MECHANISMS INVOLVED IN THE REGULATION OF HUMAN LEUKOCYTE ANTIGEN CLASS II EXPRESSION IN ESTROGEN RECEPTOR ALPHA POSITIVE AND ESTROGEN RECEPTOR ALPHA NEGATIVE BREAST CANCER CELLS

By

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ABSTRACT

Human leukocyte antigen (HLA) class II molecules [HLA-DR, HLA-DM, invariant chain (Ii)] are crucial for anti-tumor immune responses. Regulation of HLA class II occurs mainly at the transcription level through the class II transactivator (CIITA) that is induced by interferon gamma (IFN-γ). In addition, mitogen activation protein kinase (MAPK) regulates HLA class II expression at the protein and the transcriptional level in antigen presenting cells (APC) and some tumors.

Previously, our laboratory reported that tumor cell expression of HLA-DR in breast cancer correlated with decreased estrogen receptor (ER) levels and younger age at diagnosis. In this study we used established $ER\alpha^+$ and $ER\alpha^-$ breast cancer cell lines (BCCL) from ATTC and showed that, estradiol (E_2) resulted in down regulation of HLA-DR expression in MCF-7 ($ER\alpha^+$) and BT-474 ($ER\alpha^+$), but not in T47-D ($ER\alpha^+$), nor in SK-BR-3 ($ER\alpha^-$) and MDA-MB-231 ($ER\alpha^-$). Altogether, these data suggest that the E_2-ER pathway plays a significant role in HLA class II expression in breast carcinoma. We further postulated that the higher expression of HLA-DR in $ER\alpha^-$, as compared to $ER\alpha^+$ breast cancer cells is due to MAPK activation.

To study the mechanism(s) involved in the regulation of HLA class II expression in breast cancer cells, we used a cell line model of an $ER\alpha^+$ cell line, MC2 (MDA-MB-231 transfected with the wild type ERα gene) and $ER\alpha^-$ cell line, VC5 (MDA-MB-231 transfected with the empty vector). These cells were subjected to different hormonal treatments [E_2, Tamoxifen (TAM) and Fulvestrant (ICI 182,780)], cytokine treatments

(IFN-γ) and MAPK inhibitors (U0126, SP 600125 and SB 202190), to determine their effects on HLA class II regulation.

Inducible CIITA and HLA class II expression were markedly reduced at the protein and transcription levels in MC2, compared to VC5. Moreover, CIITA and IFN-γ activated sequence (GAS) luciferase activities were reduced in MC2 and were further inhibited by E_2-treatment. In parallel, E_2 decreased GAS luciferase activity in the same pattern in MCF-7 ($ER\alpha^+$) and BT-474 ($ER\alpha^+$). The MAPK pathway played a role in HLA-DR surface expression in the breast cancer cell lines (BCCL) regardless of ERα status. In aggregate, these results indicate that the E_2-ER and MAPK signaling pathways modulate HLA class II expression in BCCL.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

AD:	Activation domain
AF-1:	Activation function
AML:	Acute myeloid leukemia
Amp:	Ampicillin
AP1:	Activator protein-1
APC:	Antigen presenting cells
APS:	Ammonium persulfate
ARE:	Activation response element
Arf6:	ADP-ribosylation factor 6
ATF:	Activating transcription factor
ATP	Adenosine triphosphate
BCA:	Bicinchoninic acid
BCCL:	Breast cancer cell lines
BIGAF:	Binding -IFN- gamma activated factor
BLIMP1:	B-lymphocyte-induced maturation protein
BRG1:	Brahma-related gene 1
BSA	Bovine serum albumin
cAMP:	Cyclic adenosine monophosphate
CARD:	Ccaspase-recruitment domain
CARM1:	Co-activator-associated arginine-nethyltransferase 1
CBP:	CREB-binding protein
CCD:	Coiled-coiled domain
CDFBS:	Charcoal/dextran fetal bovine serum
CDK:	Cyclin-dependent kinases
CIITA:	Class II transactivator
CLIP:	Class II associated Ii peptide
CO-IP:	Co-immunoprecipitation
CREB:	cAMP response element binding protein
CT:	Cycle threshold
D:	Aspartic acid
DBD:	DNA binding domain
DC:	Dendritic cells
DCCR:	DharmaFECT cell culture reagent
DEPC:	Diethyl procarbonate
DHEA	Dehydroepiandrosterone
DMSO:	Dimethyl sulfoxide
DN:	Dominant negative
DTT:	Dithiothreitol
E-box:	Enhancer box
E:	Glutamic acid

E. Coli:	Escherichia coli
E_2:	Estradiol
E2A:	Early region 2a
EDTA:	Ethylene-diaminetetraaccetic acid
EGFR:	Epidermal growth factor receptor
Elk-1:	ETS domain-containing protein 1
ER:	Estrogen receptor
ERE:	Estrogen response element
ERK:	Extracellular signal-regulated protein kinases
ETS:	E-twenty six
FBS:	Fetal bovine serum
FGF:	Fibroblast growth factor
G:	Glycine
GAM:	Goat anti-mouse
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GAR:	Goat anti-rabbit
GAS:	IFN-γ activated sequence
GFP:	Green fluorescence protein
GPR:	G-Protein-coupled receptor
GTP:	Guanosine triphosphate
H:	Histidine
H3, 4:	Histone 3 and 4
HAT:	Histone acetyltransferase
HDAC:	Histone deacytylase
HEPES:	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HLA:	Human leukocyte antigen
HMT:	Histone methyltransferases
Hour:	Hr
HSP:	Heat shock protein
IBE:	Immortalized brain endothelial cells
ICAM-1:	Intercellular adhesion molecule 1
ICC:	Immunocytochemistry
ICI 182, 780:	Fulvestrant (ICI)
IFN:	Interferon
IFNAR:	IFN-alpha receptor
IFNGR:	IFN-gamma recpetor
Ii:	Invariant chain
IL:	Interleukin
IMDM:	Iscove's modified Dulbecco's medium
IP-10:	Interferon-induced protein of 10 kDa
IRF-E:	IFN-regulatory factor element
IRF:	IFN-regulatory factor
ISGF3:	Interferon stimulated gene factor 3
ISRE:	Interferon-sensitive response element
JAK:	Janus activating kinases

JH:	Janus homology domains
JIP:	JNK interacting protein
JNK:	c-Jun N-terminal kinases
K:	Lysine
Kb:	Kilobase
kDa:	Kilo Dalton
LARII:	Luciferase assay reagent II
LB	Lysogeny broth
LBD:	Ligand-binding domain
LMP:	Low molecular weight Protein
LOH:	Loss of homozygoisty
LPKS:	Leucine-proline-lysine-serine
LRR:	Leucine-rich repeats
MAP-K2:	MAPK-activated protein kinase-2
MAPK:	Mitogen-activated protein Kinase
MAPKAP:	MAPK-activated protein kinase
ME:	Mercaptoethanol
MECL:	Multicatalytic endopeptidase complex Like
MEK1	MAPK/ERK kinase 1
MEKK	MEK kinase
MEM:	Minimum essential medium
MFI:	Mean fluorescence intensity
MGB:	Minor groove binder
MHC:	Major histocompatibility complex
MIC:	MHC class I related chain
MIG:	Monokine induced by IFN
MIIC:	MHC class II loaded compartments
Min:	Minute
MKK6:	MAPK kinase 6
MLK:	Mixed-lineage protein kinase
MNK:	MAPK-interacting kinase
MSC:	Mesenchymal stem cells
MSK:	Mitogen and stress-activated protein kinase
MTA1	Metastasis associated gene 1 protein
NCoR	Nuclear receptor co-repressor
NES:	Nuclear export signal
NF-IL6:	Nuclear factor for interleukin 6
NF-Y:	Nuclear transcription factor Y
NF-κβ:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK:	Natural killer
NLRC5:	NOD-like receptor family CARD domain containing 5
NLS:	Nuclear localization sequence
NOD:	Nucleotide-binding oligomerization domain
NPI-1:	Nucleoprotein Interacting Protein 1
NR:	Nuclear Receptor

OBF:	Octamer-binding factor
OBS:	Octamer-binding site
p-TEFb:	Positive elongation factor b
P:	Proline
PA:	Proteasome activated
PAGE:	Polyacrylamide gel
PBS:	Phosphate buffer saline
pCAF:	p300/CBP-associated factor
PDL-1:	Program death ligand 1
PFA:	Paraformaldehyde
pI, pII, pIII, pIV:	CIITA promoters 1-4
PI3-K:	Phosphoinositide-3-kinase
PKC:	Protein kinase C
PLB:	Passive lysis buffer
PMSF:	Phenylmethylsulfonyl fluoride
Pol II:	Polymerase II
PRF:	Phenol red-free
PST:	Proline-serine-threonine
PTP:	Protein tyrosine phosphatase
PyK2:	Protein tyrosine kinase 2
Raf1:	Rapidly accelerated fibrosarcoma 1
RFX:	Regulatory factor X
RFXANK:	RFX-associated protein with ankyrin repeats
RFXAP:	RFX-associated protein
RIPA:	Radioimmunoprecipitation assay
RLU:	Relative luciferase unit
RNA:	Ribonucleic acid
RPMI:	Roswell park memorial institute
RQ:	Relative quantity
RSK:	Ribosomal S6 kinase
RT-PCR:	Reverse transcriptase polymerase chain reaction
S:	Serine
SAPK:	Stress activated protein kinase
SDS:	Sodium dodecyl sulphate
SEM:	Standard errors of the mean
SERM:	Selective ER modulator
SH2:	Src homology 2
Shp2:	SH2-containg tyrosine phosphatase-2
siRNA:	Small interfering RNA
SMRT	Silencing mediator for retinoid or thyroid-hormone receptors
SOCS:	Suppressors of cytokine signaling
SRC:	Steroid receptor co-activator
STAT:	Signal transducer and activator of transcription
SWI-SNF	SWItch/sucrose non-fermentable
T:	Threonine

TAA:	Tumor associated antigen
TAD:	Transcriptional activation domain
TAFII:	Tein-associated factors II 32
TAM:	Tamoxifen
TAP:	Transporter associated with antigen processing
TATA:	Thymine-adenine-thymine-adenine
TBE:	Tris/borate/EDTA
TBS	Tris-buffered saline
TE:	Tris/EDTA
TEC:	Thymic epithelial cells
TEMED:	Tetramethylethylenediamine
TFIIB:	TATA binding protein-associated transcription factors IIB
TGF-β:	Transforming growth factor beta
Th1:	T helper type 1
TM:	Transmembrane
TNF:	Tumor necrosis factor
TSA:	Trichostatin-A
USF:	Upstream stimulatory factor
UTR:	Untranslated region
V:	Volt
WB:	Washing buffer
X2BP:	X2 binding protein
XBP-1	X box-binding protein 1
Y:	Tyrosine

LIST OF APPENDICES

Chapter 1: Introduction

1.1. The major histocompatability complex

1.1.1. Overview

The major histocompatibility complex (MHC) is a group of highly polymorphic genes that spans a region of approximately 4000 kilobase (kb) on the short arm of chromosome six (6p21.3) and is referred to as human leukocyte antigen (HLA) in humans and the H-2 complex in mice. This region is comprised of a series of about 200 tightly linked pseudogenes, expressed genes and genes of unknown function (1). MHC is divided into three regions called class I, class II and class III.

The classical HLA class I genes code for HLA-A, B and C molecules which are important in presenting peptides of 8-10 amino acids in length from endogenously synthesized proteins to $CD8^+$ cytotoxic T-cells (2). HLA class I glycoproteins form heterodimers with β_2-microglobulin and are expressed in nearly all nucleated cells. The non-classical HLA genes code for HLA-E,-F,-G, and MHC class I related chain A (MICA) & MICB (3), which have a significant role in innate immunity by acting as a natural killer (NK) cell inhibitory (4, 5) and activating ligands (6) respectively.

The HLA class II region spans 1000 kb and contains numerous pseudogenes as well as the classical HLA class II genes (*HLA-DRA, DRB, DPA, DPB, DQA and DQB*), which code for class II α chain and β chain glycoproteins. Both chains form heterodimers and are important in sampling exogenous antigenic peptides, of 12-24 amino acids residues in length to $CD4^+$ T helper cells (3). Constitutive expression of HLA class II molecules is largely restricted to antigen presenting cells (APC), namely dendritic cells

(DC), B cells and macrophages. HLA class II expression is regulated in these cells in a maturation-dependent manner, such that HLA expression on DC increases with maturation, whereas B cells downregulate expression following their differentiation into plasma cells. HLA class II is also expressed on thymic epithelial cells (TEC) and activated T cells. Various cytokines such as interferon gamma (IFN-γ) may upregulate expression on most other HLA negative cell types such as fibroblasts, endothelial and epithelial cells (7-9). The class II region also encodes for the non-classical HLA-DM, which acts as peptide editor for proper antigen presentation (10, 11), and HLA-DO, which is important in negative regulation of HLA-DM (3). The transporter associated with antigen processing (TAP) and low molecular weight proteins (LMP), which are involved in HLA class I antigen presentation pathway (12), are also encoded by genes in the class II region (3).

