recognizing both estrogen



C

Time(h)	Bcl-2	Bax	Ratio (Bax/Bcl-2)
0	944.84	423.73	1:2.2
0.5	906.68	469.66	1:1.9
1	1282.42	626.16	1:2.0
2	1486.23	882.02	1:1.7

FIGURE 2. Estrogen treatment induces translocation of Bax from cytosol to mitochondria in THP-1 macrophages. *A*, Immunoblots showing Bax translocation performed on cytosolic and mitochondrial fractions obtained from 4 h lysates of THP-1 macrophage exposed to different treatments as indicated. The purity of the mitochondrial fraction was determined by probing for cytochrome *c* (Cyt C) using an anti-cytochrome *c* Ab (500 ng/ml). c, cytosolic fraction; m, mitochondrial fraction. *B*, Time-kinetic analysis of Bcl-2 up-regulation and Bax translocation through immunoblotting of cytosolic and mitochondrial fractions of THP-1 macrophages with anti-Bcl-2 (80 ng/ml) and anti-Bax Abs (500 ng/ml). Silver-stained gel (*top*) shows profiles of mitochondrial and cytosolic fractions with equal amounts of protein loaded. Immunoblot analysis (*bottom three panels*) is shown of Bcl-2, Bax, and cytochrome *c* in cytosol and mitochondrial fraction of cells after 10 nM E2 treatment collected at indicated time points. *C*, Data represents absolute values of Bcl-2 and Bax and their ratio at various time intervals after E2 treatment as determined by densitometric analysis of specific immunoreactive bands on blots, representatives of which are shown in *B*. Data are representative of three independent experiments.

receptors α and β that demonstrated expression of estrogen receptors in >95% of THP-1 macrophages (Fig. 1*F*).

Taken together, these data established the capability of E2 to induce an estrogen receptor-dependent increase in Bcl-2 mRNA and protein in human THP-1 macrophages.

E2 induces Bax translocation to the mitochondria in THP-1 macrophage

In many cell systems, the Bax to Bcl-2 ratio serve as a control point upstream of irreversible damage to cellular constituents where Bax translocation to the mitochondria from cytosol occur upon receipt of apoptotic stimuli (43, 44). In THP-1 macrophages, within 4 h of E2 exposure, translocation of Bax from cytosol to the mitochondria occurred (Fig. 2*A*, *lanes 3* and *4*). Presence of the estrogen receptor antagonist ICI 182780 during E2 treatment prevented this translocation of Bax (Fig. 2*A*, *lanes 9* and *10*). The estrogen receptors β and α agonists DPN and PPT, respectively, were able to induce Bax translocation in the absence of E2 (Fig. 2*A*, *lanes 5–8*). Over a period of 2 h after E2 treatment, there was a clear increase in the expression of mitochondria associated Bcl-2 and Bax (Fig. 2*B*), but the ratio of the two proteins remained in favor of Bcl-2 (Fig. 2*C*). Because of the concomitant increase of Bcl-2 levels the cell survival pathway was favored even after Bax translocation to mitochondria. Taken together, data showed that exposure to E2 induced a death response in the macrophages through a estrogen receptor mediated pathway.

Bcl-2 expression is dependent on intracellular Ca^{2+} concentration

There is a close relationship between Bcl-2 expression and intracellular Ca^{2+} changes because although Bcl-2 can regulate release of Ca^{2+} from the endoplasmic reticulum stores (45), Ca^{2+} is reported to be able to regulate Bcl-2 expression through the ERK

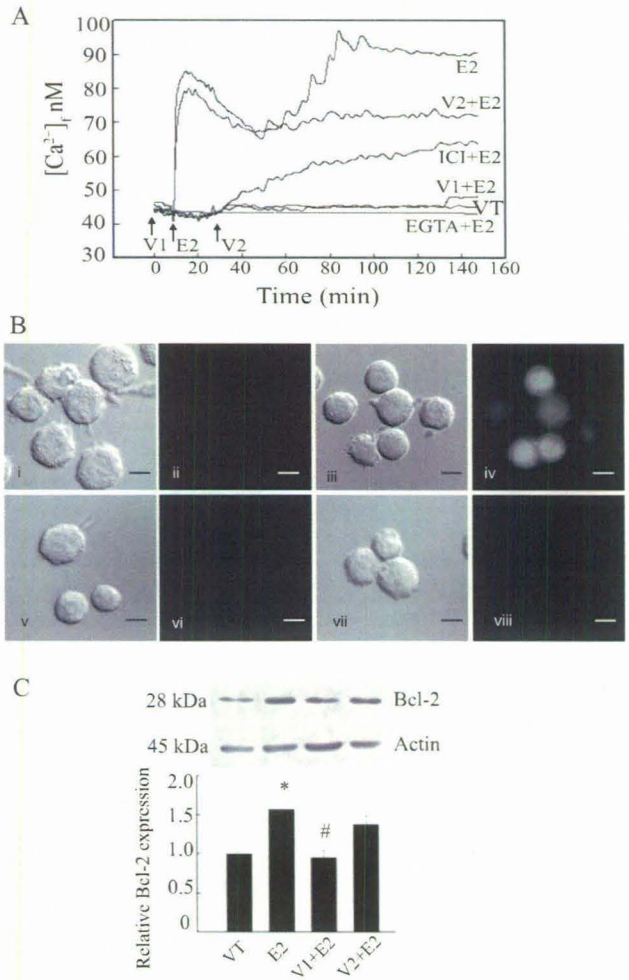


FIGURE 3. E2-induced Ca^{2+} release regulates Bcl-2 expression. *A*, Intracellular free Ca^{2-} was measured using 0.5 μM Ca^{2+} binding dye fluo-3-AM to label 10^6 cells/ml of THP-1 macrophages, and 10 nM E2 was added in the presence or absence of 20 μM verapamil added 10 min before (E2+V1) or 20 min after (E2+V2) the hormone addition, or in the presence of 1 μM ICI 182780 (E2+ICI), or 2 mM EGTA (E2+EGTA) and monitored over 150 min. VT, vehicle treated. *B*, Fluo-3-AM-labeled THP-1 macrophages showing intracellular Ca^{2+}; vehicle treated (ii), 10 nM E2 treatment (iv), 10 nM E2 treatment along with 1 μM ICI 182780 (vi) and 10 nM E2 treatment plus 20 μM verapamil (viii). Panels i, iii, v, and vii represent the respective phase contrast images. Scale bar represents 10 μm. *C*, The effect of 20 μM verapamil on THP-1 macrophage Bcl-2 levels. Verapamil treatment before addition of E2 (V1+E2), verapamil treatment 20 min after E2 addition (V2+E2), E2-treated cells (E2), and vehicle treated control (VT) show the ability of verapamil to reduce E2-induced Bcl-2 levels. Data represent the relative Bcl-2 expression levels normalized to actin as compared with vehicle treated control, calculated by densitometric analysis of multiple immunoblots as detailed in *Materials and Methods*. Error bars are ± SEM with $n = 3$ experiments in duplicate. *, $p < 0.05$ as compared with vehicle treated control, #, $p < 0.05$, ET vs V1+ET.

signaling pathway (46). Intracellular Ca^{2+} levels doubled within a minute after addition of E2 (Fig. 3*A*) and a second peak of Ca^{2+} increase occurred without any further addition of E2 at around 90 min, and this level of ~90 nM was maintained till 140 min, the time point at which the last measurement was made (Fig. 3*A*). Presence of the ICI 182780 (Fig. 3*A*, ICI+E2) could attenuate the increase in Ca^{2+} (Fig. 3*A*), indicating that Ca^{2+} modulation was an estrogen receptor-dependent phenomenon. The source of Ca^{2+}

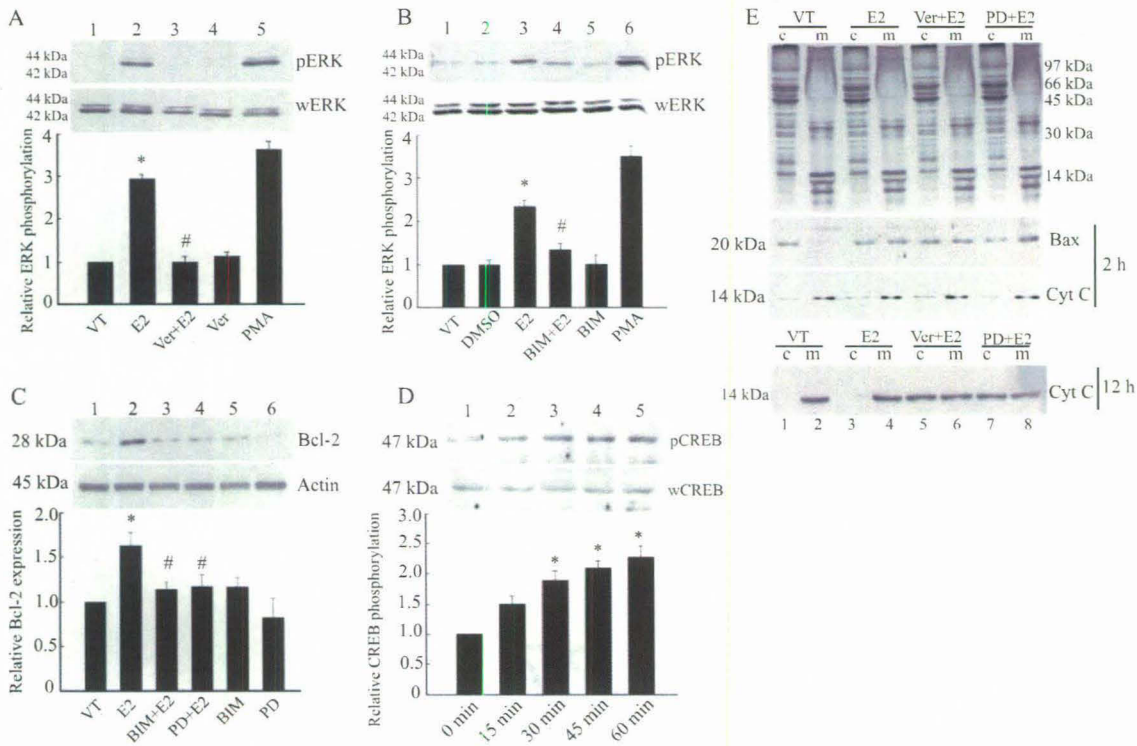


FIGURE 4. E2 induces up-regulation of Bcl-2 by a Ca^{2+}-dependent ERK phosphorylation mechanism. *A*, THP-1 macrophages were preincubated with 20 μM verapamil for 10 min before a 5 min treatment with 10 nM E2. Whole cell lysates were prepared, and Western blots were probed using specific Abs against phosphorylated ERK1/2 (1/1000) or whole ERK1/2 (900 ng/ml). Blot shows distinct ERK phosphorylation on E2 treatment (*lane 2*). Cells treated with PMA (100 nM) were used as a positive control for ERK phosphorylation. *B*, THP-1 macrophages were preincubated with 1 μM BIM, a specific PKC inhibitor for 30 min before a 5 min treatment with 10 nM E2. Western blots of whole cell lysates prepared from cells collected at 5 min after E2 treatment were probed using specific Abs against phosphorylated ERK1/2 or total ERK1/2. DMSO treatment (*lane 2*) was used as a control as BIM was dissolved in DMSO. Cells treated with PMA (100 nM) were used as a positive control. *C*, THP-1 cells were preincubated with 25 μM PD98059 for 10 min or 1 μM BIM for 30 min before treatment with E2 for 6 h. A clear reduction in Bcl-2 levels was observed with BIM (*lane 3*) and PD (*lane 4*) on Western blots. Actin was used as loading control. *D*, CREB phosphorylation (pCREB) was analyzed by Western blotting of extracts obtained from cells treated with or without 10 nM E2 for 15–60 min as indicated. Data in *A–D* represent densitometric analysis. Error bars are $\pm$ SEM with $n = 3$ experiments in duplicate. *, $p < 0.05$, vehicle treated vs E2 treated; #, $p < 0.05$, groups compared with E2 treatment. *E*, Silver-stained gel (*top*) with equal loading of mitochondrial (m) and cytosolic (c) fractions representing protein loading for blots. The panels below represent immunoblots probed with anti-Bax (500 ng/ml) and anti-cytochrome *c* (500 ng/ml) Abs showing blots at 2 h after cells were treated with only E2 (*lanes 3* and *4*) or with 20 μM verapamil and E2 (ver+E2) (*lanes 5* and *6*) or 25 μM PD98059 with E2 (PD+E2) (*lanes 7* and *8*). Cytochrome *c* release at 12 h is shown for the same groups.