HLA class III regions contain 57-60 structural genes, most of which code for proteins that have important molecular functions in the immune response. These include tumor necrosis factor (TNF)-α, lymphotoxin and complement factors C2 and C4 (2).

The genes encoded in each of these sub-regions play an important role in the adaptive immune system, development of autoimmunity, and response to tumor antigens. Since this thesis concerns HLA class II molecules and breast carcinoma, this part of the review will focus on HLA class II genes.

1.1.2. Structure of human leukocyte antigen class II molecules

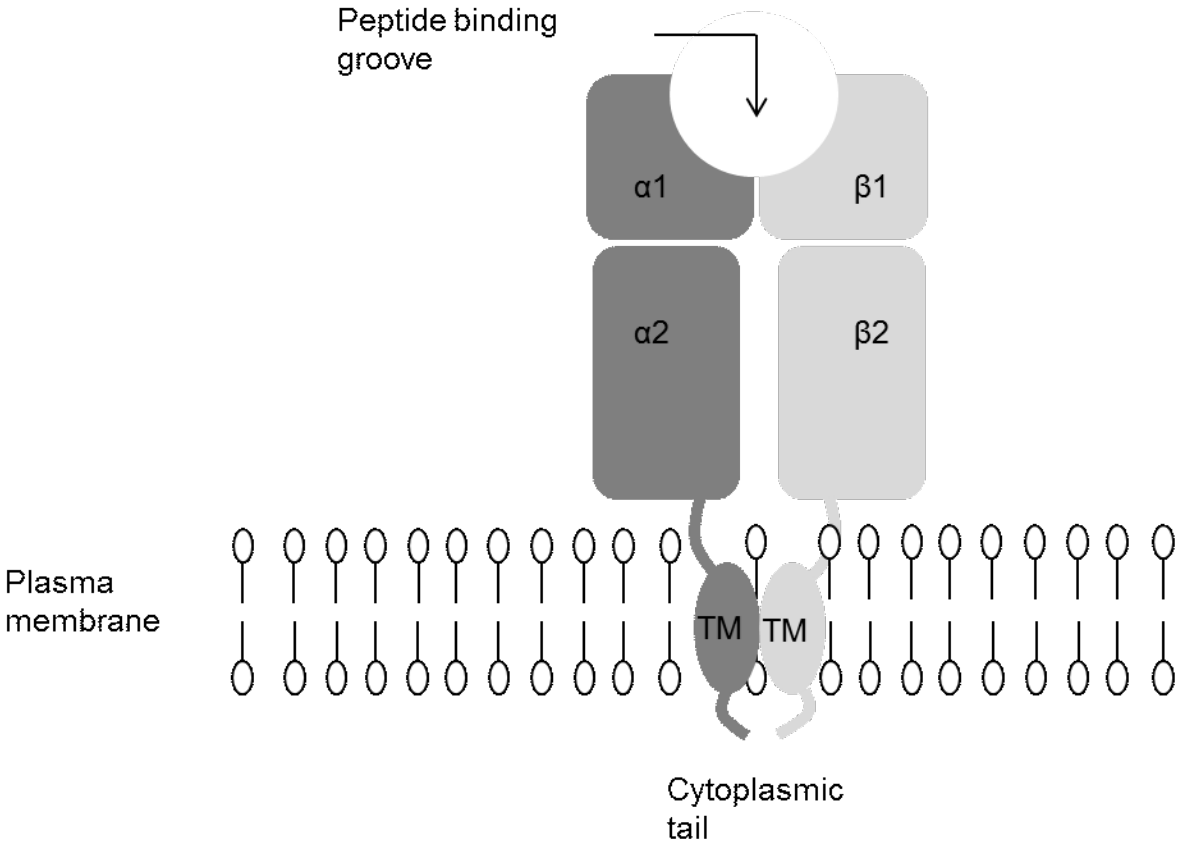
The HLA class II molecule is a heterodimer, composed of four external domains, two in each chain (α1, α2 and β1, β2), a transmembrane (TM) piece and a short intra-cytoplasmic domain (Figure 1.1). The peptide-binding groove is composed of two alpha helices above a beta-pleated sheet represented by α1 and β1 domains and is open-ended to accommodate long peptides (12-24 amino acid residues). The amino acid sequence around the peptide-binding groove is the most variable site in the HLA molecule, leading to a high degree of peptide-binding specificity. HLA-DRα is considered monomorphic, with absent functional variation in the mature *HLA-DRA* alleles. Thus, the polymorphism of HLA-DR is mainly through the HLA-DRβ chain with about 1200 alleles listed in IMGT/HLA Database Version Report-3.11.0 (2013-01) (13). In contrast, both α and β chains are responsible for the polymorphism of HLA-DP and HLA-DQ while little polymorphism is reported for the non-classical HLA-DM and HLA-DO (3, 14).

1.1.3. Human leukocyte antigen class II antigen presentation pathway

Alpha and beta chains of HLA class II molecules are synthesized in the rough endoplasmic reticulum along with a third molecule, the invariant chain (Ii) (Figure 1.2), that is important in the HLA class II presentation pathway. The Ii has several functions; firstly, it acts as a co-chaperone and ensures proper folding and stability of the α and β chains of HLA class II molecules. This occurs through the formation of a nine subunit complex consisting of three class II α and β dimers associated with one Ii trimer $(\alpha\beta)_3Ii_3$ (15). Secondly, Ii binds to the peptide-binding groove of the HLA class II during its

Figure 1.1 Human leukocyte antigen class II molecule structure.

Human leukocyte antigen (HLA) class II molecules consist of two glycoprotein trans-membrane chains (α and β), which are non-covalently linked. The HLA class II molecules are anchored in the plasma membrane by the transmembrane (TM) domain and cytoplasmic tail. Adapted from (14).


Peptide binding groove
α1
β1
α2
β2
Plasma membrane
TM
TM
Cytoplasmic tail

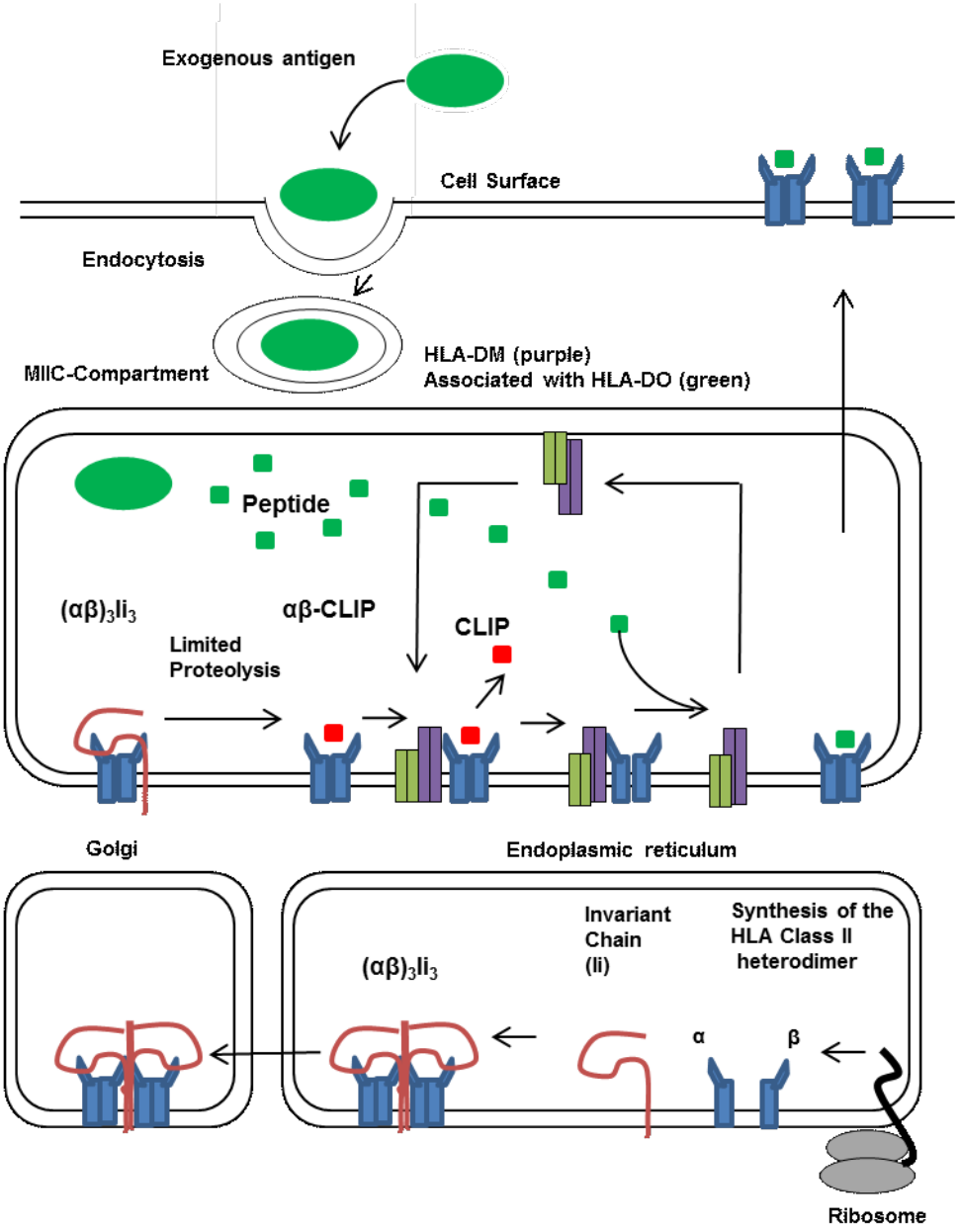
assembly in the endoplasmic reticulum, thereby preventing non-specific peptide binding (16). Lastly, Ii molecules harbor sorting signals in its cytoplasmic tail which play a significant role in transportation of $(\alpha\beta)_3Ii_3$ complex to the Golgi apparatus (17).

Ii transits from the Golgi apparatus to the endocytic compartment, including early endosomes, late endosomes and lysosomes. Lysosomes fuse with the late endosome-like compartment to form MHC class II loaded compartments (MIIC) that contain abundant HLA class II complexes (17). In the MIIC, limited proteolysis of Ii occurs by the lysosomal protease, cathepsin S, leaving a class II associated Ii peptide (CLIP) in the peptide-binding groove (18). CLIP is exchanged for antigenic peptides, a process that is catalyzed by HLA-DM molecules, which function as a "peptide editor", exchanging CLIP and low affinity binding peptides from the peptide-binding groove, with high affinity peptides (19). HLA-DM is always found in close association with HLA class II molecules in MIIC compartment with very small amounts detected on the cell surface (20). Besides being a "peptide editor", HLA-DM stabilizes unbound class II molecules, thus, preventing HLA-DR from aggregating (21). HLA-DM negative cells do not undergo this peptide exchange and CLIP remains bound within the groove (22), leading to unstable HLA/peptide complex.

HLA-DO is a heterodimer encoded by *HLA-DOB* and *HLA-DNA* genes (now referred to as *HLA-DOA*) and acts as a negative modulator of HLA-DM (23, 24). The action of HLA-DO is maintained by stably associating with HLA-DM, thus, inhibiting the catalytic action of HLA-DM on class II peptide loading. Additionally, HLA-DO affects the peptide repertoire that is ultimately presented to the immune system by HLA class II molecules (25). Recently, it was shown by X-ray crystallography that HLA-DO can act

Figure 1.2 Biology of antigen presentation by human leukocyte antigen class II.

Human leukocyte antigen (HLA) class II heterodimers are synthesized in the endoplasmic reticulum and form a peptide-binding groove that is bound by Invariant chain (Ii). Ii associates with HLA class II to form $(\alpha\beta)_3Ii_3$ complex. $(\alpha\beta)_3Ii_3$ complexes move into MHC class II loading compartment (MIIC) where Ii is degraded by cathepsin S with the class II associated Ii peptide (CLIP) fragment remaining (orange). HLA-DM, which is associated with HLA-DO, assists in exchange of CLIP for a high affinity stable peptide (green). HLA-DM and DO dissociate from the complex and loaded HLA class II are transported to the cell surface to interact with $CD4^+$ helper T cells. Adapted from (26).