that contributed to the intracellular increase was of extracellular origin because presence of EGTA (Fig. 3*A*, E2+EGTA) during estradiol treatment prevented both Ca^{2+} peaks (Fig. 3*A*). To determine the route of Ca^{2+} influx, we used voltage gated Ca^{2+} channel blockers like pimozide for T-type channels (47) and verapamil for L-type channels (48) and Na^+/Ca^{2+} exchange blockers like benzamil (49) and bepridil (50). Pimozide, bepridil or benzamil could not prevent Ca^{2+} influx (data not shown), but verapamil was able to reduce both Ca^{2+} peaks when added at two different time points, one before addition of E2 (Fig. 3*A*, V1) and another just before the second increase of Ca^{2+} (Fig. 3*A*, V2), suggesting the involvement of L-type Ca^{2+} channels. Microscopically, a clear increase in labeling with Ca^{2+} binding fluorescent dye fluo-3-AM was evident in the E2 treatment group (Fig. 3*B*, iv) as compared with vehicle treated controls (Fig. 3*B*, ii). Cells treated with E2 in the presence of ICI 182780 (Fig. 3*B*, vi) and verapamil (Fig. 3*B*, viii) did not show any detectable increase in Ca^{2+} levels in the cells, the constitutive levels of Ca^{2+} being nondetectable by fluorescence microscopy. To establish whether this increase in Ca^{2+} was related to Bcl-2 expression, verapamil was used to prevent Ca^{2+} entry after E2 treatment. Verapamil added before initiation of E2 treatment (Fig. 3*C*, V1+E2) inhibited Bcl-2 increase significantly as compared with that induced by E2 alone (Fig. 3*C*, E2). However, addition of verapamil just before the second peak of Ca^{2+} (Fig. 3*C*, V2+E2) could not produce statistically significant inhibition of Bcl-2 levels. Collectively, these experiment established that E2 was able to induce a biphasic Ca^{2+} increase through L-type Ca^{2+} channels and the first peak of Ca^{2+} was linked to Bcl-2 increase.

ERK phosphorylation regulates Bcl-2 expression

Prior knowledge on the effects of Ca^{2+} on ERK phosphorylation (46) prompted us to check the effect of E2 on ERK. ERK phosphorylation occurred within 5 min of E2 exposure (Fig. 4*A*, *lane 2*) and was dependent on intracellular Ca^{2+} levels because verapamil could prevent ERK phosphorylation (Fig. 4*A*, *lane 3*). To check upstream events to ERK phosphorylation, BIM, a protein kinase C (PKC) inhibitor, was used at 1 μM concentration (51) and it was able to inhibit ERK phosphorylation (Fig. 4*B*, *lane 4*). PD98059, a selective pharmacological antagonist that inhibits MEK-1, which phosphorylates and activates ERK, was able to partially inhibit estrogen-induced Bcl-2 increase (Fig. 4*C*, *lanes 3* and *4*) when used at a dose of 25 μM (52). Therefore, a link between PKC pathway, phosphorylation of ERK, and Bcl-2 increase could be established. Downstream to ERK phosphorylation, CREB phosphorylation occurred (Fig. 4*D*), suggesting that possibly CREB

could mediate ERK-induced effects and that ERK consequently acts as a prosurvival protein inducing Bcl-2 increase. Because along with Bcl-2 increase, there was a concomitant translocation of Bax, it was of interest to see whether the PKC-ERK pathway was involved in Bax translocation. E2-induced Bax translocation (Fig. 4*E*, *lanes 3* and *4*) could not be prevented by either verapamil (Fig. 4*E*, *lanes 5* and *6*) or PD98059 (Fig. 4*E*, *lanes 7* and *8*), showing that Bax translocation was not dependent on Ca^{2+} or ERK phosphorylation. Because the primary function of Bax is to facilitate cytochrome *c* release into the cytosol from the mitochondria, both mitochondrial and cytosolic fractions of E2-treated and untreated cells in the presence of verapamil and PD98059 were checked. In both cases, at around 12 h, a distinct cytochrome *c* release into the cytosol was observed that was not visible at 2 h (Fig. 4*E*). Collectively, these data suggest that E2 mediates increase in Bcl-2 levels via the Ca^{2+}-PKC-ERK signaling pathway, but Bax translocation was independent of this signaling cascade.

E2 does not affect human peripheral blood MDM viability while it up-regulates Bcl-2

In view of the results obtained with THP-1 macrophages, we used human peripheral blood MDM as a cellular system to validate the effects of E2 in primary cells. Exposure of human peripheral blood MDM to E2 did not induce any loss of viability. The percentage of survival was as follows: vehicle treated, 95 ± 2; 1 nM E2 treatment, 93 ± 3; 10 nM E2 treatment, 95 ± 2; 100 nM E2 treatment, 96 ± 1; and 1 μM E2 treatment, 95 ± 1. Considering our results with THP-1 macrophage, we checked Bcl-2 levels and >1.5 fold up-regulation of Bcl-2 protein was observed after E2 treatment in human peripheral blood MDM (Fig. 5*A*, *lane 2*) as compared with constitutive Bcl-2 levels (Fig. 5*A*, *lane 1*) and presence of estrogen receptor antagonist ICI 182780 prevented Bcl-2 increase from constitutive levels by 75% (Fig. 5*A*, *lane 3*). To further confirm action through estrogen receptors and elucidate the receptor subtypes involved, DPN and PPT were used and both treatments increased Bcl-2 expression (Fig. 5*A*, *lanes 4* and *5*) in the absence of E2, suggesting involvement of both estrogen receptors β and α. Also, to directly demonstrate the presence of estrogen receptors in human peripheral blood MDM, immunocytochemistry was performed with an Ab that recognizes both estrogen receptors α and β and analyzed by flow cytometry, which showed that >95% of human peripheral blood MDM express estrogen receptors (Fig. 5*B*).

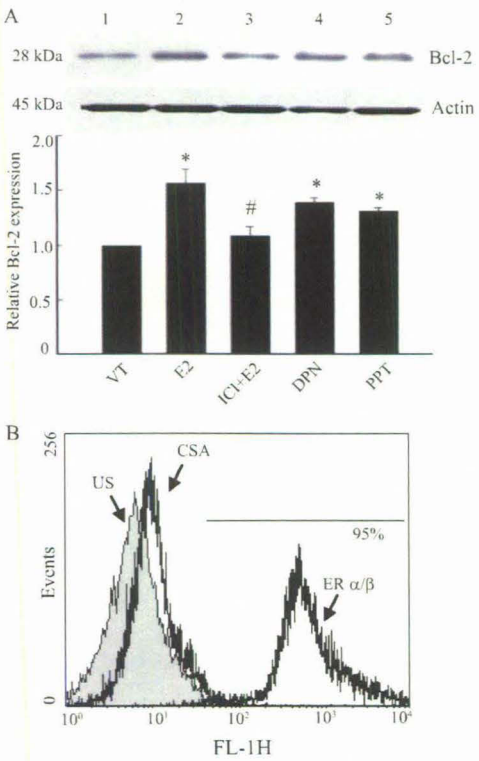


FIGURE 5. E2 induces Bcl-2 up-regulation in human MDM. *A*, Human MDM were treated with 10 nM E2 only (E2), E2 with 1 μM ICI 182780 (ICI+E2), or 100 nM DPN or PPT in the absence of E2, and whole cell extracts were probed for Bcl-2 expression by Western blotting. Actin was used as loading control. Data represent relative Bcl-2 expression levels normalized to actin as compared with vehicle treated control, calculated by densitometric analyses of multiple immunoblots as detailed in *Materials and Methods*. Error bars are ± SEM from $n = 3$ experiments in duplicate. *, $p < 0.05$ for *lane 1* vs *2*; #, $p < 0.05$, *lane 2* vs *3*. *B*, Estrogen receptor expression was analyzed by flow cytometry on human peripheral blood MDM immunostained with anti-estrogen receptor Ab reactive to both estrogen receptors α and β. The marker represents the percentage population that stain positive for the receptors. CSA, control secondary Ab; US, unstained cells.

E2 induces THP-1 macrophage cell death in Bcl-2 knockdown conditions

Because E2 initiated a concomitant translocation of Bax along with Bcl-2 increase, the question was how the cells would behave under Bcl-2 knockdown conditions. For this experiment, several routes of Bcl-2 inhibition in the presence of E2 were exploited. L-type Ca^{2+} channel blocker verapamil, PKC inhibitor BIM, and MEK inhibitor PD98059 were used to reduce Bcl-2 levels and cell fate was followed. Because the described treatments could have global effects affecting other pathways, Bcl-2 inhibition through the use of siRNA against Bcl-2 was used during E2 treatment. Fig. 6, *A* and *B*, demonstrates Bcl-2 decrease in the presence of Bcl-2 siRNA in THP-1 macrophages and human peripheral blood MDM, respectively. A 60% down-regulation of constitutive Bcl-2 in THP-1 macrophages (Fig. 6*A*, *second lane*) as compared with cells transfected with negative control siRNA (Fig. 6*A*, *first lane*) could be achieved. The transfection efficiency was ~95% estimated by fluorescence microscopic analysis of Cy3-labeled negative control siRNA. In E2-treated THP-1 macrophages, Bcl-2 up-regulation could be significantly knocked down with siRNA against Bcl-2 (Fig. 6*A*, *last lane*) as compared with E2 treatment in the presence of negative control siRNA (Fig. 6*A*, *third lane*). In human peripheral blood MDM, ~30% down-regulation of constitutive Bcl-2 was achieved with Bcl-2 siRNA (Fig. 6*B*, *second lane*) as compared with cells treated with negative control siRNA (Fig. 6*B*, *first lane*). The transfection efficiency of siRNA was ~95% as detected by observing fluorescence of Cy3-labeled negative control siRNA. As observed in THP-1 macrophages, siRNA against Bcl-2 was able to knockdown E2 induced Bcl-2 up-regulation significantly in human peripheral blood MDM (Fig. 6*B*, *fourth lane*) as well as compared with E2 treatment in the presence of negative control siRNA (Fig. 6*B*, *third lane*).

Analysis of viability in the presence of agents that inhibited Bcl-2 increase, namely verapamil, BIM, or PD98059, during E2 treatment showed a significant increase in cell death as compared with only E2 group (Fig. 6*C*). When the number of THP-1 macrophages entering the death pathway after estrogen exposure was estimated in a siRNA-mediated Bcl-2 knockdown condition by calculating cells showing phosphatidylserine exposure, nuclear propidium iodide staining, or both, then ~53%, 63%, and 82% of the cells tested positive at 2, 4, and 6 h (Fig. 6*D*, iv–vi), respectively, as compared with 1% in the absence of estrogen exposure (Fig. 6*D*, iii). Human peripheral blood MDM behaved similarly,

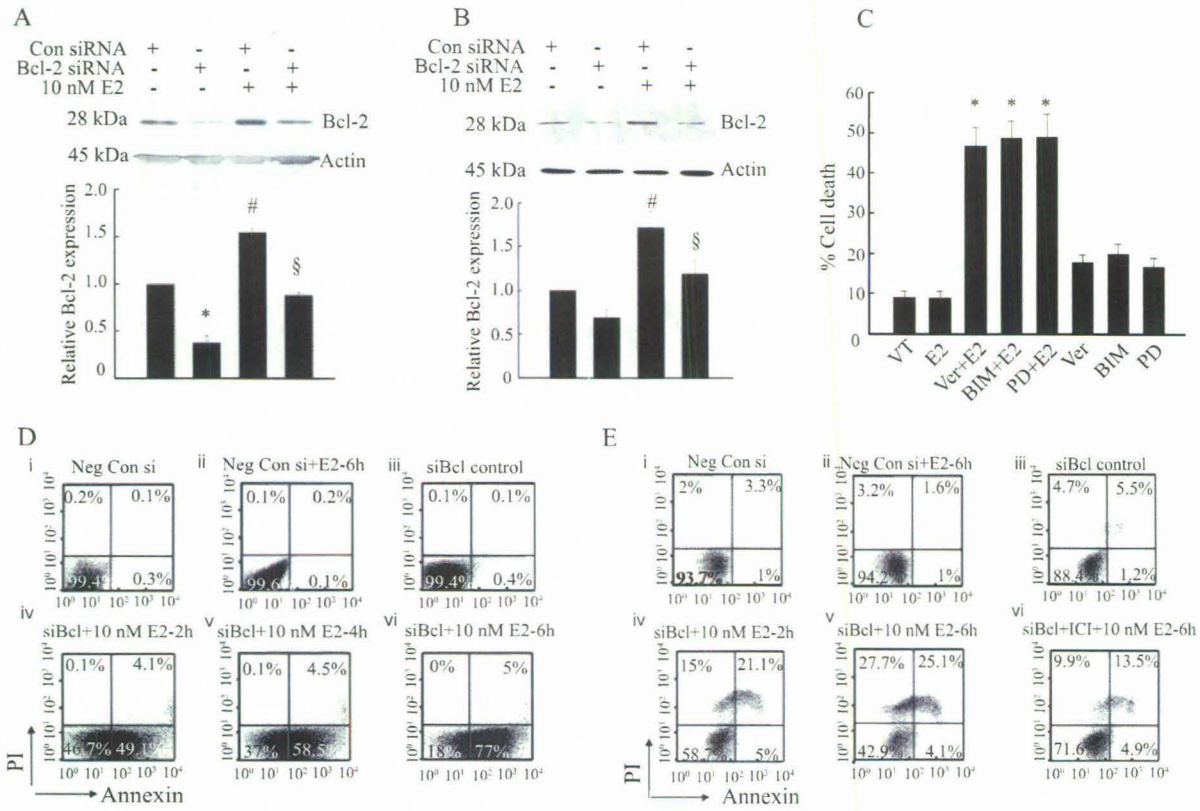


FIGURE 6. Inhibition of E2-induced Bcl-2 up-regulation results in cell death. The Bcl-2 siRNA-mediated Bcl-2 knockdown efficiency is shown on Western blots of extracts of THP-1 macrophages (*A*) or human peripheral blood MDM (*B*) treated with 10 nM E2 or without E2 and probed with mouse monoclonal anti-Bcl-2 Ab (80 ng/ml). Negative control siRNA was used as a target gene knockdown specificity control. Data represent the relative Bcl-2 expression levels normalized to actin as compared with vehicle treated control, calculated by densitometric analyses of multiple immunoblots as detailed in *Materials and Methods*. Error bars are ± SEM with $n = 3$ experiments in duplicate. *, $p < 0.05$ *first lane* vs *second*; #, $p < 0.05$, *first lane* vs *third*; §, $p < 0.05$, *third lane* vs *fourth*. *C*, THP-1 macrophages were preincubated with 20 μM verapamil or 25 μM PD98059 for 10 min or 1 μM BIM for 30 min before treatment with 10 nM E2 for 12 h. Cell death was analyzed by fluorescence microscopy using propidium iodide (1 μg/ml) staining. Data are representative of three independent experiments. Error bars are ± SEM with $n = 3$ experiments. *, $p < 0.05$ for E2 vs Ver+E2, BIM+E2, and PD+E2. *D*, Flow cytometric analysis of THP-1 viability by simultaneous Annexin V and propidium iodide staining of cells transfected with negative control siRNA and treated with (ii) or without (i) E2, Bcl-2 siRNA treated without (iii) or with 10 nM E2 for 2 h (iv), 4 h (v), and 6 h (vi). *E*, Flow cytometric analysis of THP-1 viability by simultaneous Annexin V and propidium iodide staining of cells transfected with negative control siRNA treated with (ii) or without (i) E2, Bcl-2 siRNA treated without (iii) or with 10 nM E2 for 2 h (iv), 6 h (v), or in the presence of ICI 182780 (vi). The *y*-axis represents propidium iodide labeling and the *x*-axis represents Annexin V labeling. The percentage shown represents cells analyzed that lie within each quadrant.

and the number of human peripheral blood MDM entering the death pathway after estrogen exposure was 40% and 57% at 2 and 6 h (Fig. 6*E*, iv and v), respectively, as compared with 10% in the absence of estrogen exposure (Fig. 6*E*, iii). ICI 182780 could prevent cell death after E2 exposure (Fig. 6*E*, vi). This result clearly showed that Bcl-2 knockdown makes the THP-1 macrophages and human peripheral blood MDM susceptible to the death-inducing effects of E2.

Discussion

The ability of macrophages to respond to estrogen plays an incisive role in macrophage function (9–14), and in this study we establish the mechanism by which estrogen regulates the macrophage mitochondrial death pathway. Our report illustrates the function of two Bcl-2 family members with disparate biological properties, namely Bax and Bcl-2 (26) in regulating estrogen-induced effects on macrophage survival. The ability of estrogen to influence macrophages would depend on the presence of functional receptors unless the hormone acts through a receptor independent pathway (40). Because both THP-1 macrophages and human peripheral blood MDM contained functional receptors proven by the ability of estrogen agonists to mimic E2 action on Bcl-2 and Bax and estrogen antagonists to prevent such action, the cells were therefore competent to respond to E2 via receptors. Clearly, the effect of E2 on Bcl-2 was a genomic effect mediated through a PKC-ERK signaling pathway because there was an actual increase in Bcl-2 transcript level. It was unlikely that estrogen responsive elements in *bcl-2* gene (53) were directly involved in responding to E2 because interference with the events of Ca^{2+} influx, PKC activation, and ERK phosphorylation could prevent E2 induced Bcl-2 increase. Changes in cellular Ca^{2+} induced by any stimuli are an important event for a cell in terms of its survival (54). There is a close relationship between Bcl-2 and Ca^{2+} because although Ca^{2+} can mediate Bcl-2 increase, Bcl-2 can also regulate cellular Ca^{2+} through manipulation of endoplasmic reticulum Ca^{2+} stores (45). Bcl-2-induced modulation of Ca^{2+} was excluded by the inability of siRNA-mediated Bcl-2 knockdown to affect E2-induced Ca^{2+} elevation (data not shown), but experiments with L-type Ca^{2+} channel blocker that resulted in a reduction of Ca^{2+} and, consequently, inhibited Bcl-2 increase suggested a situation like hippocampal neurons in which estrogen activates rapid Ca^{2+} influx (55). Interestingly, there were two peaks of Ca^{2+} increase

both of which could be inhibited by verapamil, but although inhibition of the first resulted in inhibition of Bcl-2, lowering of the second peak did not prevent Bcl-2 increase, suggesting that involvement of the second peak of Ca^{2+} in Bcl-2 expression is unlikely and increased Ca^{2+} at that point in time could be serving some other purpose.

The augmentation of Bcl-2 levels by E2 in both THP-1 macrophages and human peripheral blood MDM suggested the creation of a favorable condition for the cells to survive. Similar instances of estrogen-induced Bcl-2 up-regulation by ~1.3-fold have been demonstrated to protect B cells from BCR-mediated apoptosis (56). Increase in Bcl-2 under estrogen exposure is also observed in neurons (57) and MCF-7 breast cancer cells (58). Although Bcl-2 must ensure cell viability as demanded by certain conditions of stress, cell death is facilitated by translocation of Bax from cytosol to the mitochondria (59). In the mitochondria, Bax could either be overwhelmed by the amount of Bcl-2 present and the equilibrium will shift to Bcl-2 ensuring survival or in low Bcl-2 conditions Bax will prevail and facilitate release of cytochrome *c* ensuring death (59). As Bax translocation was independent of Ca^{2+} increase and activation of the PKC pathway, this provided us with an opportunity to investigate Bcl-2 function without interfering with Bax translocation. The crucial role of estrogen induced Bcl-2 increase was obvious from experiments in which L-type Ca^{2+} channel blocker verapamil restricted Ca^{2+} influx, and the consequent inhibition of the PKC pathway prevented ERK phosphorylation resulting in Bcl-2 decrease but did not inhibit Bax translocation as a result of which cell death ensued. Although the above data confirmed the importance of the altered Bax/Bcl-2 level for survival, both PKC and ERK activity could involve other pathways as well. Therefore, to unequivocally prove the involvement of Bcl-2 in macrophage survival at the time of estrogen exposure, we used Bcl-2 siRNA to knockdown Bcl-2 levels during E2 treatment, and our studies establish that Bcl-2 up-regulation is an absolute requirement for macrophage survival in the presence of estrogen. The reduced number of late apoptotic cells observed in THP-1 macrophages as compared with human peripheral blood MDM could be attributed to higher expression of antiapoptotic proteins in THP-1 (60) due to its leukemic nature.

Clearly, E2 shows two distinct set of effects. Although increase in Ca^{2+} leads to ERK phosphorylation resulting in an increase in Bcl-2, which is an antiapoptotic signal, a distinct proapoptotic signal in the form of translocation of Bax from cytosol to mitochondria was generated by the Ca^{2+} signaling-independent but estrogen receptor-dependent pathway (Fig. 7). Therefore, in macrophages, estrogen shows a dichotomous effect and depending on other factors that could influence Bax or Bcl-2 proteins in a given circumstance, a survival or a death pathway would be chosen.

How might our findings be integrated into a model of macrophage behavior in high estrogen microenvironments? From a pathological point of view, there are two possible scenarios in which this estrogen mediated cell survival could be of relevance. Optimally, macrophage survival at tumor or inflammatory sites during chemotherapy is essential during the orderly process of cell death to restrict harm to host tissues (61). Our report provides a new insight into macrophage function in response to estrogen where the hormone is able to generate a cytoprotective response in macrophages through manipulation of the Bcl-2 proteins. This function will be crucial under conditions of Bcl-2 down-regulation (31–35) when macrophage population will be depleted if Bcl-2 knockdown is attempted in tumors of estrogen targets. On the contrary, in many tumors, tumor-associated macrophages play a protumorigenic role by secreting growth factors and angiogenic factors and current antitumor strategy includes targeted destruction of these cells in which case macrophage killing through Bcl-2 down-regulation would be beneficial for tumor therapy. From a physiological point of view, estrogen-induced Bcl-2 up-regulation is obligate for human macrophage survival and hence necessary for the manifestation of the immunomodulatory effects of estrogen on macrophages.

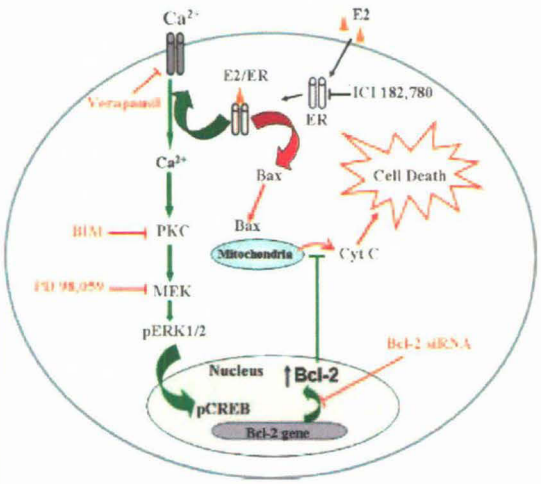


FIGURE 7. Schematic showing intracellular signaling pathways involved in estrogen action on human macrophages. The model diagram illustrates the dichotomous effect of estrogen on human macrophage survival. The pathway highlighted in green represents the antiapoptotic signals and those highlighted in red represent the proapoptotic signals activated by estrogen. Estrogen-induced increase in Ca^{2+} leads to ERK phosphorylation and consequently CREB phosphorylation, resulting in an increase in Bcl-2. Translocation of Bax from cytosol to mitochondria is Ca^{2-} signaling-independent but estrogen receptor-dependent. Interference with Bcl-2 increase through inhibition of Ca^{2+} (verapamil), PKC (BIM), phosphorylation of ERK (PD98059), and Bcl-2 mRNA degradation via Bcl-2 siRNA leads to cell death in presence of estrogen due to elevated levels of Bax.