Exogenous antigen
Cell Surface
Endocytosis
MIIC-Compartment
HLA-DM (purple)
Associated with HLA-DO (green)
Peptide
$(\alpha\beta)_3Ii_3$
αβ-CLIP
CLIP
Limited
Proteolysis
Golgi
Endoplasmic reticulum
Invariant
Chain
(Ii)
Synthesis of the
HLA Class II
heterodimer
$(\alpha\beta)_3Ii_3$
α
β
Ribosome

as a competitive inhibitor for HLA-DM. HLA-DO binds to HLA-DM in the same site involved in HLA class II interaction, which results in prevention of association of HLA-DM with HLA class II molecules (27). Ultimately, HLA class II molecules, which are properly loaded with the stable peptide complexes, are then transported to the plasma membrane for presentation to $CD4^+$ T cells (15).

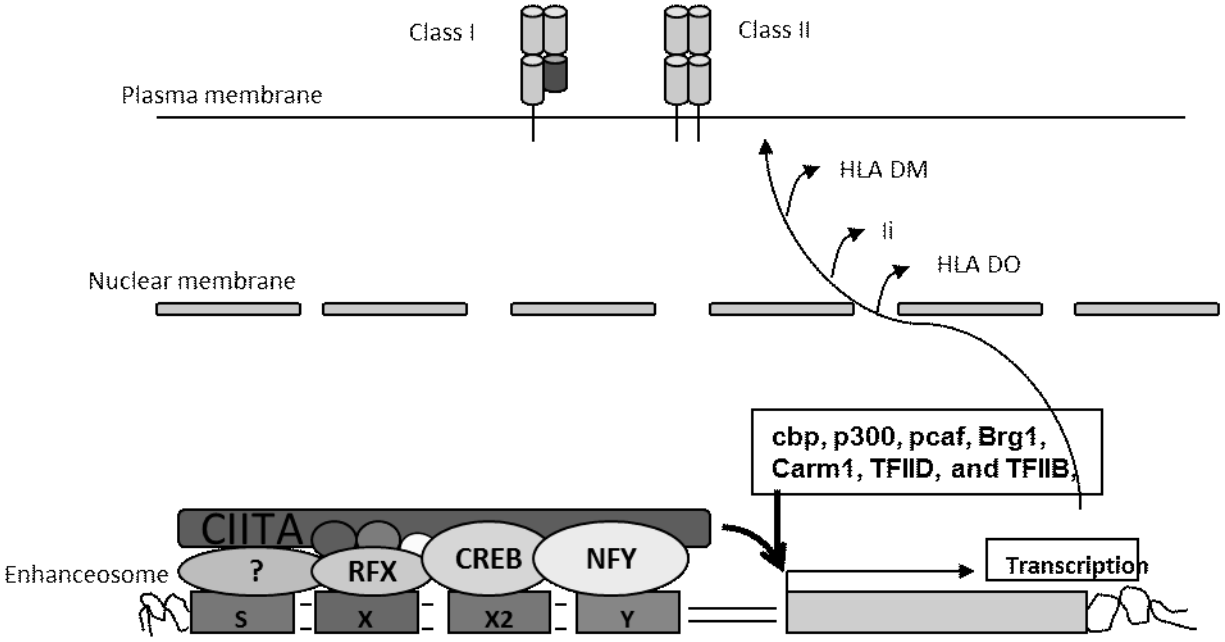
1.1.4. Regulation of human leukocyte antigen class II gene expression

Regulation of HLA class II gene expression occurs mainly at the transcriptional level, by a highly conserved regulatory module that is located in the promoter of genes encoding the α-chain and β-chain of all HLA class II molecules and Ii. This HLA class II specific regulatory module, known as the SXY module, consists of four sequences: the S, X, X2 and Y boxes (28) (Figure 1.3). This module is bound by transcription factors to form the multi-protein complex known as the HLA class II enhanceosome (29). A trimeric complex composed of regulatory factor X 5 (RFX5), RFX-associated ankyrin-containing protein (RFXANK; RFX-B) and RFX-associated protein (RFXAP), referred to as RFX, is bound to the X box (30-33). X2 binding protein (X2BP) and cAMP response element binding protein (CREB) are bound to the downstream X2 box (34). A trimeric complex composed of nuclear transcription factor YA (NF-YA), NF-YB, and NF-YC, together known as NF-Y, is bound to the Y box (35). The transcription factor that binds to the S-box remains unclear, although it has been shown that RFX could bind to the S box in vitro (36).

Although all the previously described transcription factors are ubiquitously and

Figure 1.3 Regulation of human leukocyte antigen class II expression.

Human leukocyte antigen (HLA) class II is regulated at the transcriptional level by binding of regulatory factor X (RFX), cAMP response element-binding (CREB), nuclear transcription factor Y (NF-Y) to the S, X, Y module present in the promoter of HLA class II genes. These form a landing pad for class II transactivator (CIITA), which recruits more transcription machinery and aids in HLA class II expression. Adapted from (37).



Gene encoding class II Molecules

constitutively expressed, most of the cells lack the ability to induce HLA class I expression on their own. Yet, they form the enhancesome complex, which acts as a landing pad for the class II transactivator (CIITA) (38-40) (Figure 1.3).

1.1.5. Class II transactivator overview

The CIITA is a non-DNA binding protein that acts as a transcriptional integrator by connecting transcription factors bound to the HLA class II promoter with components of the general transcriptional machinery. CIITA interacts with general transcription factors thymine adenine thymine adenine (TATA) binding protein-associated transcription factors IIB (TFIIB), tein-associated factors II 32 and II 70 ($TAF_{II}32$ and $TAF_{II}70$), which help transcription initiation (41, 42); positive elongation factor b (p-TEFb), which promotes transcription elongation and promoter clearance (42, 43); and cAMP response element-binding (CREB) protein (CBP) involved in chromatin remodelling (44, 45). Moreover, CIITA interacts with cyclin-dependent kinases 7 (CDK7) and CDK9, and facilitates their ability to phosphorylate the carboxy-terminal domain of RNA polymerase II (Pol II), thereby starting promoter clearance and mRNA synthesis (46).

CIITA expression directly correlates with expression of class II genes, thus, the term “master regulator” of HLA class II genes is usually used to describe CIITA (47). Furthermore, CIITA regulates other genes that facilitate the class II antigen-processing pathway that contains S-X-Y boxes in their promoter (48). It was previously thought that CIITA contribute to the expression of HLA class I through interaction with a region in the promoter, which shows sequence similarity to the S-X-Y module (49-51). Recent data

showed that nucleotide-binding oligomerization domain (NOD) like receptor family CARD domain containing 5 (NLRC5) acts in a manner similar to CIITA and binds to transcription factors bound to those regulatory elements (52).

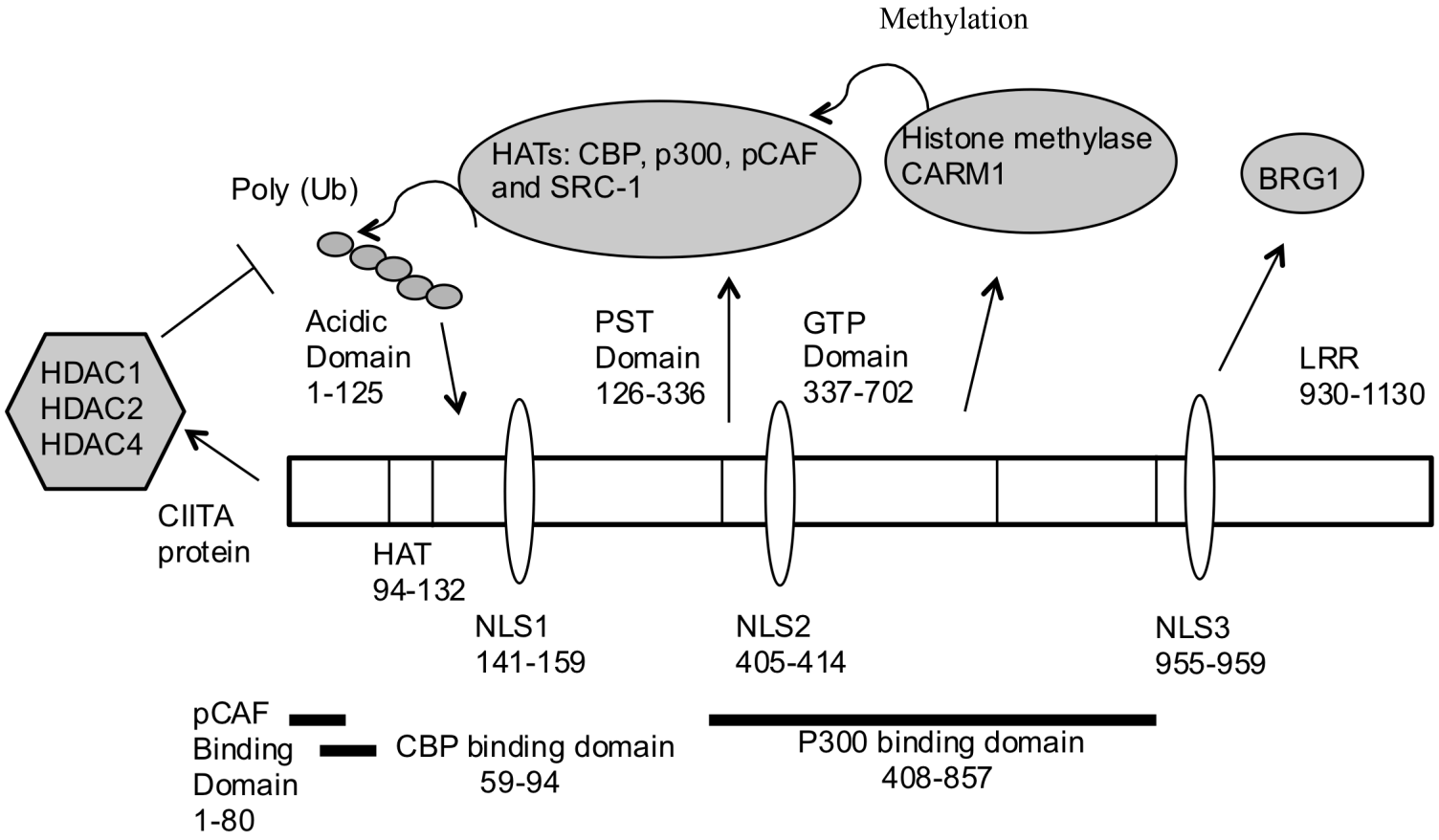
1.1.6. Class II transactivator structure

CIITA belongs to a family of large proteins known as NOD protein family, caspase-recruitment domain (CARD), transcription enhancer, R (purine)-binding, pyrin, leucine-rich repeats (LRRs), which make up the CATERPILLER protein family. This family is characterized by an amino (N) terminal domain, which is the variable region, a carboxy (C) terminal region containing LRP (53, 54) and a region in between known as NOD (Figure 1.4).

There are some features of CIITA protein that are unique from the CATERPILLER family members. The N-terminal transcription activation domain (AD) (residues 1-125) is rich in acidic amino acids. The proline-serine-threonine (PST) rich domain (residues 126-336) encloses several phosphorylation sites (55-57) and harbors an intrinsic histone acetyltransferase (HAT) domain (58). The guanosine triphosphate (GTP) binding domain (residues 337-702) is located in the center of the CIITA protein and is involved in protein self-association and nuclear import. This segment also contains the p300 binding (37), and provides the interaction surface for histone acetylase CBP and RFXANK (59-61). Lastly, the C terminus contains the LRP domain (residues 930-1130), which has the same role as GTP-binding domain (62, 63). Three nuclear localization signals (NLS) have been identified in the CIITA protein: the N-terminal (residues 141-159); the GTP domain (residues 405-414); and the LRP (residues 955-959) (62, 64, 65).

Figure 1.4 Structure and epigenetic control of class II transactivator protein.

Class II transactivator (CIITA) consist of four domains (Acidic, proline-serine-threonine (PST), guanosine triphosphate (GTP) and leucine rich repeat (LRR) domains) and 3 nuclear localization sequences (NLS1, NLS2, NLS3). Multiple epigenetic mechanisms are involved in CIITA regulation, which include histone deacytylase (HDAC), histone acetyltransferase (HAT), histone methylase and Brahma-related gene 1 (BRG1). Bars underneath the CIITA scheme represent the HAT binding site. Adapted from (38, 66).