Disclosures

The authors have no financial conflict of interest.

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Oestrogen modulates human macrophage apoptosis *via* differential signalling through oestrogen receptor-α and β

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Abstract

Human macrophages express oestrogen receptors and are therefore competent to respond to the hormone present in their microenvironment, which is implicated in sexual dimorphism observed in several immune and autoimmune phenomena. An earlier study from this laboratory demonstrated 17β-oestradiol (E2) induced apoptosis in macrophages derived from human peripheral blood monocytes and THP-1 acute monocytic leukaemia cell line, when Bcl-2 was down-regulated; however, the involvement of E2 receptor subtypes in the modulation of death pathways in these cells remain unknown. Using macrophages derived from THP-1 human acute monocytic leukaemia cells as a model, we demonstrate that plasma membrane associated oestrogen receptor (ER) -α participate in E2 induced Bcl-2 increase, through activation of the mean arterial pressure-kinase (MAPK) pathway whereas cytosolic ER-β transmits signals for the pro-apoptotic event of Bax translocation. The mechanistic basis of Bax translocation comprised of ER-β mediated increase in intracellular pH, facilitated by activation of the Na^+–H^- exchanger. Intracellular alkalinization accompanied by concomitant Bcl-2 increase and Bax migration does not cause cellular apoptosis; however, siRNA mediated down-regulation of ER-α during E2 exposure leads to inhibition of Bcl-2 increase and consequently apoptosis due to the unopposed action of mitochondrial Bax. In summary, this study underscores the importance of integrative signalling modality from multiple oestrogen receptor pools in modulating oestrogen effects on human monocyte-derived macrophage apoptotic signalling pathway, which opens new vistas to explore the use of selective oestrogen receptor modulators in apoptosis-based therapies.

Keywords: oestrogen • macrophage • oestrogen receptor • Bcl-2 • Bax • apoptosis • alkalinization

Introduction

Macrophages derived from the differentiation of monocytes express steroid hormone receptors and are therefore sensitive to the hormones present in their microenvironment. The study of steroid hormone action on modulation of human macrophage function is of significant interest because these versatile cells are involved in the regulation of immune response and consequently are relevant to pathogenesis of many diseases. The ovarian steroid oestrogen is able to exert pleiotropic effect on macrophages, including modulation of the death pathway, for example, it exerts paradoxical effects on human U937 macrophages where cell death is induced by oestrogen [1], but the same hormone accords protection to these cells from TNF-α induced apoptosis [2]. Similar effect is exerted on murine osteoclasts, where oestrogen exposure leads to caspase-dependent apoptosis [3, 4]. A previous study from this laboratory demonstrated 17β-oestradiol (E2) induced apoptosis in macrophages derived from human peripheral blood monocytes and THP-1 acute monocytic leukaemia cell line, when Bcl-2 was down-regulated [5]. It is well established that the survival of a cell in response to certain apoptotic stimuli depends on the critical ratio of mitochondrial Bcl-2 and Bax. Consequent to sensing of apoptotic stimuli, Bax, which exists as an inactive monomer in the cell cytoplasm, migrates to the mitochondria to interact with the existing mitochondrial Bcl-2 and the resulting interaction determines the fate of the cell, higher or lower ratio of Bcl-2/Bax being anti-apoptotic or pro-apoptotic [6], respectively. For the change of location of Bax, a conformational alteration occurs in the Bax protein, leading to exposure of the mitochondrial targeting sequence resulting in its translocation to the outer mitochondrial membrane. Multiple mechanisms have been proposed as instrumental in initiating Bax translocation, which include neutralization of several pro-survival proteins by BH3 only members of the Bcl-2 family such as Noxa and Puma [7],

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phosphorylation events mediated by kinases [8, 9], down-modulation of clusterin [10], intracellular acidification [11] or alkalinization [12] and caspase-dependent cleavage of Bax [13]. Because modulation of Bcl-2 and Bax could form part of a strategy for manipulation of cell survival, it is important to establish the associated processes that lead to such changes.

Although it is known that E2 can influence cell death pathways in human macrophages, the involvement of oestrogen receptor-α (ER-α) and -β (ER-β) expressed at multiple subcellular locations [14] in these cells in mediating death or survival signals is not known. It is important to understand the relative contribution of the two receptors on effects elicited by oestrogen because signalling through separate subtypes could have diverse outcome [15]. This is evident from the distinct phenotypes obtained with ER-α and ER-β knockout mice [16], and in addition, evidence for differential receptor activity come from studies showing overlapping but exclusive sets of downstream target genes for the two subtypes [17]. The classical model of ER action is where ligand bound ER interacts with oestrogen response elements in target genes and initiates transcription by modulating co-repressors and co-activators. Conversely, ERs can also interact with other transcription factors like activating protein-1 and stimulating protein-1 to initiate transcription [18, 19]. In addition to the above mode of actions, oestrogen may elicit effects through genomic or non-genomic mechanisms by binding to oestrogen receptors localized on the plasma membrane of target cells [20] and activate mean arterial pressure-kinase (MAPK) signalling [21] or induce intracellular Ca^{2+} fluxes [22]. Elicitation of a particular event in response to E2 could be dependent on the relative concentrations of the two ER subtypes, for example, U937 monocytes expressing mostly ER-β are sensitive to oestrogen induced apoptosis; however, after differentiation to macrophages, receptor population expressed is predominantly ER-α, as a result of which the apoptosis inducing effect of oestrogen becomes ineffective [23].

The purpose of this study was to investigate the involvement of the two ER subtypes in mediating apoptosis associated events in human macrophages using THP-1 monocyte derived macrophages as a model system. We show that THP-1 human macrophage survival is compromised in the presence of E2 if ER-α but not ER-β receptor levels are down-regulated. This is because E2 signalling *via* ER-α mediates the anti-apoptotic event of Bcl-2 up-regulation, whereas ER-β signals for the pro-apoptotic event of Bax translocation to the mitochondria *via* Na^+-H^+ exchanger mediated intracellular alkalinization.

Materials and methods

Cell lines and culture

THP-1, a human acute monocytic leukaemia cell line, and MCF-7, a human breast carcinoma cell line (ATCC, Manassas, VA, USA), were maintained in RPMI-1640 (Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with 10% FCS (Biological Industries, Kibbutz Beit Haemek, Israel). Differentiation of THP-1 monocytes to macrophages was induced by treatment with 10 ng/mL PMA for 36 hrs. Forty eight hours prior to experimentation, the cells were transferred to phenol-red free RPMI supplemented with 10% dextran-coated charcoal stripped FCS to remove all extraneous sources of oestrogen.

Reagents

E2 (cyclodextrin encapsulated), E2 cojugated to BSA (E2-BSA), E2-BSA conjugated to FITC (E2-BSA-FITC), PD98,059, nigericin, amiloride and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). ICI 182,780 was obtained from Tocris Cookson (Bristol, UK). Negative control siRNA was purchased from Ambion (Austin, TX, USA), whereas Bcl-2, ER-α and ER-β siRNAs were obtained from Dharmacon (Lafayette, CO, USA). The siRNA transfection reagent Transpass R2 was purchased from New England Biolabs (Ipswich, MA, USA). All reagents for Western blotting and ECL development were from Amersham Biosciences (Piscataway, NJ, USA). SNARF (5-(and-6)-carboxy SNARF®1-AM), Sodium Green™ tetra-acetate, secondary antimouse IgG conjugated to Alexa fluor 488 and Hoechst 33342 nuclear dye were purchased from Molecular Probes (Eugene, OR, USA). Antibodies for oestrogen receptor α/β, oestrogen receptor-α and actin were procured from Calbiochem (Darmstadt, Germany), whereas antibodies against phospo-ERK and whole-ERK were from StressGen Biotechnologies (Victoria, BC, Canada). Anti-Bcl-2, anti-Bax and anti-cytochrome c antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-GAPDH antibody was from Ambion (Austin, TX, USA), whereas anti-histone dimethyl lysine antibody was purchased from Upstate (VA, USA). Secondary antimouse and anti-rabbit IgG conjugated to horseradish peroxidase were procured from Jackson Immunoresearch (Cambridgeshire, UK). The Vybrant apoptosis detection system was purchased from Promega (Madison, WI, USA). Anti-clusterin antibody was a kind gift from Dr. C. Yan Cheng of the Population Council, NY, USA. All other chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned otherwise.

Intracellular pH measurement

Intracellular pH measurement was performed with the long-wavelength fluorescent pH indicator carboxy SNARF-1 AM following manufacturer's protocol. Briefly, the cells (10^6 /ml) were resuspended in serum-free RPMI and Q2
incubated with a final concentration of 1 μM SNARF-1 AM, diluted from a stock solution of 1 mM in DMSO for 15 min. at room temperature. Cells were washed and incubated for 20 min. at room temperature for complete de-esterification of AM esters. *In situ* calibration of SNARF-1 AM was performed with the ionophore nigericin at 10 μM concentration in a high-K^+ buffer to equilibrate intracellular pH with that of the controlled extracellular medium. Appropriate groups were subjected to different treatments, and fluorescence measurements were commenced in a spectrofluorometer (Perkin Elmer, Waltham, MA, USA), followed by kinetic analysis. The pH was calculated from the fluorescence measurements using the following formula:

$$pH = pK_a - \log [\{(R - R_B)/(R_A - R)\} \times (F_{B(\lambda 2)}/F_{A(\lambda 2)})],$$

where pK_a of carboxy SNARF-1 AM is 7.5. R is the ratio of fluorescent intensities (F) measured at two emission wavelengths, 580 (λ1) and 640 nm (λ2), with fixed excitation at 514 nm. The subscripts A and B represent the limiting values at the acidic and basic end-points of the titration, respectively. Na^+-free and HCO_3^--free buffer were prepared as described by Khaled *et al.* [24].

Intracellular Na^+ measurement

For intracellular Na^+ measurement, cells (10^6 /mL) were labelled for 20 min. at room temperature with the cell permeable fluorescent Na^+ indicator Sodium Green™ tetra-acetate, diluted to 1 μM in RPMI 1640 from a 5 mM stock solution made in DMSO. After washing the cells to remove excess probe, kinetic fluorescent measurements were carried out with a spectrofluorometer at an excitation of 480 nm and emission of 520 nm (BMG Fluostar Optima, BMG technologies, Offenburg, Germany). *In situ* Calibration was accomplished by using the indicator in solutions of precisely known free Na^+ concentration in the presence of the pore forming antibiotic gramicidin (10 μM). Intracellular Na^+ was calculated using the following equation:

$$[Na^+]_{free} = K_d (F - F_{min}/F_{max} - F),$$

where K_d of the dye is 5.7 mM at 37°C, F is the fluorescence of the experimental sample, F_{min} is fluorescence in the absence of Na^+ and F_{max} is fluorescence in the presence of saturating concentrations of Na^+.

siRNA transfection

THP-1 macrophages were transfected with specific siRNAs using Transpass R2 transfection reagent as described previously [5]. Briefly, Bcl-2 siRNA (15 pmol), ER-α and ER-β siRNA (100 pmol) or negative control siRNA (pre-designed siRNA with no known target genes) at similar concentrations were added to transfection reagent TranspassR2, diluted in serum-free medium, and incubated for 20 min. to allow the formation of transfection complexes. The formed complexes were added to 10^5 cells/well grown in 24-well plates and incubated for 6 hrs, following which fresh complete medium was added. Transfection efficiency was estimated by observing Cy3 fluorescence of the negative control siRNA with a Nikon TE2000-E fluorescence microscope using a tetramethyl rhodamine filter (530–580 nm). For all transfections, target protein knockdown was assessed 24 hrs after transfection by probing extracts of transfected cells on Western blots with anti-Bcl-2 and anti ER-α/β antibody.

Subcellular fractionation

THP-1 macrophages were allowed to swell for 10 min. in hypotonic buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5) followed by homogenization with a Dounce homogenizer (50 strokes). Immediately after cell lysis, the mitochondria were stabilized by addition of mitochondrial stabilization buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, pH 7.5; 2.5 mM EDTA, pH 7.5), and the homogenate was centrifuged at 1300 × *g* for 15 min. to isolate the nuclear fraction. The post-nuclear supernatant was further centrifuged at 17,000 × *g* for 15 min. in an ultracentrifuge (Optima XL-100K, Beckman) to isolate the mitochondria. The post-mitochondrial supernatant was centrifuged at 100,000 × *g* for 1 hr to obtain the membranous fraction as a pellet and the supernatant as the cytosol. The homogeneity of the obtained fractions was determined by Western blotting with probes specific for each fraction.

Cell viability assay

To assess cell viability, PI dye exclusion assay was performed by incubating the cells with 1 μg/ml PI for 5 min. at 37°C, followed by one wash with ice-cold PBS. The cells were analysed under a Nikon TE2000-E fluorescence microscope using Nikon G2A filter cube. The percentage cell death was calculated as the number of cells with PI positive nuclei as against the total number of cells. Annexin-V-PI staining was performed as described previously [5], and data acquisition was performed on a BD-LSR flow cytometer equipped with a 488 nm air-cooled argon ion laser. The acquired data was analysed using WinMDI software (Microsoft v.2.9).

Reverse transcription-polymerase chain reaction

Total RNA was isolated using TRIzol reagent (GIBCO, CA, USA), and cDNA was synthesized as described previously [5]. The specific primers used were

ER-α (sense) GTGGGAATGATGAAAGGTGG; ER-α (antisense) TCCAGAGACTTCAGGGTGCT

ER-β (sense) TGAAAAGGAAGGTTAGTGGGAACC; ER-β (antisense) TGGTCAGGGACATCATCATGG

Actin(sense),5′-GTGGGGCGCCCCAGGCACCA-3′; Actin(antisense), 5′CTCCTTAATGTCACGCACGATTTC-3′.

PCR was performed after determining the cycle number in which a linear amplification of serially diluted template could be achieved. The PCR products were then resolved on 1.5% agarose gel and visualized by ethidium bromide staining and quantitated by densitometry.

Immunocyotochemistry

Cells were fixed with 4% formaldehyde for 20 min., followed by several washes with ice-cold PBS. Saponin (0.1%) was used for cell permeabilization, and 3% normal goat serum was used as a blocking reagent to reduce non-specific binding. The permeabilized cells were incubated with primary antibody recognizing ER-α/β (1 : 100) followed by incubation with secondary antimouse IgG conjugated to Alexa fluor 488 (1 : 200). For live-cell staining, all incubations were performed at 4°C with anti-ER-α antibody in addition to reagents as described above. For nuclear labelling of cells, Hoechst 33342 was used. All stainings were visualized using a Nikon TE2000-E fluorescence microscope using appropriate filter blocks, and the image acquisition was carried out with a high-resolution Retiga *Exi* camera (Q-imaging, Surrey, BC, Canada), the mask of co-localization was created and the co-efficient of colocalization was calculated using Image-Pro Plus software (Media cybernetics, Silver Spring, MD, USA).

SDS-PAGE and Western blot

Whole cell extracts were prepared by treating the cells with lysis buffer (0.125 M Tris, 4% SDS, 20% glycerol and 10% 2-ME), and protein estimation was performed with CBX protein assay kit (G-Biosciences, St. Louis, MO, USA). Lysates were resolved on 12% SDS-PAGE gel, following which they were transferred onto nitrocellulose membrane as described before [5]. Non-specific binding sites were blocked by incubating the blots in 5% non-fat skimmed milk with 0.05% PBS-Tween 20 for 1 hr. Primary (1: 5000) and secondary antibody (1 : 10,000) incubations were carried out for 1 hr each, and immunoreactivity was visualized by enhanced chemiluminescence using ECL reagent, as described previously [5].

Densitometry and statistical analysis

Quantitative assessment of reaction intensity in Western blots was performed using a UVP gel-documentation instrument, and the data were

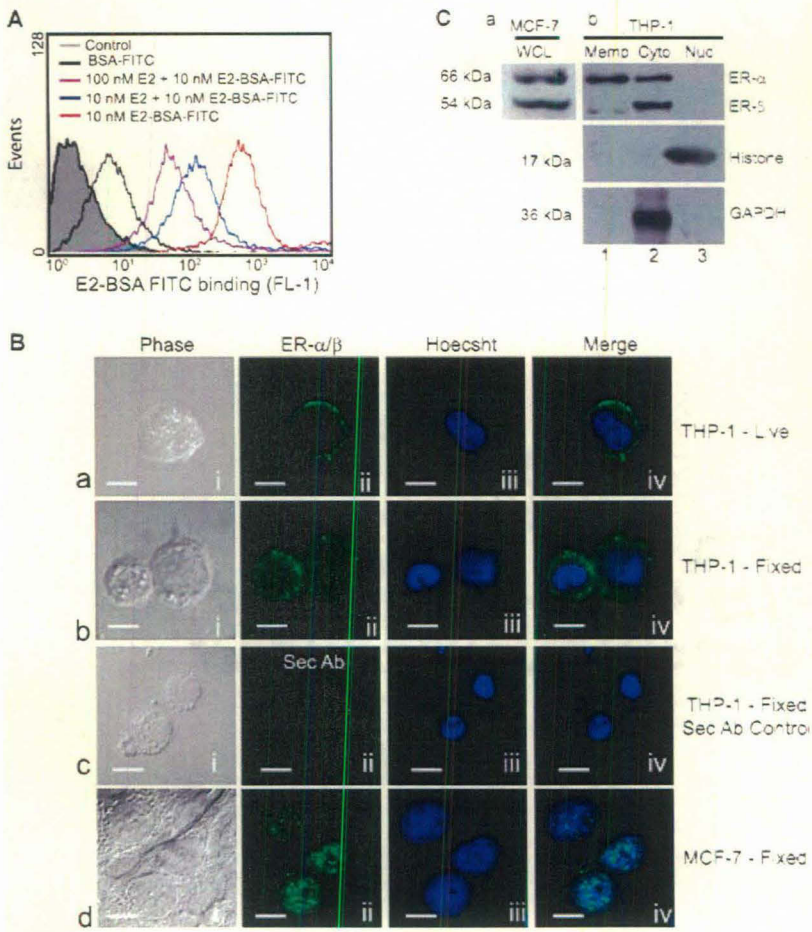

Fig. 1 Human macrophages express oestrogen receptor α and β on the plasma membrane and cytoplasm. (**A**) The histogram represents flow-cytometric analysis of live THP-1 macrophages incubated with E2 conjugated to BSA-FITC (red line) or co-incubation with different concentrations of E2 (blue, 10 nM E2 and pink, 100 nM E2 lines) under similar conditions. The area shaded grey represents unlabelled cells, and the black line represents cells labelled with only BSA-FITC. Note the distinct shift in staining of the cells treated with E2-BSA-FITC demonstrating recognition of cell surface localization of ERs. (**B**) Indirect immunofluorescence on live as well as formaldehyde-fixed cells stained with anti-ER-α/β antibody. (**a**) THP-1 live; (**b**), THP-1 fixed; (**c**), secondary antibody control, (**d**) fixed MCF-7 cells – (i), nomarski image; (ii), ER-α/β staining; (iii), nuclear staining with Hoechst 33342; (iv), overlap of (ii) and (iii). The MCF-7 cells (**d**, i–iv) used as positive controls show presence of nuclear receptors. The bar represents 10 μm. All data are representative of at least three independent experiments. (**C**) Western blots of subcellular fractions of THP-1 cells probed with anti-ER-α/β showing presence of both forms in the cytoplasm (**b**, Cyto, lane 2), predominantly ER-α in membrane fraction (**b**, Memb, lane 1) and absence of receptors in nuclear fraction (**b**, Nuc, lane 3). (**a**) MCF-7 cell extracts show the presence of both ER-α and ER-β. Western blot for histone and GAPDH was performed to assess the homogeneity of the obtained nuclear and cytoplasmic fractions, respectively.

analysed with the LabWorks image analysis and acquisition software (v4.0.0.8; UVP, Upland, CA, USA). At least three Western blots per experiment were quantitated to arrive at the average value of the signal. All measurements were normalized to internal loading controls. Data are expressed as mean ± standard error (SE) unless mentioned. Comparisons were made between different groups using unpaired Student's t-test. The values were considered to be significantly different at $P < 0.05$. All analysis was performed on data acquired from three or more independent experiments.

Results

Human macrophages express both oestrogen receptor-α and β at multiple subcellular locations

E2 initiates cellular signalling pathways *via* interaction with its receptors expressed primarily as two subtypes – the ER-α and ER-β [25] – found in the nucleus, plasma membrane or cytosol, the distribution varying with different cell types [26]. Human macrophages are known to express both ER-α and ER-β [27, 28], however, the subcellular localization of these receptors is not known. In the current study, membrane bound E2 receptors were detected on viable differentiated THP-1 macrophages by labelling live cells at 4°C with membrane impermeable E2-BSA linked to FITC (E2-BSA-FITC), and flow cytometric analysis showed an obvious shift in fluorescence labelling intensity in these cells compared with those labelled with only BSA-FITC used as a control (Fig. 1A). Decreased fluorescence readings obtained with cells incubated with E2-BSA-FITC in the presence of unconjugated E2 compared with cells exposed to only E2-BSA-FITC confirmed specificity of this binding (Fig. 1A).

Further analysis of presence of ERs in different subcellular locations in these cells showed surface labelling of ERs on live cells (Fig. 1B, a, i–iv) with an anti-E2 receptor antibody raised against common epitopes on ER-α and ER-β receptor proteins, thus corroborating the above data obtained with E2-BSA-FITC.

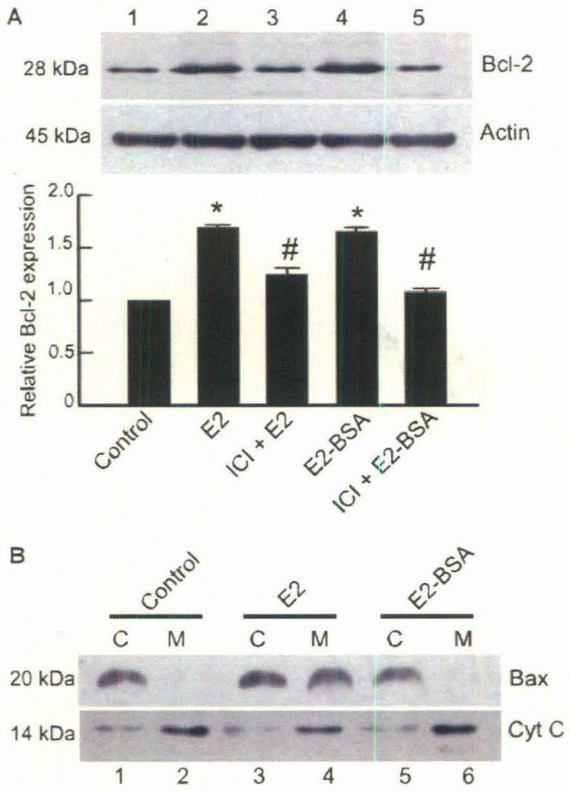

Fig. 2 Regulation of Bax and Bcl-2 is mediated by E2 receptors localized at different sub-cellular locations. (**A**) Extracts of THP-1 cells treated with E2 (10 nM)(lane 2) and E2-BSA for 6 hrs (10 nM)(lane 4) show Bcl-2 up-regulation. Presence of ICI 182,780 (1 μM) during E2 (lane 3) and E2 BSA (lane 5) treatment inhibited Bcl-2 up-regulation. Same blots were stripped and reprobed for actin, which was used as a loading control. The bar graph represents densitometric measurements of Bcl-2 expression relative to control and normalized to actin ($n = 3$). The error bars represent ± SEM. * $P < 0.05$ compared with control. # $P < 0.05$ compared with E2 or E2-BSA treated group. (**B**) THP-1 macrophages were exposed for 6 hrs to E2 (lanes 3 and 4) or E2 BSA (lanes 5 and 6) and subcellular fractions of mitochondria (M) and cytosol (C) were analysed for the localization of Bax. Note the absence of Bax translocation in the E2-BSA treated groups. The blot was stripped and reprobed for cytochrome c to determine the homogeneity of mitochondrial fractions. Note the heavy presence of cytochrome c in the mitochondrial fraction. All data are representative of at least three independent experiments. Cyt C: cytochrome c.