Methylation
HATs: CBP, p300, pCAF
and SRC-1
Histone methylase
CARM1
BRG1
Poly (Ub)
HDAC1
HDAC2
HDAC4
Acidic
Domain
1-125
PST
Domain
126-336
GTP
Domain
337-702
LRR
930-1130
CIITA
protein
HAT
94-132
NLS1
141-159
NLS2
405-414
NLS3
955-959
pCAF
Binding
Domain
1-80
CBP binding domain
59-94
P300 binding domain
408-857

1.1.7. Class II transactivator regulation

1.1.7.1. Epigenetic regulation

CIITA is involved in activation and regulation of HLA class II genes through different mechanisms, which result in covalent modifications of nucleosomes and are known as epigenetic regulation (Figure 1.4). Epigenetic regulations include histone acetylation, methylation, phosphorylation, and ubiquitination, which result in chromatin remodeling and either enhance or repress gene transcription (66). CIITA plays a significant role in histone 3 (H3) and H4 acetylation at HLA class II promoters through recruitment of multiple histone acetyl transferases (HATs). HATs involved in class II activation, include cyclic AMP responsive-element-binding protein (CBP), p300/CBP-associated factor (pCAF), and steroid receptor co-activator 1 (SRC1) (67). Similarly, CIITA is involved in histone methylation through recruitment of histone methyltransferases (HMTs), including co-activator-associated arginine-methyltransferase 1 (CARM1), which is important in H3 methylation at arginine 17 in class II promoters. This epigenetic modification is required for constitutive and IFN-γ induced class II expression (68). Another important epigenetic control of HLA class II expression is recruitment of brahma-related gene 1 (BRG1), which is an adenosine triphosphate (ATP)ase that drives chromatin–remodeling complex SWItch/sucrose non-fermentable (SWI-SNF) and has been linked to IFN-γ mediated induction of CIITA (69). Moreover, ubiquitination through a degradation independent pathway may enhance the activation of CIITA by increasing its association with HLA class II transcription factors and gene promoters (70).

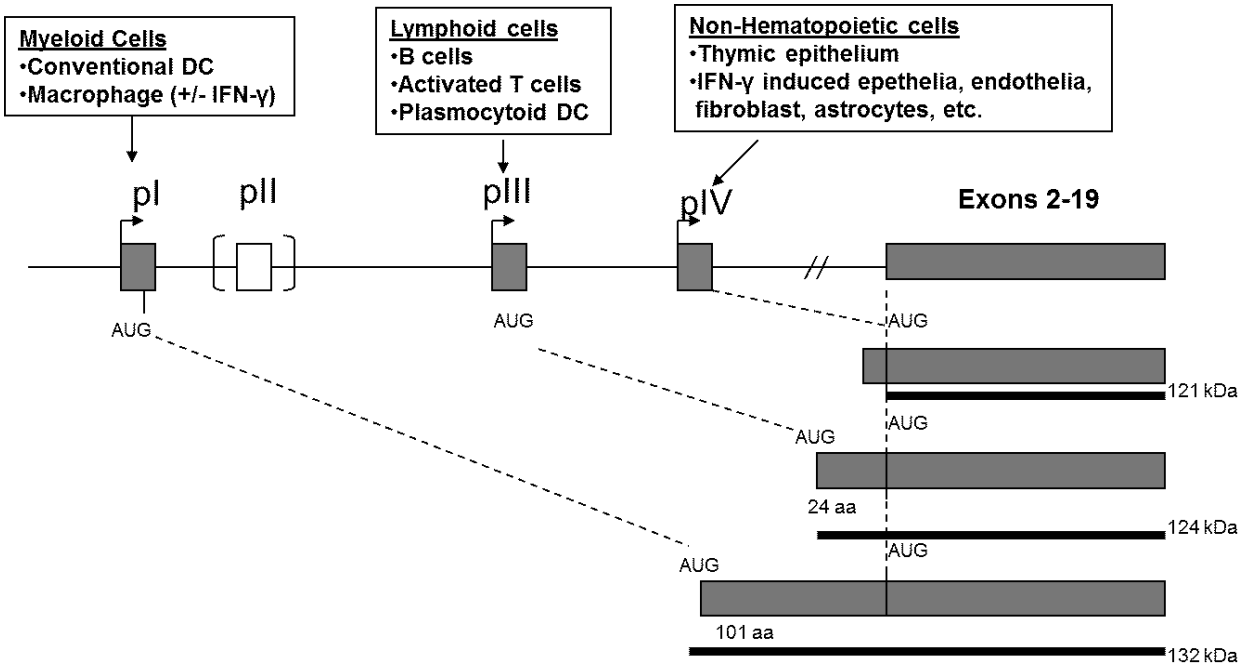
Although CIITA acts as a gene activator, it may result in gene silencing, through recruitment of histone deacytylase (HDACs) and demethylases. This process is reversible by using drugs such as trichostatin-A (TSA) that inhibit HDAC and results in enhancing HLA class II gene transcription (71-73). Silencing of *CIITA* through epigenetic mechanism may occur in tumor cells and result in immune escape (discussed in Chapter 7) (74).

1.1.7.2. Regulation of the gene encoding class II transactivator

As previously mentioned, regulation of CIITA occurs mainly at the transcriptional level; however, additional changes may occur through post-transcriptional modifications or by modulating protein stability (75, 76). Transcriptional activation of *CIITA* is driven by a large regulatory region, which spans over 12 kb and contains four independent promoters, known as pI, pII, pIII and pIV (Figure 1.5). Promoter I, pIII, and pIV are highly conserved between human and mouse. Promoter II (pII) is not expressed in mice and its function in humans remains unknown (77). Each promoter is followed by a unique exon 1 that is spliced with the other shared exons (2-19 exons) to form three isoforms of the *CIITA* mRNA (I, III, and IV). In type IV *CIITA* mRNA translation is initiated in exon 2. On the other hand, translation is initiated in exon 1 in type I and III *CIITA* mRNA, which results in *CIITA* transcripts with a specific N terminal protein sequence of 101 and 24 amino acids respectively. As a result, three CIITA isoforms [121, 124, and 132 kilo Dalton (kDa)] are produced which differ in their N terminal end (77, 78).

Figure 1.5 Regulation of class II transactivator.

Class II transactivator (CIITA) is under the control of four promoters. Activation of these promoters results in splicing of first exon to the shared downstream exons (exons 2-19). This results in the formation of three different isoforms that differ in their first exon but share exon 2-19. pII is of unknown significance. Adapted and modified from (37).


Myeloid Cells
•Conventional DC
•Macrophage (+/- IFN-γ)
Lymphoid cells
•B cells
•Activated T cells
•Plasmocytoid DC
Non-Hematopoietic cells
•Thymic epithelium
•IFN-γ induced epethelia, endothelia, fibroblast, astrocytes, etc.
pI
pII
pIII
pIV
Exons 2-19
AUG
AUG
AUG
121 kDa
AUG
AUG
24 aa
124 kDa
AUG
AUG
101 aa
132 kDa

Promoter I (pI) is a myeloid cell specific promoter and drives CIITA in conventional DC and IFN-γ activated macrophages. Promoter III (pIII) is a lymphoid cell specific and drives CIITA in B cells, activated T cells, and plasmacytoid DC (77). Lastly, promoter IV (pIV) is responsible for IFN-γ inducible expression of CIITA in non-bone marrow-derived cells (endothelial, epithelial, fibroblasts and astrocytes) (79), and responsible for constitutive expression in thymic epithelial cells (TEC) (80).

1.1.7.2.1. Molecular regulation of promoter I of class II transactivator

The molecular regulation that controls the DC specific activity of pI remains unknown. A 400 bp promoter region has been previously described to contain sequence motifs for essential binding sites for known transcription factors. These include activating protein 1 (AP1), CAAT, nuclear factor for interleukin 6 (NF-IL6) and early region 2a (E2A). None of these promoter-binding sites have been confirmed through functional studies (81).

1.1.7.2.2. Molecular regulation of promoter III of class II transactivator

Molecular regulation that underlies the activation of CIITA transcription from pIII has been studied in various cell lines. Promoter III is a 320 bp promoter region that contains five sequence motifs, activation response element 1 (ARE1), ARE2, enhancer box (E-box), E-twenty six-interferon-sensitive response element (ETS-IRSE), and IFN-γ activated sequence (GAS) (77, 82, 83) (Figure 1.6). ARE1 and ARE2 are the main binding sequences for pIII in B-cell lines (83, 84). Transcription factors, acute myeloid

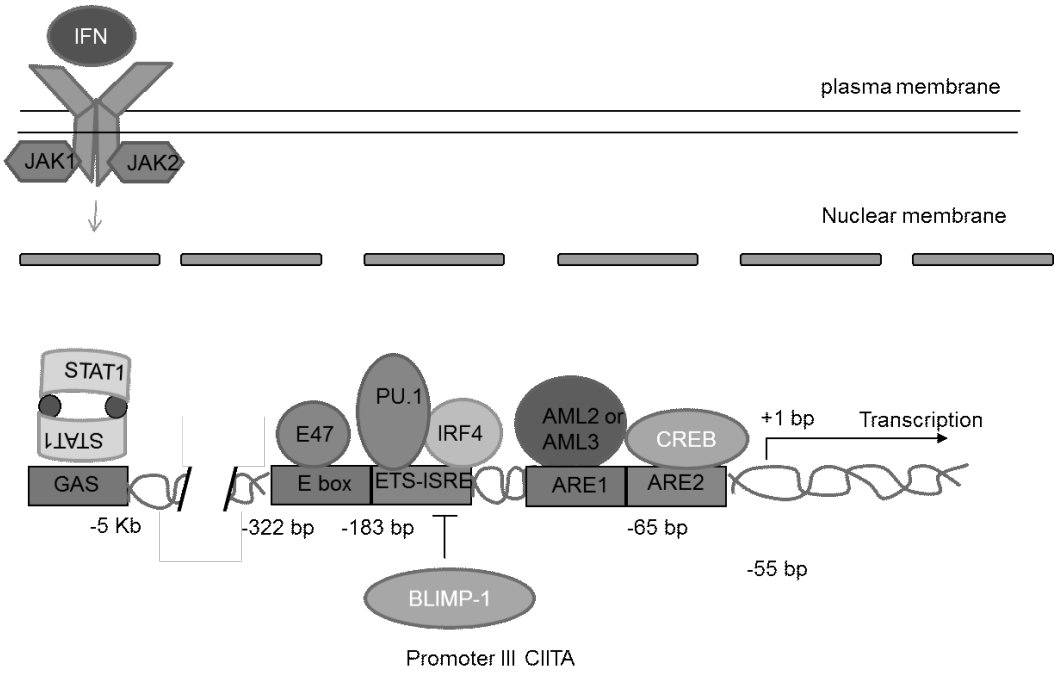
leukemia 2 (AML2) and CREB, bind to ARE1 and ARE2 respectively (37). Upstream of the ARE motif are the E-box motif, which serves as a binding site for the E47 transcription factor, and the ETS-IRSE motif binding site, which recruits the PU.1 and IFN-regulatory factor (IRF) 4 transcription factors (85). During the maturation of B-cells to plasma cells, the *CIITA* gene is switched off by the binding of B-lymphocyte-induced maturation protein 1 (BLIMP1) to the ISRE site in pIII of CIITA (86, 87). An additional distal enhancer about five kb from the pIII regulatory element has been found to be activated by signal transducer and activator of transcription 1 (STAT1) in response to the classical IFN-γ mediated induction. Activation of this enhancer element is only cell specific in human glioblastoma and fibrosarcoma cell lines and not conserved in mice (88, 89).

1.1.7.2.3. Molecular regulation of promoter IV of class II transactivator

Since pIV is responsible for IFN-γ expression of CIITA, it depends on IFN-γ signaling pathway for its activation. A conserved 120 bp promoter region that contains three cis-acting sequences, a GAS, an E box, and (IRF) element (IRF-E), all function in synergy and are important for induction of pIV (37) (Figure 1.7). Binding of IFN-γ to its receptor activates and phosphorylates Janus activating kinases (JAK) 1 and JAK2, which lead to phosphorylation, dimerization, and nuclear import of STAT1. STAT1 and upstream stimulatory factor 1 (USF1) cooperatively bind to the GAS sequences and the adjacent E box motif respectively, which are present in the pIV regulatory region (80). STAT1 also activates the expression of IRF1, which binds to IRF-E and results in

Figure 1.6 Molecular regulation of promoter III of class II transactivator.

Promoter (pIII) is controlled by a 320 bp promoter region, which includes several regulatory binding sites [enhancer box (E-box), E-twenty six-interferon-sensitive response element (ETS-IRSE), activation response element 1 (ARE1) and ARE2]. An additional distal enhancer, interferon gamma activated sequence (GAS), which is present approximately 5 kb from transcription ignition site, is activated by signal transducer of transcription 1 (STAT1) in response to the classical interferon gamma (IFN-γ)-mediated induction pathway. Adapted from (37).


IFN
plasma membrane
JAK1
JAK2
Nuclear membrane
STAT1
STAT1
GAS
E47
PU.1
IRF4
E box
ETS-ISRE
AML2 or
AML3
CREB
ARE1
ARE2
+1 bp
Transcription
-5 Kb
-322 bp
-183 bp
-65 bp
-55 bp
BLIMP-1
Promoter III CIITA

subsequent induction of CIITA expression (90).

Promoter III (pIII) is induced directly by STAT1 phosphorylation, while pIV requires IRF1 in addition to STAT1 for proper induction of CIITA, which may explain the enhanced response of pIV to IFN-γ (89). Activation of STAT1 is an immediate response of IFN-γ signaling, while activation of IRF1 is a delayed response, because IRF1 protein is synthesized first and later binds to the IRF-E site in pIV of the *CIITA* gene. Therefore, STAT1 mediates a faster IFN-γ response via pIII and a slower response via pIV (90).

1.1.8. Expression of human leukocyte antigen class II in breast cancer

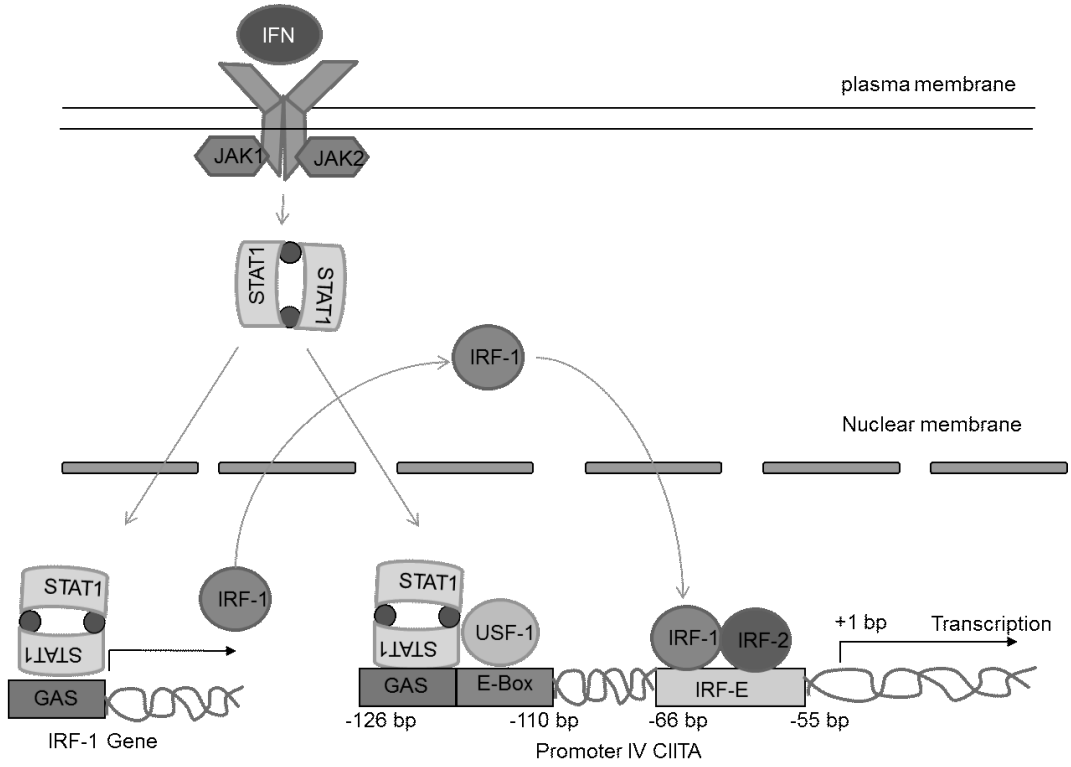
1.1.8.1. Overview

Resting breast epithelial cells do not express HLA class II, whereas, lactating breast cells express HLA class II (91, 92), in which prolactin has been claimed for this upregulation (93). Furthermore, HLA class II is upregulated in approximately 50% of breast cancer lesions (94). An important research question, which has not been completely answered yet, is why and how breast cancer cells within the tumor microenvironment upregulate HLA class II molecules?

A considerable number of studies have reported that HLA class II can be induced in various breast cancer cell lines by IFN-γ (95), interleukin (IL)-1α, IL-1β, estradiol (E_2) (96), tumor necrosis factor -α (TNF-α) (97), and IL-4 (98). These suggest a mechanism by which breast tumors can upregulate HLA class II through release of various cytokines in the tumor microenvironment from infiltrating cells of the immune system and secretion

Figure 1.7 Molecular regulation of promoter IV of class II transactivator.

Promoter IV (pIV) is activated in response to the classical interferon-γ (IFN-γ)-mediated signaling pathway. The promoter region spans 120 bp and contains an IFN-γ activated sequence (GAS) binding site, an enhancer box (E-Box) and interferon regulatory factor element (IRF-E) binding site. Interferon regulatory factor 1 (IRF1) induction is dependent on IFN-γ and thus explains the reason for the delayed kinetics of pIV CIITA. Adapted from (37).


IFN
plasma membrane
JAK1
JAK2
STAT1
STAT1
IRF-1
Nuclear membrane
STAT1
IRF-1
STAT1
USF-1
IRF-1
IRF-2
+1 bp
Transcription
GAS
GAS
E-Box
IRF-E
-126 bp
-110 bp
-66 bp
-55 bp
IRF-1 Gene
Promoter IV CIITA

of hormones from adipose tissues (99, 100). In addition, non-neoplastic breast epithelial cells adjacent to lymphocytic infiltrating cells also upregulte HLA-DR, thus suggesting that HLA class II is modulated in benign breast epithelium by cytokines (101). Alternatively, molecular changes induced as a result of hyperplasia in benign breast epithelium may suggest a different mechanism of HLA class II modulation independent of cytokines modulation (91, 102, 103). Differential HLA class II expression has been reported in breast cancer with HLA-DR more frequently expressed followed by DP and then DQ (101, 104). HLA-DM is rarely expressed and *DRB* alleles are differentially expressed in HLA-DR tumor (105, 106). This modulation of HLA class II expression may be due to promoter polymorphisms. Moreover, discrepancies between DRB promoter activity and of *DRB* mRNA levels suggest that *DRB* gene expression is regulated at both transcriptional and post-transcriptional levels (107). Thus, cross talk between signaling pathways activated during tumor progression may have a role in transcriptional and post-transcriptional regulation of HLA-DR expression on breast cancer cells.

1.1.8.2. Significance of human leukocyte antigen class II expression in breast cancer

The significance of HLA class II expression on breast carcinoma cells is contradictory, and previous studies have not provided a clear association between HLA class II expression and disease outcome (102, 108). The absence of any association could be a result of the lack of co-stimulatory molecule expression by tumor cells, which are required to initiate an optimal $CD4^+$ T-cell response (109). More importantly, the expression of co-inhibitory molecules such as programmed cell death ligand 1 (PD-L1)

by some tumours can result in inhibition of T cell activation, as reviewed by Driessens et. al. (2009) (110). Co-stimulatory molecules expressed in APC are necessary to initiate a proper immune response in lymphoid tissues (111). However, if an antitumor immune response results in effector T cells homing to the tumour site, they may be activated in the absence of co-stimulation (110).

There is significant experimental evidence to support a role for tumor cell HLA class II antigen expression in induction of anti-tumor immunity. It has been shown that HLA class II antigen positive breast carcinoma cells can generate and present the relevant tumor associated antigen (TAA) derived peptides and activate effector $CD4^+$T-cells (112-114). This occurs in a HLA class II antigen restricted manner and results in elimination of the tumor both in mice (115-117) and in humans (112-114).

The expression of HLA class II co-chaperone molecules (Ii and HLA-DM) in breast carcinoma plays a significant role in the outcome and the prognosis (106). Ii is expressed in a greater subset of breast carcinomas than HLA-DR, and is associated with a poor prognosis (118, 119). High levels of Ii attached to the groove of the HLA class II molecules may prevent TAA presentation to $CD4^+$ T cells and, thus, decreased ability to induce allogeneic $CD4^+$ T cells and result in autologous $CD4^+$ T cells stimulation instead.

Coordinate expression of HLA-DM, with DR and Ii on tumor cells in breast cancer tissues is associated with high tumoral IFN-γ, better prognosis, and higher survival rate (106). As previously noted, HLA-DM acts as a peptide editor (120), in which CLIP is replaced by peptides derived from TAA that have undergone enzymatic proteolysis in the endocytic pathway. Thus, improved survival of patients with coordinate expression of

HLA class II and HLA-DM over patients with discordant HLA class II tumors (HLA-DR^+, Ii^+, HLA-DM^-) may be due to differences in antigen presentation, which result in recruitment of a different subset of T cells and a different response. Therefore, the expression of HLA-DM by tumor cells may be an indicator of improved survival in breast carcinoma. Furthermore, tumor cells that coordinately express HLA class II and co-chaperones may function as effective APC, in activating effector T helper type 1 (Th1) cells, resulting in effective antitumor immunity. Alternatively, high levels of IFN-γ from Th1 response may be required for coordinate expression of HLA class II and co-chaperones. Thus, the interaction of these factors together with hormones and cytokines especially IFN-γ in tumor microenvironment may result in modulation of HLA class II antigen expression by tumor cells.

1.2. Interferon-gamma signaling

1.2.1. Interferons

Since their initial discovery, interferons (IFNs) are identified as agents that interfere with viral replication (121). They are sub-classified into type I (IFN-α, IFN-β), type II (IFN-γ) and type III (IFN-λ) based on receptor binding (122). The main sources of IFN-γ are cells of the innate immune system (NK cells and NKT cells), which provide the first line of defence against pathogens, and cells of adaptive immune system including $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T-lymphocytes (123). IL-12 initially secreted by APC during early infection controls IFN-γ production by attracting NK cells to the site of infection and promotes IFN-γ production by the same cells.

1.2.2. Interferon gamma receptor

IFN-γ binds to IFN-γ receptor (IFNGR) (123). IFNGR is comprised of two IFNGR1 chains, which are responsible for ligand binding, and two IFNGR2 chains, which are responsible for signal transduction (124). IFN-γ signaling is mainly regulated by IFNGR2 being tightly regulated according to the cell's needs, while IFNGR1 is constitutively expressed and present in excess (125, 126). Both receptors are deficient in kinase/phosphatase activity and they must bind to the other members of IFN-γ machinery to facilitate signal transduction. The cytoplasmic domain of IFNGR1 contains binding sites for JAK1 and STAT1. The JAK1 binding site leucine, proline, lysine, serine (LPKS) is located at residues 266–269 and the STAT-binding site tyrosine, aspartic acid, lysine, proline, histidine (YDKPH) is located at residues 440–444. The Y^{440} phosphorylation site is phosphorylated during signal transduction to allow STAT1 recruitment to the receptor. The remaining residues ^{441}DKPH^{444} are responsible for the binding specificity (127, 128). The cytoplasmic domain of IFNGR2 contains binding sites for the JAK2. IFN-γ was thought to bind to IFNGR2 in the presence of IFNGR1, indicating that IFNGR2 binds only to the complex IFNGR1: IFN-γ, (129, 130); however, newer techniques have shown that the receptor is assembled before binding to the ligand (131).

1.2.3. Janus kinase

JAKs are a family of four non-receptor tyrosine kinases, JAK1, JAK2, JAK3 and TYK2, which selectively activate STATs (132). JAK protein structure is composed of seven regions, which are highly conserved, Janus homology domains (JH)1-JH7. JH1 is

important for the kinase activity. JH2 represents a pseudokinase domain, which aids in JH1 catalytic activity (Figure 1.8). The amino-terminal (JH3-JH6) domains have been involved in receptor association (133) and JH7 is of unknown function. JAK1 and JAK2 are involved in IFN-γ signaling where JAK1 is important in ligand binding to the receptor, whereas, JAK2 is important in signal transduction (134).