Intracellular receptors were detected by staining fixed and permeabilized cells with the same antibody that demonstrated the presence of receptors within the cytosol and the membrane but not in the nucleus (Fig. 1B, b, i–iv). Figure 1B, c, i–iv shows absence of fluorescence in secondary antibody controls. MCF-7 cells, where E2 receptors are predominantly nuclear [29], were used as positive controls and showed distinct nuclear staining with the same antibody (Fig. 1B, d, i–iv). Cytosolic ERs dimerize and migrate to the nuclei upon ligand engagement [18]; this phenomenon was used as a control for cytosolic receptors, and Figure S1 shows that upon E2 exposure the cytosolic ERs migrate to the nuclei (E2, b and d) compared with control (Control, b and d) and E2-BSA treatment (E2-BSA, b and d). Measurement of the mask of colocalization clearly shows significant colocalization of ER receptor staining with nuclear staining after E2 treatment (E2, e) compared with vehicle treated controls (Control, e) and after E2-BSA treatment (E2-BSA, e).

To determine the subtype specific distribution of receptors, Western blots of subcellular fractions of THP-1 cells were probed with the same antibody as above, and it recognized both receptor types in the cellular cytosolic fraction (Fig. 1C, b, lane 2) whereas the membranous fraction primarily showed reactivity for ER-α subtype, the expression of ER-β being low (Fig. 1C, b, lane 1). The nuclear fraction did not show any reactivity, suggesting the absence of nuclear oestrogen receptors in this particular cell type (Fig. 1C, b, lane 3). Total extract of MCF-7 cells known to express both ER-α and ER-β was used as positive control (Fig. 1C, a). Collectively, the above data demonstrated the presence of ERs on the cell surface as well as in the cytosol of THP-1 monocyte derived macrophages, nucleus being devoid of such receptors. Both ER-α and ER-β are present intracellularly, whereas the plasma membrane appears to be primarily populated by ER-α.

E2 modulates Bcl-2 and Bax *via* different sets of receptors in THP-1 cells

To investigate if the receptors at different subcellular locations transmit similar or different signals for modulation of the mitochondrial apoptotic pathway, both membrane permeable and impermeable E2 were used. E2-BSA, where E2 is conjugated to BSA through a six atom hydrocarbon tether [30] restricting its diffusion through the plasma membrane, was used to distinguish signals originating from membrane bound receptors only [20, 30].

Equivalent increase in Bcl-2 levels was obtained with membrane permeable E2 as well as membrane impermeable E2-BSA at 6 hrs (Fig. 2A, lanes 2 and 4). Pure E2 receptor antagonist ICI 182,780 inhibited this increase (Fig. 2A, lanes 3 and 5), confirming that in both cases, E2 receptors were involved. In contrast to its ability to modulate Bcl-2, E2-BSA was unable to exert any effect on subcellular localization of Bax (Fig. 2B, lanes 5 and 6), whereas unconjugated E2 was able to stimulate Bax translocation (Fig. 2B, lanes 3 and 4). The inability of E2-BSA to induce migration of Bax from cytosol to the mitochondria unlike free E2 suggested that Bax migration was independent of membrane receptor mediated signalling.

Based on the above data, attempt was made to identify receptor subtypes involved in mediating the above responses. ER-α and ER-β mRNA and protein were selectively down-regulated with siRNA for test the effect of this down-regulation on Bcl-2 and Bax and, eventually, cell death. Figure 3A shows the RT-PCR for ER-α mRNA performed with primers specific for ER-α, where lane 2 shows decrease in ER-α mRNA when siRNA to ER-α was used but

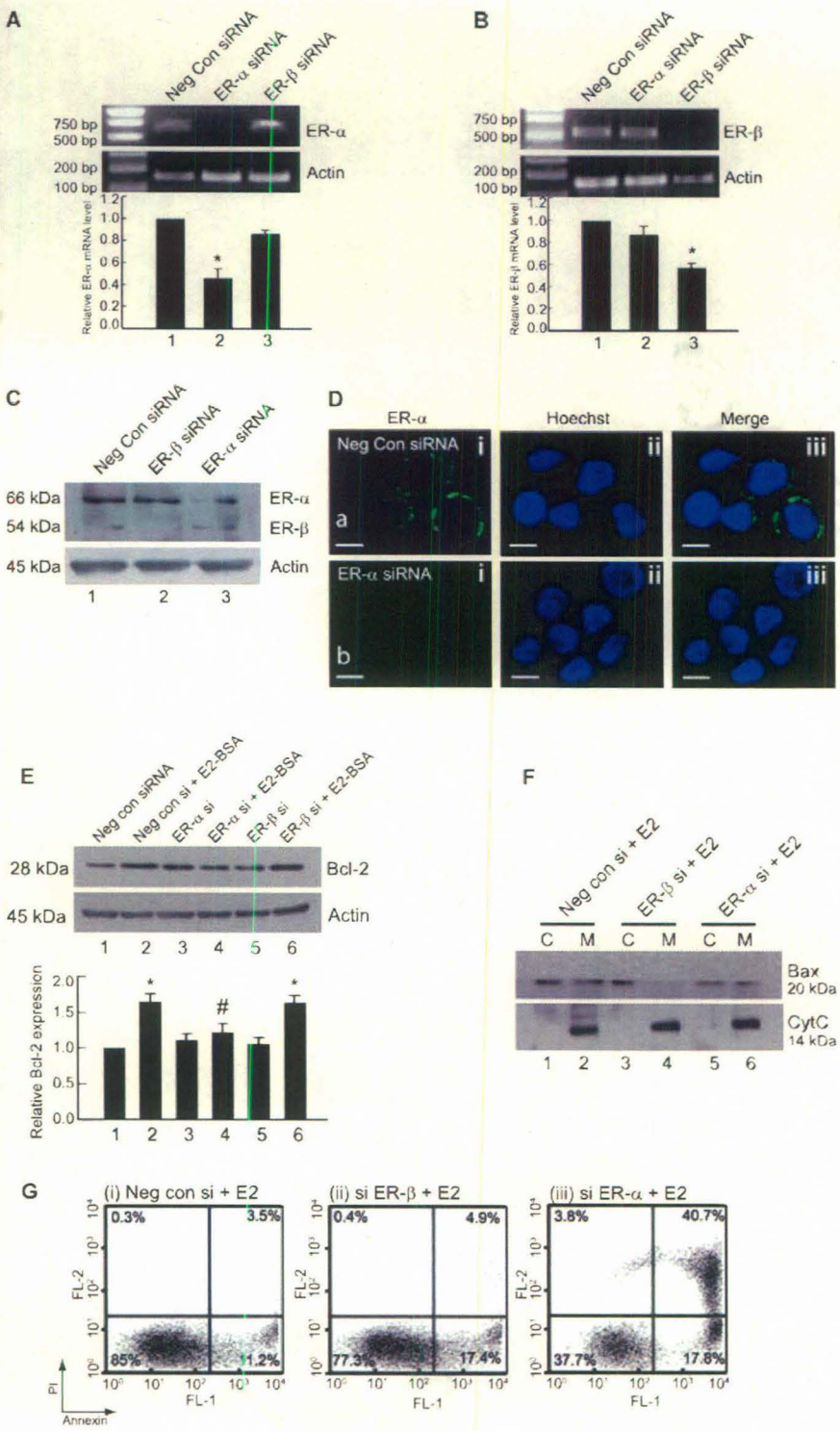

Fig. 3 Bcl-2 increase and Bax translocation are regulated by distinct oestrogen receptor subtypes. **(A)** RT-PCR for ER-α mRNA. Down-regulation of ER-α mRNA at 24 hrs of treatment by siRNA against ER-α (lane 2) in THP-1 cells as visible by amplification of mRNA for ER-α by RT-PCR. Note that there is no down-regulation of ER-α mRNA in cells transfected with ER-β siRNA (lane 3). **(B)** RT-PCR for ER-β mRNA. Down-regulation of ER-β mRNA by siRNA against ER-β (lane 3) at 24 hrs of treatment as visible by amplification of mRNA for ER-β by RT-PCR. Note that there is no down-regulation of ER-β mRNA (lane 2) in cells transfected with ER-α siRNA. **(C)** Western blot analysis on whole cell lysates for expression of ER-α and ER-β in THP-1 cells transfected with negative control siRNA (lane 1), ER-β siRNA (lane 2) or ER-α siRNA (lane 3), 24 hrs after transfection. Note the down-regulation of ER-α and ER-β after treatment with respective siRNAs. The blot was stripped and reprobed for actin, which was used as an endogenous loading control. **(D)** Live cell fluorescence microscopic analysis of ER-α expression on the plasma membrane by immunostaining with an ER-α specific antibody in negative control siRNA transfected (**a**, i–iii) and ER-α siRNA (**b**, i-iii) transfected THP-1 macrophages at 24 hrs. The blue stain (ii) is labelling with the nuclear dye Hoechst 33342. Note the loss of membrane ER-α expression in ER-α siRNA transfected cells (**b**, i-iii). The bar represents 10 μm. **(E)** Analysis of Bcl-2 expression by Western blotting in THP-1 macrophages transfected with negative control siRNA (Neg con siRNA), ER-α siRNA (ER-α si) or ER-β siRNA (ER-β si) for 24 hrs and subsequently treated with or without 10 nM E2-BSA for 6 hrs. Note the reduction in Bcl-2 when ER-α siRNA and E2-BSA (lane 4) was used compared with ER-β siRNA and E2-BSA, respectively (lane 6). Western blot for actin was used as loading control. Bar graph is densitometric representation of the relative Bcl-2 expression compared with negative control siRNA transfected cells. * $P < 0.05$ compared with the respective control groups. # $P < 0.05$ compared with negative control siRNA transfected cells treated with E2-BSA. **(F)** Western blot analysis for subcellular localization of Bax was performed in cells transfected with negative control siRNA (lanes 1, 2), ER-β siRNA (lanes 3, 4) or ER-α siRNA (lanes 5, 6) for 24 hrs, followed by treatment with 10 nM E2 for 6 hrs. Note the decrease in Bax translocation in lanes 3 and 4. The blots were stripped and reprobed for cytochrome c, which served as a control to determine the homogeneity of the obtained mitochondrial and cytosolic fractions. All data are representative of at least three independent experiments. **(G)** Flow-cytometric analysis of Annexin-V-PI staining in ER-α (iii) or ER-β (ii) knockdown cells, treated with respective siRNAs for 24 hrs, followed by exposure to 10 nM E2 for 6 hrs. Note that ER-α down-regulation in the presence of E2 shows high number of apoptotic cells. Cells in the lower left quadrant represent viable cells. Neg Con si: negative control siRNA; siER-α: ER-α siRNA; si ER-β: ER-β siRNA.

no decrease with ER-β siRNA (Fig. 3A, lane 3). Figure 3B, shows the RT-PCR for ER-β mRNA performed with ER-β specific primers, where lane 3 shows down-regulation of ER-β mRNA levels with ER-β siRNA but not with ER-α siRNA (lane 2). Negative control siRNA did not show any interference with RNA levels of either receptors (Fig. 3A and B, lanes 1). Status of protein levels of ER-α and ER-β after ER-α siRNA (Fig. 3C, lane 3) and ER-β siRNA (Fig. 3C, lane 2) transfection shows that protein levels of both receptors were significantly down-regulated with respective siRNA treatment. A distinct reduction of surface receptor population was observed with siRNA for ER-α (Fig. 3D, b, i-iii) compared with a negative control siRNA (Fig. 3D, a, i-iii), as visualized by immunostaining of treated and untreated cells with a specific anti-ER-α antibody.