1.2.4. Signal transducer and activator of transcription 1

STAT1 is a member of the signal transducers and activators of transcription family. Crystallography of STAT1 identified one divergent domain, carboxy-terminal transcriptional activation domain (TAD) and five conserved domains: amino-terminal domain (N); the coiled-coiled domain (CCD); the DNA binding domain (DBD); the linker domain; and the Src Homology 2 (SH2)/tyrosine activation domain (132) (Figure 1.8). N-terminal dimerization aids in binding to GAS elements (135), interaction with the transcriptional co-activator CBP/p300 (136), and regulates nuclear translocation (137). The CCD is important for protein-protein interaction and for nuclear export of STAT1 (138, 139). The DBD recognizes bases in the most proximal half of the GAS element. The linker domain joins the DBD with the SH2/dimerization domain, which plays an important role in receptor recruitment, dimerization and DNA binding (140-142).

1.2.5. Interferon regulatory factors

IRF gene family is an important intermediate for type I and type II IFN signaling with nine members identified. IRF1, IRF2 and IRF9 take part in IFN-γ signaling. IRF9 was initially discovered as a DNA-binding subunit of the transcription factor interferon

stimulated gene factor 3 (ISGF3), and was previously termed p48/ISGF3 (143). Although IRF1 and IRF2 mRNAs are ubiquitously expressed, IRF1 is significantly upregulated by IFN stimulation.

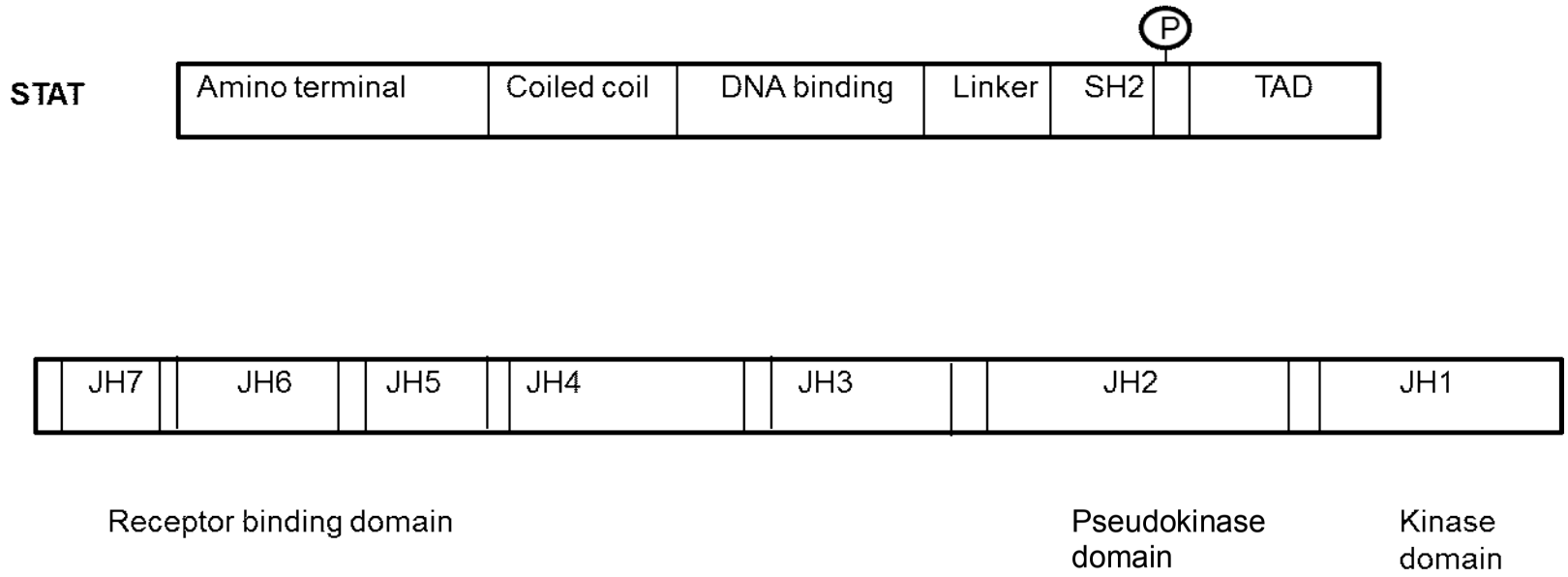
IRF1 and IRF2 are 62% homologous with most of the sequence homology occurring in their amino terminal regions (144). IRF1 binds to the IRF-E site in the promoter region of many target genes and results in their induction. In addition, the binding specificity of IRF1 overlaps with the ISRE binding site. In this manner, IRF1 has the ability to activate a full range of IFN-inducible genes. Although IRF2 also binds to the IRF-E site, it largely functions to oppose transcription of IRF1-inducible genes through its transcriptional repressor domain (145). The IRF1 protein is very unstable with a half-life of 30 min whereas, IRF2 protein is more stable with a half-life of 8 hr. These early experiments indicate that IRF1 acts as transcriptional activator and IRF2 functions as a repressor for the IFN-γ genes (146). This role is true in most IFN-inducible genes, however, IRF2 has a positive regulatory function when together with IRF1, it binds to IRF-E in CIITA promoter (37).

1.2.6. Interferon gamma signaling

IFN-γ is a biologically active compound formed by the non-covalent association of two polypeptide subunits, thus, two IFNGR1 bind to one IFN-γ dimer (147). IFNGR1 and IFNGR2 are assembled before ligand binding and are pre-associated with JAK1 and JAK2 respectively, which are bound to the intracellular domain of the receptor (123). Binding of IFN-γ to IFNGR results in activation, which produces conformational changes

Figure 1.8 Schematic cartoon of signal transducer and activator of transcription and Janus kinase structure.

Janus kinase (JAK) harbor seven regions of conserved homology, Janus homology domains (JH)1–JH7. Signal transducer and activator of transcription (STAT) also harbour several conserved domains, N-terminal domain (NH2), a coiled-coil domain, the DNA binding domain (DBD), a linker domain, an Src Homology 2 (SH2) domain, and a tyrosine activation domain (circled P) and transcription activating domain (TAD) domain. Adapted from (26).


P
STAT
Amino terminal
Coiled coil
DNA binding
Linker
SH2
TAD
JAK
JH7
JH6
JH5
JH4
JH3
JH2
JH1
Receptor binding domain
Pseudokinase domain
Kinase domain

in the receptor. Thus, JAK2 and subsequently JAK1 become phosphorylated. The activated JAK1 next phosphorylates tyrosine 440 (Y^{440}) on each IFNGR1. Phosphorylated Y^{440} acts as a docking site for the SH2 domain of the STAT1. Next STAT1 is phosphorylated on Y^{701} by JAK2 (147, 148), permitting STAT1 homodimerization and separation from the receptor. A subsequent phosphorylation on serine 727 (S^{727}) occurs, which allows the STAT1 dimer to translocate to the nucleus and initiate gene transcription. STAT1 S^{727} phosphorylation does not influence the STAT1 homodimer formation, however, it is important for STAT1-mediated transcription (149, 150). The STAT1 dimer associates with importin-α-1 nucleoprotein interacting protein 1 (NPI-1), which facilitates nuclear translocation of STAT1 through the nuclear pores (151). The nuclear membrane forms an efficient barrier to the inactivated STAT1, and entry usually requires activation and dimerization (152). The STAT1 homodimer initiates gene transcription by binding to GAS, in the promoter regions of genes. The number of GAS binding sites in the promoter of the genes determines the transcriptional activity of these genes (153). Phosphorylation of IFNGR, JAK and STAT occurs within 1 min of IFN-γ stimulation (154). However, nuclear entry of STAT1 is observed at 15 min and finished by 30 min of IFN-γ stimulation, reviewed in (123) (Figure 1.9). Thus, the whole process of early gene transcription takes between 15-30 min. Early IFN-γ regulated genes are mainly transcription factors (e.g, IRF1) which, when transcribed, are able to drive the regulation of the next wave of late transcription: example, intercellular adhesion molecule 1 (ICAM-1); CIITA; monokine induced by IFN (MIG)). Recent studies have shown that other factors such as activation of other signaling pathways and the presence of certain

kinases alters responses to IFN-γ signaling (155). Thus IFN-γ signaling is activated in a cell type specific manner.

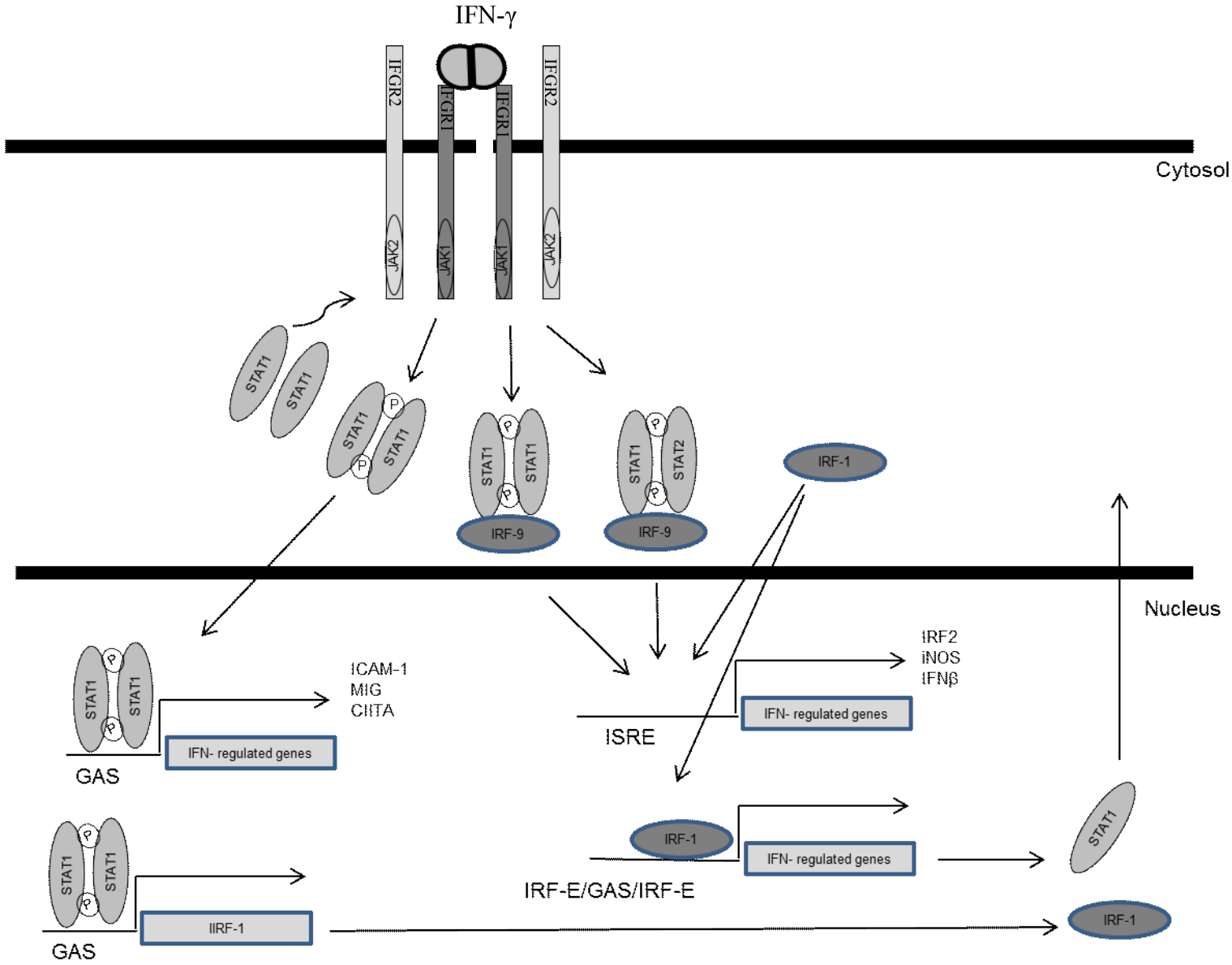
1.2.7. Regulation of interferon gamma signaling

Dissociation of IFNGR from its ligand occurs in the endosomal pathway after internalization of the complex followed by recycling of the IFNGR1 back to the cell surface, after which the ligand is degraded (156). Furthermore, IFN-γ signaling may result in receptor degradation, and decreased cell surface expression (157). Suppressors of cytokine signaling-1 (SOCS-1) protein act as a feedback inhibitor of IFN-γ signaling by interfering with JAK 1/2 tyrosine kinase activity (158). SOCS-1 also facilitates degradation of different components of the IFN-γ signaling pathway by binding and targeting them to the ubiquitin-proteasome pathway (159). SOCS-3 is another protein, which negatively inhibits IFN-γ signaling, but not as efficiently as SOCS-1 (160).

Downregulation of IFN-γ signaling can also result from protein tyrosine phosphatase (PTPs), such as SH2-containg tyrosine phosphatase-2 (Shp2), which dephosphorylate JAK 1/2 and IFNGR1 (161). Nuclear dephosphorylation of STAT1 plays an important role in inhibiting STAT1 signaling. Binding of STAT1 to the DNA masks an important nuclear export signal (NES) at residues 197–205. However, when STAT1 is dephosphorylated by nuclear PTP, this allows the release of STAT1 from DNA and exposes NES (162). An export receptor chromosome region maintenance/exportin 1 (CARM1) recognises NES-containing proteins and facilitates STAT1 translocation through the nuclear pore to the cytoplasm (163).

Figure 1.9 Schematic presentation of interferon gamma signaling.

Activation of IFN-gamma receptor (IFNGR) by interferon gamma (IFN-γ) results in conformational changes, which result in Janus kinase (JAK) activation and subsequent signal transducer and activater of transcription 1 (STAT1) phosphorylation, dimerization and translocation to the nucleus where it binds to the interferon-γ activated site (GAS) binding sites. STAT1:STAT1: Interferon regulatory factor (IRF)9 complex and STAT1:STAT2:IRF9 (ISGF3) complex can be activated in response to IFN-γ signaling. Both of these complexes bind to Interferon-sensitive response element (ISRE) binding sites in the promoter of the IFN-γ induced genes. Two sets of genes can be activated by IFN-γ, early (e.g IRF1) and late e.g Intercellular Adhesion Molecule 1 (ICAM-1), class II transactivator (CIITA), monokine induced by IFN (MIG). Adapted from (123).


IFN-γ
IFGR2
IFGR1
IFGR1
IFGR2
JAK2
JAK1
JAK1
JAK2
Cytosol
STAT1
STAT1
P
STAT1
STAT1
P
P
STAT1
STAT1
P
IRF-9
STAT1
STAT2
P
IRF-9
IRF-1
Nucleus
STAT1
STAT1
P
P
ICAM-1
MIG
CIITA
IFN- regulated genes
GAS
IRF2
iNOS
IFNβ
IFN- regulated genes
ISRE
STAT1
STAT1
P
P
IIRF-1
GAS
IRF-1
IFN- regulated genes
IRF-E/GAS/IRF-E
STAT1
IRF-1

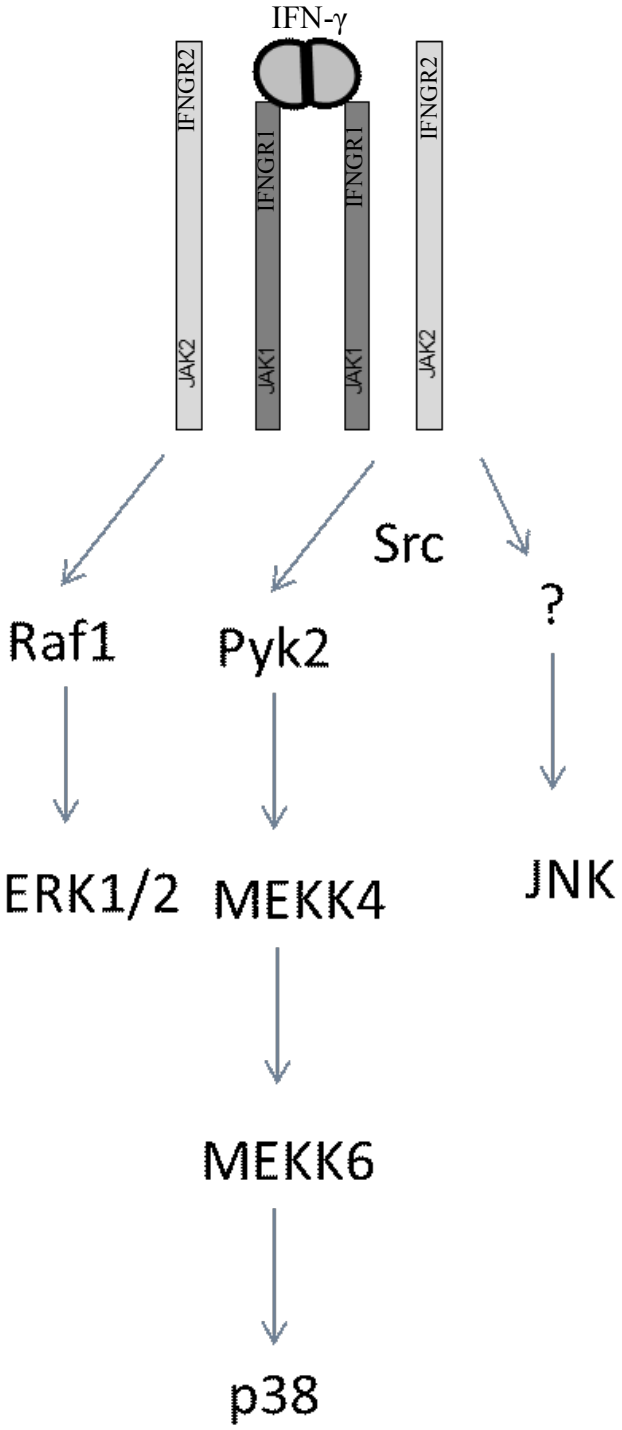
1.2.8. Non-classical interferon gamma signaling

IFN-γ signaling occurs independently of JAK/STAT1 signaling. Evidence arises from experiments on STAT1-deficient mice, which are more resistant to viral challenges than IFNα/β/γ receptor deficient mice, suggesting that IFN-γ can signal in the absence of STAT1 (164). Moreover, microarray studies showed that 30% of IFN-γ stimulated genes are upregulated in the absence of STAT1 (165). For example, other members of the STAT family such as STAT3 and STAT5 have been shown to be upregulated by IFN-γ in the absence of STAT1 activation (166-168). There are other pathways that act fully in parallel to JAK/STAT1 signaling, For example: epidermal growth factor receptor (EGFR); phosphoinositide-3-kinase (PI3-K/AKT); protein kinase C (PKC); calcium/calmodulin kinase II; and mitogen activated protein kinase (MAPK) have been shown to phosphorylate STAT1 on S^{727}, reviewed in (169). Activation of these kinases in response to IFN-γ is further evidence for non-JAK/STAT signaling pathways.

IFN-γ has been shown to activate MAPK in various types of cells through different pathways (170, 171) (Figure 1.10). For example, IFN-γ activates rapidly accelerated fibrosarcoma 1 (Raf1) which is found in close association with JAK2, resulting in phosphorylation of extracellular signal-regulated kinase (ERK) in an MAPK/ERK Kinase 1 (MEK1) independent manner (172, 173). ERK activation occurs by two mechanisms, a rapid JAK-STAT1 independent activation which occurs within 30 min and leads to AP1 activation (171), and a slower mechanism, which involves STAT1 S^{727} phosphorylation activation and occurs 2 hr after IFN-γ activation (170). IFN-γ activates the p38 pathway through recruitment of MyD88 which has been shown to

stimulate MAPK kinase 6 (MKK6)/p38 (174). In addition, p38 can be activated by c-Src at the IFNGR, which results in activation of calcium dependent pyk2 kinase and, in turn, activates MEK Kinase (MEKK4/MKK6/p38 MAPK pathway) (175). IFN-γ has been shown to selectively activate c-Jun N-terminal kinases (JNK) pathway that is important for genes associated with antigen presentation in macrophages, (176).

Figure 1.10 Schematic presentation of the non-classical interferon gamma signaling. Interferon gamma (IFN-γ) signaling can activate multiple pathways including the mitogen activated protein kinase (MAPK) [extracellular signal-regulated protein kinases 1 and 2 (ERK 1/2), p38 and Jun N-terminal kinase (JNK)] pathways through the activation of rapidly accelerated fibrosarcoma 1 (Raf1), protein tyrosine kinase (PyK2), and Src.


IFN-γ
IFNGR2
IFNGR1
IFNGR1
IFNGR2
JAK2
JAK1
JAK1
JAK2
Src
?
Raf1
Pyk2
ERK1/2
MEKK4
JNK
MEKK6
p38

1.3. Mitogen activated protein kinase signaling

1.3.1. Overview

Mitogen-activated protein kinase (MAPK) signaling pathways are a group of family related kinases that link gene regulation in the nucleus in response to external stimulus, thereby controlling cellular functions such as proliferation, differentiation, migration and apoptosis (177, 178) (Figure 1.11). MAPKs are activated by mitogenic stimuli such as stress, growth factors, cytokines, and hormones, which generally convey their signaling through hormone receptors, growth factor receptors, tyrosine kinases, and through the Ras and Rho families of small GTPases, which participate in the process of signal initiation (179).

The classical MAPK activation consists of a cascade of three consecutive protein kinases which start with the phosphorylation of a mitogen-activated kinase kinase kinase (MAPKKK) that phosphorylates serine and threonine in a second mitogen activated kinase kinase (MAPKK) which then phosphorylates threonine and tyrosine in a third mitogen-activated kinase (MAPK) (180, 181). Components of the signaling cascade are required to be colocalised within the cell for efficient signal transduction. This occurs by direct protein-protein binding or binding through scaffold proteins (182). MAPKs are highly evolutionarily conserved and phosphorylation of both threonine and tyrosine residues are required for full activation (183). The pattern of phosphorylation is threonine -X-tyrosine, where X stands for any amino acids and results in forming tri-peptide motif. In mammals, there are many distinctive subfamilies of MAPKs that can be grouped together based on amino acids present between the two phosphokinase sites: the threonine (T)-glutamic (E) –tyrosine (Y) tripeptide motif usually identifies the extracellular signal-

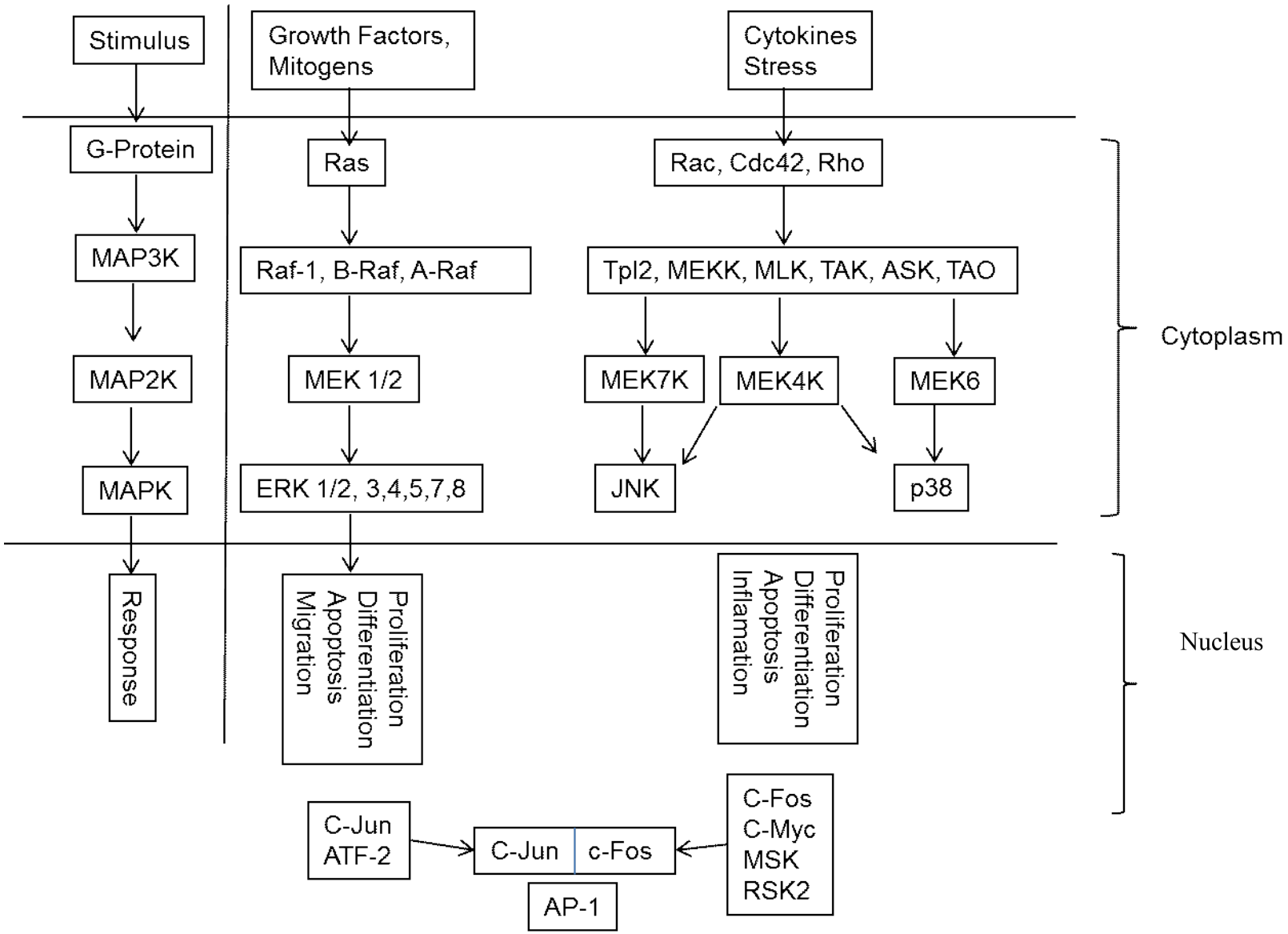
regulated protein kinases (ERK 1/2), ERK3s (ERK 3, ERK 4), ERK 5 [(ERK 5) (also known as Big Map Kinase), ERK 7s (ERK 7, ERK 8); the T-glycine (G)-Y usually identifies the c-Jun N-terminal kinases (JNK 1/2/3), and threonine-proline-tyrosine usually identifies the p38s MAPKs (p38α, p38β, p38γ, p38δ), (184, 185). The specificity of MAPK activation depends on the tri-peptide motifs present in the activation loop and the scaffold protein, which bind signaling molecules, thereby ensuring proper cell response and regulating the duration and intensity of MAPK signal output.