Next, the effects of ER-α and ER-β down-regulation on Bcl-2 and Bax modulation were examined. E2-BSA was able to increase Bcl-2 in the presence of negative control siRNA (Fig. 3E, lane 2) but not when the cells were transfected with ER-α siRNA (Fig. 3E, lane 4), thus linking the involvement of ER-α in Bcl-2 increase. This treatment with ER-α siRNA, therefore, creates a condition within the cell, which is pro-apoptotic in nature because presence of E2 will induce Bax translocation, and in the absence of Bcl-2, Bax will induce apoptosis. In contrast, the knockdown of ER-β during E2-BSA exposure did not affect Bcl-2 increase (Fig. 3E, lane 6), showing the absence of any effect of membrane associated ER-β on Bcl-2 levels. Knockdown of ER-β (Fig. 3F, lanes 3 and 4) but not of ER-α (Fig. 3F, lanes 5 and 6) in the presence of E2 resulted in inhibition of Bax migration, thereby creating an anti-apoptotic condition because Bcl-2 will not have to counteract the effect of translocated Bax to the mitochondria. The translocation was complete in cells transfected with negative control siRNA (Fig. 3F, lanes 1 and 2). Together, the above data demonstrate a dichotomous effect of E2 on the components of the mitochondrial cell death pathway mediated through the two ER subtypes, ER-α mediating Bcl-2 increase and ER-β arbitrating Bax translocation.

Arguably, the modulation of the two receptor levels leading to changes in the pro-apoptotic Bax and anti-apoptotic Bcl-2 would affect cell survival. Annexin-V/PI staining showed that ER-β knockdown in the presence of E2 did not induce any apoptotic death (Fig. 3G, ii), whereas ER-α knockdown resulted in about 40% late apoptotic cells and 17% early apoptotic cells at 6 hrs after E2 exposure (Fig. 3G, iii). This corroborated our findings that ER-α but not ER-β was involved with the survival pathway, and interference with this pathway resulted in increased cell death in the presence of E2.

Bcl-2 modulation is mediated through ERK phosphorylation whereas Bax translocation is dependent upon intracellular alkalinization

Downstream to ER engagement by E2-BSA and E2, inhibition of ERK phosphorylation by MEK inhibitor PD 98,059 resulted in abrogation of Bcl-2 up-regulation (Fig. 4A, lane 5 and 3). Furthermore, E2-BSA was able to induce phosphorylation of ERK in as early as 10 min. (Fig. 4B, lane 4), which could be inhibited by ICI 182,780 (Fig. 4B, lane 5). Therefore, this indicated the competence of the membrane associated ERs to transmit Bcl-2 up-regulation signals. Following the observation that cytosolic ER-β was involved in Bax translocation, the mechanisms that lead to this change in subcellular localization was investigated. Clusterin

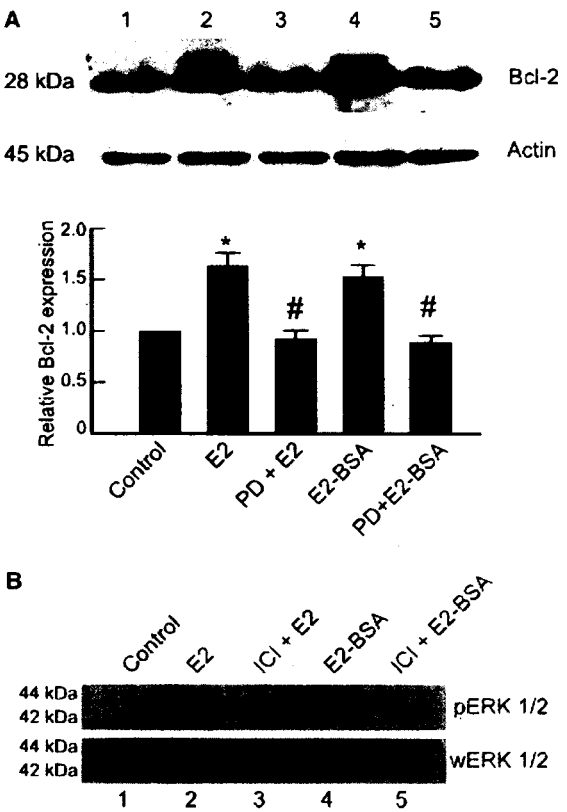

Fig. 4 E2-BSA regulates Bcl-2 increase through ERK phosphorylation. (**A**) THP-1 macrophages were treated with 10 nM E2 or E2-BSA for 6 hrs with or without pre-incubation with the MEK inhibitor PD 98,059 (25 μM, 30 min. prior to E2 addition), and extracts were probed for Bcl-2 levels by Western blotting. The bar graph represents densitometric measurements of Bcl-2 expression relative to control and normalized to actin (n = 3). The error bars represent ± SEM. * $P < 0.05$ compared with control. # $P < 0.05$ compared with E2 or E2-BSA treated group. PD, PD 98,059 (MEK inhibitor). (**B**) Figure shows detection of phosphorylated ERK1/2 as an early response by Western blotting in THP-1 macrophages treated with E2 and E2-BSA for 10 min. with or without incubation with ICI 182,780. The blot was stripped and reprobed for whole ERK1/2, which served as a loading control.

and cytosolic pH statuses were checked, as these are known to mediate Bax translocation [10, 11, 12]. E2 exposure did not suppress the expression of secretory form of clusterin in THP-1 cells (Fig. 5A), which is the pro-survival form, thereby ruling out its involvement in E2-induced Bax translocation as opposed to earlier reports in fibrosarcoma and prostate cancer cells, where suppression of clusterin was shown to induce Bax translocation [10]. Also, the level of pro-apoptotic nuclear clusterin remained unaltered (Fig. 5A).

In other cellular systems like B cells and thymocytes, it has been demonstrated that Bax translocation could occur in response to a pH change [11, 12, 31]. As shown in Figure 5B, E2 treatment resulted in an increase in intracellular pH from a basal level of 7.5 to about 7.7–7.8, but this increase was inhibited by the E2 receptor antagonist ICI 182,780 (Fig. 5B). Involvement of membrane-associated receptors was ruled out, as E2-BSA was unable to effect any change in pH. When pH change was prevented by placing the cells in high-K^+ buffer of desired pH in the presence of nigericin, an activator of K^+/H^+ antiporter, E2 was not able to induce Bax translocation (Fig. 5C, lane 3 and 4). On the contrary, the translocation was complete when cells were maintained at a pH of 7.7 (Fig. 5C, lane 5 and 6), suggesting that E2-induced intracellular alkalinization acted as a signal for Bax translocation. In contrast, E2-induced Bcl-2 up-regulation was unaffected by pH alterations (Fig. 5C). To ascertain if translocation of Bax could occur whenever there was a pH change independent of other stimuli, an increase in intracellular pH was induced by nigericin treatment in high-K^+ buffer in the absence of E2, and a change of pH to 7.7 resulted in Bax translocation but not when pH was maintained at 7.5 (Fig. 5D, lanes 5, 6 and 3, 4, respectively). These data point out that an increase in intracellular pH was sufficient to induce the translocation of Bax, independent of other signalling pathways that might be activated by E2. Also, siRNA mediated down-regulation of ER-β prevented E2 induced pH change (Fig. 5E), providing evidence that signals generated through ER-β was capable of altering the pH and also supports that data presented above that signals for Bax translocation is mediated through ER-β.

Becuse intracellular pH is maintained by the co-ordinated activity of a number of ion channels and their respective ions, the most important of which are the Na^+–H^+ exchangers (NHE) and the HCO_3^- transporters, involvement of each of these elements in the E2-induced increase in intracellular pH was investigated. NHE functions in the maintenance of intracellular pH by pumping out intracellular H^+ for extracellular Na^+, and hence its activity is indicated by both an increase in intracellular Na^+ and alkalinization of the cytoplasm due to expulsion of H^+ ions. When THP-1 macrophages were suspended in a Na^+-free media, E2 was unable to induce a pH

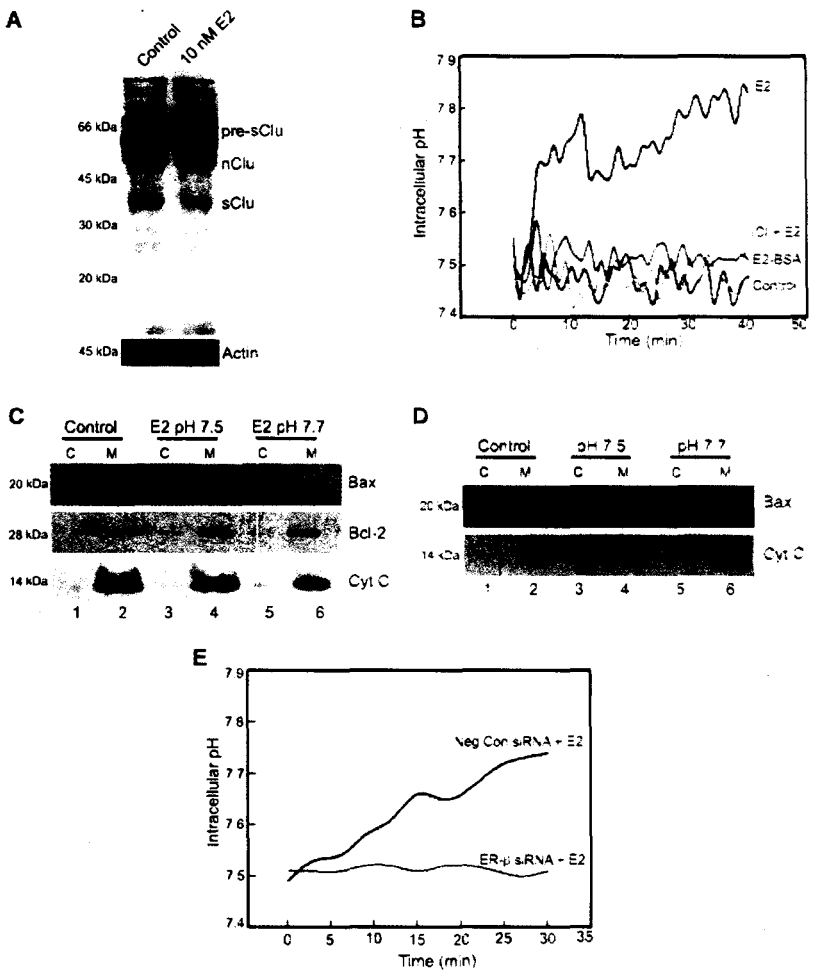

Fig. 5 Bax translocation is dependent on E2-induced change in intracellular pH mediated *via* ER-β. **(A)** Western blot analysis of clusterin expression after E2 treatment indicates no change in the expression of the different isoform levels. Pre-sClu: precursor to secretory clusterin; nClu: nuclear clusterin; sClu: secretory clusterin. **(B)** Intracellular pH measurement was performed with the pH sensitive dye SNARF-1 AM. The graph represents the calculated intracellular pH of THP-1 macrophages exposed to various drug treatments as indicated over a time-course of 40 min. Note that although E2 Q4 increased the intracellular pH, this was prevented in the presence of antioestrogen ICI 182,780 but E2-BSA but was unable to alter intracellular pH. ICI: ICI 182,780. **(C)** Appropriate groups of THP-1 macrophages were resuspended in high-K^+ buffer of pH 7.5 or 7.7. The control group was left untreated whereas the other two groups were treated with 1 μM nigericin and 10 nM E2 for 6 hrs. The cytosolic (C) and mitochondrial (M) fractions of the appropriate groups were probed for Bax and Bcl-2 expression by immunoblotting. Western blotting for cytochrome c was performed to determine the homogeneity of the obtained fractions. Note the lack of Bax migration in cells maintained at basal pH (lanes 3 and 4). Cyt C: cytochrome c. **(D)** Appropriate groups of THP-1 macrophages were resuspended in high-K^+ buffer of pH 7.5 or 7.7 and permeabilized with 1 μM nigericin to maintain the intracellular pH the same as that of the extracellular medium. Lysates of cytosolic (C) and mitochondrial (M) fractions were probed for Bax and cytochrome c by Western blotting. Note the migration of Bax at pH 7.7. Cyt C: cytochrome c. **(E)** Intracellular pH measurement in cells transfected with negative control siRNA and ER-β siRNA following treatment with 10 nM E2. Note the inhibition of increase in pH with knockdown of ER-β. Neg Con si: negative control siRNA; ER-β si: ER-β siRNA.

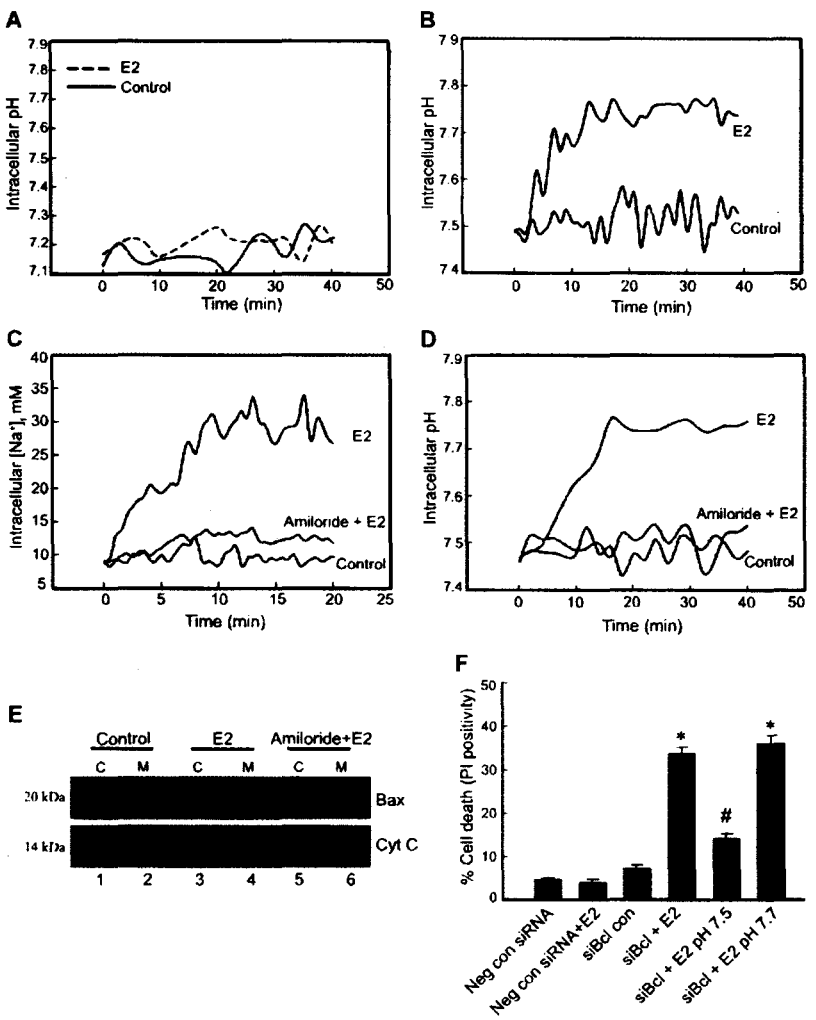

Fig. 6 Oestrogen increases intracellular pH by activation of Na^+–H^+ exchanger. THP-1 macrophages were resuspended in Na^+-free buffer (**A**) or HCO_3^--free buffer (**B**), and intracellular pH was measured using SNARF-1 AM dye after treatment with 10 nM E2 over a time period of 40 min. (**C**) Intracellular Na^+ was monitored in THP-1 macrophages using the fluorescent dye Sodium Green™ that shows a distinct increase in Na^+ in response to E2. Amiloride, a Na^+–H^+ exchanger inhibitor, was used at a concentration of 2 μM 10 min. prior to E2 treatment, which was able to inhibit the increase in intracellular Na^+ levels. (**D**) Intracellular pH measurement in THP-1 macrophages exposed to E2 and pre-incubated with or without the Na^+–H^+ exchanger inhibitor amiloride (2 μM) shows an abrogation of E2 induced increase in pH in the presence of amiloride. (**E**) Lysates of cytosolic (C) and mitochondrial (M) fractions prepared from the above groups after 6 hrs of incubation, and probed for Bax and cytochrome c on Western blots show the absence of Bax translocation to the mitochondria in the presence of amiloride (lanes 5 and 6). Cyt C: cytochrome c. (**F**) Cell viability. THP-1 macrophages were transfected with negative control siRNA or Bcl-2 siRNA, and 24 hrs post-transfection cells were subjected to appropriate treatments with E2 as indicated. All cells were resuspended in high-K^+ buffer and appropriate groups, where intracellular pH was to be maintained at 7.5 or 7.7, were treated with 1 μM nigericin. E2 treatment was given for 6 hrs and viability was analysed by propidium iodide dye exclusion method performed with fluorescence microscopy. The bar graph represents the percentage cell death in the various treatment groups. * $P < 0.05$, compared with cells transfected with negative control siRNA and treated with E2. # $P < 0.05$ compared with Bcl-2 siRNA transfected cells treated with E2. Neg Con si: negative control siRNA; siBcl: Bcl-2 siRNA.

change indicating that influx of Na^+ was essential for alkalinization (Fig. 6A). HCO_3^- was not required for E2-induced intracellular alkalinization because E2 was able to induce a pH change in a HCO_3^- free media (Fig. 6B). A possible role for NHE in mediating cellular alkalinization was indicated by an increase in intracellular Na^+ in response to E2 as observed by an increase in Sodium Green™ fluorescence (Fig. 6C). This was further confirmed when amiloride, an NHE inhibitor lowered Na^+ levels and also prevented alkalinization of the cytoplasm (Fig. 6C and D). If amiloride could prevent intracellular alkalinization, ideally then amiloride should be able to prevent translocation of Bax if pH increase and Bax translocation were linked. Western blots of cytosolic and mitochondrial fraction obtained from cells treated with E2 in the presence of amiloride showed absence of Bax translocation to mitochondria (Fig. 6E, lanes 5 and 6) compared with cells treated with E2 only (Fig. 6E, lanes 3 and 4). In summary, the above data suggest that E2 signals, through ER-β, to induce intracellular alkalinization *via* activation of NHE, which results in Bax translocation.

To confirm that alkalinization-induced change in Bax translocation is the major pro-apoptotic event induced by E2, Bcl-2 knockdown macrophages were treated with E2, a situation that normally precipitates cell death (Fig. 6F, siBcl + E2), but when these cells are maintained at pH of 7.5, the apoptosis inducing effect of E2 was abrogated (Fig. 6F, siBcl +E2, pH 7.5).

Discussion

Insights into the role of oestrogen in macrophage survival and associated mechanisms are of great relevance because the findings would have direct bearing on the development of tumour targeting therapies [32]. Our earlier study showed that E2 was able to induce apoptosis in human macrophages when Bcl-2 was down-regulated [5]. The work described in this manuscript explores the involvement of E2 receptor subtypes localized in distinct subcellular compartments in the regulation of mitochondrial death pathway in human THP-1 macrophages. We demonstrate that (i) signals for Bcl-2 increase is primarily mediated through membrane associated ER-α; (ii) the translocation of Bax to mitochondria is mediated *via* signalling through intracellular ER-β receptors and (iii) the E2-induced Bax translocation is dependent on intracellular alkalinization mediated through activation of Na^+/H^+ exchangers.

Ratio of Bcl-2/Bax is crucial in maintaining cell viability under certain conditions, and therefore the relative involvement of the ERs in regulating this ratio was important to examine. Recognition of ER-α binding sites on live cells by specific anti-ER-α antibody and knockdown of surface ER-α by siRNA for ER-α clearly confirmed the presence of surface localized ER-α in THP-1 cells, which is in agreement with a growing body of evidence showing the presence of membrane associated ERs in various cell types [33, 34]. Interestingly, the surface located ER-α emerges as the major transducer of survival signal during E2 treatment, as demonstrated by the ability of E2-BSA, the membrane impermeable form of E2, to up-regulate Bcl-2, as well as abrogation of this effect upon siRNA mediated knockdown of surface localized ER-α resulting in cell death. Although we show that membrane associated ER-α is sufficient to transduce the survival signal, the relative contribution of cytosolic ER-α in the survival response could not be determined due to the non-availability of specific inhibitors to intracellular *versus* the membrane receptors. The anti-apoptotic role of ER-α as noted in our studies is in concurrence with other reports that implicate ER-α in mediating E2 induced protective role during H_2O_2 induced apoptosis in murine skeletal muscle C2C12 cells [35] or in human osteosarcoma cell line [36]. The downstream events after engagement of E2 on membrane associated ER-α involved the activation of MAPK pathway for an induction of Bcl-2 increase because E2-BSA was competent to phosphorylate ERK, and MEK inhibition could inhibit Bcl-2 up-regulation. The functional role of surface receptors in mediating survival signals was of interest because surface receptors are amenable to selective manipulation by cell impermeable agonists, providing opportunities to exploit the surface receptors for induction or inhibition of specific cellular functions.

In contrast to E2-induced Bcl-2 increase, Bax translocation was independent of membrane bound receptors because E2-BSA, which interacted with surface receptors, was unable to induce Bax migration. Because knockdown of ER-β but not of ER-α resulted in abrogation of Bax translocation, it indicated the importance of ER-β in death inducing arm of the mitochondrial apoptotic pathway. This particular function has not been demonstrated in cells of monocytic origin, but mediation of pro-apoptotic events by ER-β is known in cells of non-myeloid lineage like breast and colon cancer cells [37, 38]. As Bax translocation is the primary event that initiates changes pertaining to cell death, the mechanism of translocation consequent to ER- β mediated signalling by E2 is of importance. It is known that translocation of Bax to the mitochondria is linked to alteration in its conformation resulting in the exposure of its N-terminal or BH-3 domain [39, 40], which is under the control of various physiological factors, possibly of different natures [41]. Prior knowledge that a change in pH could trigger Bax movement [11, 12, 31] prompted us to focus on the possibility of E2 inducing a pH change in THP-1 cells. Because Bax translocation could be initiated upon intracellular alkalinization in the presence or in the absence of E2, movement of Bax was likely to be facilitated by any stimulus capable of altering cellular pH. Importantly, the consequence of Bcl-2/Bax ratio changes would affect cell survival, and a substantial decrease in cell death was observed after E2-induced pH change was blocked at the time of Bcl-2 knockdown, presumably due to lack of Bax translocation to the mitochondria, thus validating the observation that Bax migration to mitochondria due to pH change in the absence of concomitant Bcl-2 up-regulation is responsible for increased apoptosis. Q5 Q6

Therefore, intracellular alkalinization was an important event, and this appeared to be mediated by NHE because the process was Na^+-dependent and could be inhibited by amiloride, a NHE inhibitor. A number of studies show that E2 can alter NHE functions [42] through a NHE regulatory factor (NHE-RF), which is a primary response gene under ER control [43]. However, the rapid increase in pH in response to E2 observed in our system makes transcriptional regulation through NHE-RF unlikely. The mechanism of NHE involvement remains unknown.

In summary, this study highlights the importance of oestrogen signalling through distinct ER subtypes in modulating the mitochondrial death pathway of human monocyte derived macrophages. The observations raise interesting possibilities of exploring the use of selective oestrogen receptor modulators specific for ER-α or ER-β or those which could signal exclusively through the membranous or cytoplasmic pool of receptors to manipulate death pathway in human macrophages. For example, estren, which is an oestrogen agonist signalling selectively on the membranous ER with no known transcriptional

effects *via* the classical ER mechanism [44], could be used for generating anti-apoptotic effects. The development and use of such agonists and antagonists could be utilized to target specific receptor population in target cells to achieve desired therapeutic effects like manipulation of death pathways in favour or against cell survival.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1 Translocation of oestrogen receptors into the nucleus upon E2 treatment. Sub-cellular distribution of ERs consequent to treatment with 10 nM E2 or E2-BSA for 2 h is shown by immunofluorescence using an antibody, which recognizes both ER-α and ER-β. The panel stained green represents the ER-α/β staining (b), the blue staining represents nuclear staining with Hoecsht 33342 nuclear dye (c). The merge of images in panel b and c is shown in panel d. The panel "coloc mask" (e) represents the area within the cell showing colocalization of estrogen receptors with the nucleus. The value within the panel "coloc mask" represents the coefficient of colocalization of ER- α/β staining with the nuclear staining. Panel a represents phase contrast images. The bar represents 10 μm.

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