Once MAPKs are activated, they dissociate from their scaffolding proteins and translocate from the cytoplasm to the nucleus after phosphorylating a number of downstream proteins. These include transcription factors, co-regulators and protein kinases such as ribosomal S6 Kinase (RSK), MAPK-interacting kinase (MNK) families, MAPK-activated protein kinase (MAPKAP) and the mitogen and stress-activated protein kinase (MSK) family (180, 181). MAPK also inhibits the activity of transcription factors through increasing their retention in the cytoplasm by phosphorylation. In this case dephosphorylation of these factors is required for their nuclear localisation (186).

1.3.2. Extracellular signal-regulated protein kinase pathway

The ERK 1/2, previously known as p44, MAPK1 and p42, MAPK3, respectively, were the first MAPKs revealed in mammalian cells and they have been widely studied (187). ERK 1/2 are activated by different mechanisms, the most common is through the small guanosine triphosphate (GTP) loading of the Ras GTPase, which is initiated at the plasma membrane. In turn, activated Ras recruits the MAPKKK cascade (Raf-1, B-Raf

Figure 1.11 Schematic diagram of mitogen activated protein kinase (MAPK) signaling. All MAPK signaling starts with an external stimulus, which leads to a series of MAPK events in the cytoplasm and ends with transcription factor activation and translocation to the nucleus where genes responsible for cellular function are affected. Adapted from (188).


Stimulus
Growth Factors, Mitogens
Cytokines Stress
G-Protein
Ras
Rac, Cdc42, Rho
MAP3K
Raf-1, B-Raf, A-Raf
Tpl2, MEKK, MLK, TAK, ASK, TAO
MAP2K
MEK 1/2
MEK7K
MEK4K
MEK6
MAPK
ERK 1/2, 3,4,5,7,8
JNK
p38
Cytoplasm
Response
Proliferation Differentiation Apoptosis Migration
Proliferation Differentiation Apoptosis Inflamation
Nucleus
C-Jun ATF-2
C-Jun
c-Fos
C-Fos C-Myc MSK RSK2
AP-1

and A-Raf) to the plasma membrane in order to induce their activation (189). The mechanism of Raf activation is not fully understood but may occur through PKC (190) or mixed-lineage kinase-3 (MLK3) (191). Next, the signal is conveyed to the MAPKKs, MEK1 and MEK2 (MEK1/2), by phosphorylation of two S residues in activation loops. Thereafter, the activated MEK1/2 activates MAPKs, ERK 1/2 by phosphorylating the T and Y residues in the T-E-Y domain in their activation loop (192). ERKs stimulate the activity of numerous nuclear transcription factors by phosphorylation to regulate gene expression. Activator protein 1 (AP1) is one example of an ERK induced transcription factor. AP1 is a member of the Jun family of transcription factors (c-Jun, JunB, and JunD) that form homodimers or heterodimers within the same group or form complexes with Fos family members (c-Fos, FosB, Fra1 and Fra2), thus, forming the AP1 protein complex (193). ERKs aid in the induction of AP1 complex by two mechanisms: first, through activation of ETS domain-containing protein 1 (Elk-1), which binds to the promoter of c-Fos and induces its expression (194), and second, by direct phosphorylation of c-Fos, which results in post-translation modification that increases stability and activity (195, 196).

1.3.3. The c-Jun N terminal kinase pathway

JNK was primarily identified as a 54 kDa protein and known as stress activated protein kinase (SAPK). It is important for dual phosphorylation of the N-terminal transactivating domain of c-Jun at S^{63} and S^{73} (197). The JNKs are encoded by three genes (JNKs 1-3), also called MAPKs 8, 9, and 10, respectively. Each JNK gene is subject to differential RNA splicing to produce 46 kDa and 54 kDa polypeptides (198).

JNK1 and JNK2 are ubiquitously expressed, but JNK3 is expressed only in the brain, heart and testis (188). JNK is triggered by various stress stimuli and mitogens (197). For example, after activation, the stress and other stimuli convey their signals to members of the Rho family of GTPases such as CdcC42 and Rac1 (199), which next activate MAPKKK group of kinases, specifically, the MLK1-4 group. MLK3 is primarily involved in JNK signaling and phosphorylates MAPKK (200).

MKK4 and MKK7 are two MAPKK that have been identified in JNK signaling, which can activate JNK by dual phosphorylation on T and Y (201). Scaffold proteins play a major role in accomplishing signal specificity through uniting several key components of signaling pathways into functional modules. For example, the scaffolding protein, JNK interacting protein 1 (JIP), forms a molecular compound with JNK and its upstream activators, thus, enabling its rapid activation (202).

Next, JNK translocates to the nucleus and phosphorylates activating transcription factor (ATF) 2, which binds to the regulatory element within the promoter region of early response genes such as c-Jun. In addition, JNK activates c-Jun protein, which forms a complex with c-Fos and together form the AP1 complex. AP1 acts as a transcription factor and specifically binds to a response element in the promoter region of different growth regulated genes such as c-myc (202) and pIII CIITA (203).

1.3.4. p38 Pathway

The p38 cascade is another MAPK pathway that shows extensive cross talk with the JNK pathway (204). Five isoforms of p38 have been described: p38α, p38β, p38β2, p38γ and p38δ (205). Although, all p38s share the tri-peptide dual phosphorylation motif,

T-E-Y, in their activation loop, significant differences are found among these members (206). Different p38 isoforms show cell specificity with p38α, mainly expressed in leukocytes, p38β, in the heart and brain, and p38γ, highly expressed in skeletal muscles (207). p38 activation occurs by a number of external stimuli which include stress, endotoxin, osmotic stress, as well as inflammatory stimuli such as TNF-α, IL-1, and ultraviolet exposure (205).

After receptor activation, the signals are conveyed via adaptor proteins, small GTPases, to further activate MAPKKK and are generally analogous to those active in the JNK cascade. The signals induce phosphorylation and activation of the MAPKK components of the p38 cascade, which are commonly MKK3, MKK6 (208) and MKK4 (209). All p38 isoforms can be activated at this point. Once active, p38 proteins translocate from cytosol to the nucleus where they can activate several substrates. The main substrates for p38 are MAPK-activated protein kinase-2 (MAP-K2) and MAP-K3, which are essential for activating heat shock protein-27 (HSP-27), thus, preventing apoptosis (207). p38 also phosphorylates the transcription factors activating transcription factor 2 (ATF-2) and specificity protein 1 (SP1), which are essential in upregulating anti-inflammatory proteins (210).

1.3.5. Mitogen activated protein kinase and human leucocyte class II regulation

One of the mechanisms by which MAPK regulates gene expression is through activation of the AP1 transcription factor (211). Martins and her colleagues in 2007 showed that activation of CIITA in melanoma cells requires MAPK activation and

binding of the AP1 transcription factor to the pIII regulatory element of CIITA (203). This finding was supported by the following experiments (1) site directed mutagenesis of the AP1 binding site in the pIII regulatory element of CIITA significantly inhibited promoter activity. (2) pIII CIITA luciferase activity was decreased by MAPK-JNK dominant negative (DN) forms and (3) pIII CIITA mRNA was downregulated by U0126 or DN forms of Mek1 and MAPK-JNK. Additionally, in dendritic cells, binding of AP1 was required for activation of the HLA class II promoter (212). Thus, AP1, which is formed as a result of Fos and Jun activation from MAPK signaling, plays an important role in HLA class II regulation in different cells through different mechanisms.

MAPK upregulates HLA class II expression in macrophages through regulation of IFN-γ mediated gene expression in a selective way. JNK1 upregulates CIITA and genes encoding MHC II molecules through post-transcriptional regulation of mRNA stability. Slight effects were observed for ERK-1/2, whereas p38 has no effect on MHC II regulation (176).

In contrast, some studies point out a negative regulatory effect of MAPK on HLA II expression in immortalised brain endothelial (IBE) cells. Estrogen, through the non-genomic pathway, activates the JNK MAPK pathway, which leads to hypoacetylation of H3 and H4 and decreases CBP levels on the MHC II promoter. This results in transcriptional inhibition of MHC II with no effect on CIITA level. (213).

ERK 1/2 was shown to negatively regulate CIITA activation independent of IFN-γ by increasing CIITA nuclear export through activation of the nuclear export factor, CRM1 in COS7 cells transfected with wild type CIITA cDNA (214). This result points

towards the importance of MAPK in regulating CIITA activity according to the cell's needs.

1.3.6. Mitogen activated protein kinase and estrogen receptor alpha expression in breast cancer

Several mechanisms have been identified to explain the lack of ERα expression in in about 30% of breast cancers. Hypermethylation of ERα promoter was identified as a first mechanism, yet was observed only in 25% of $ER\alpha^-$ cases (215). Genetic alteration of the ERα gene has been suggested by a number of studies, accounts for the minority of cases. Therefore, these studies indicate that further mechanisms are involved in producing the $ER\alpha^-$ phenotype in breast cancer (216, 217). In a recent study, Brinkman et al, 2009 indicated that overexpression of growth factor receptors especially the erB family: EGFR (EGFR/erB1/HER1) and erB2 (erB2/neu/HER2) are inversely correlated with $ER\alpha^-$ phenotype breast cancer (218). EGFR is activated by extracellular growth factors. Once activated, EGFR undergoes dimerization and activation of its kinase function, which leads to activation of downstream signaling pathway such as ERK (219). erB2/neu/HER2 does not have an associated ligand, but can bind with ligand activated EGFR to form a heterodimer. This results in similar downstream signaling as ligand-activated EGFR (220).

Several studies have confirmed the inverse correlation between EGFR and ERα expression using double staining immunohistochemistry (221-224). Experimentally it was shown that activation of MAPKs in $ER\alpha^+$ MCF-7 cell line by stably transfecting the cells with EGFR, erbB-2, c-Raf, and MEK1 resulted in loss of ERα (225). Furthermore,

blocking MAPK signaling in the same cells resulted in restoration of ERα expression (225). These studies have been extended to an established $ERα^-$ breast cancer cell line, MDA-MB-231, in which a combination of MAPK inhibitors and demethylating agent results in re-expression of ERα and restoration of responses to anti-estrogen (226). Microarray analysis revealed 400 genes were modulated with MAPK hyper-activation in four $ERα^+$ breast cancer cell lines (227). ERα gene (*ESR1*) and a number of other estrogen-inducible genes were downregulated with MAPK activation. Together, these data suggest that hyper-activation of MAPK contribute to the downregulation of ERα and strongly support the idea that these mechanisms are reversible.

1.4. Estrogen receptor signaling

1.4.1. Overview

Estrogen receptors (ER) are expressed in around 80% of breast cancer cases that are diagnosed by immunohistochemistry; they are referred to as "ER-positive" or hormone dependent tumors, and require estrogen to progress (228). Persistent stimulation of breast ductal epithelium by estrogen increases cell proliferation and survival, which contributes to the development of cancer (229). Furthermore, the risk is increased in individuals with prolonged exposure to endogenous estrogen, as in women who experienced early menarche or late menopause (230).

Estradiol (E_2) is the main estrogen during the reproductive years, synthesized from testosterone and androstenedione, by the enzyme aromatase and regulated by 17β – hydroxysteroid dehydrogenase inter-conversion between E_2 and the less active hormone, estrone (231). In spite of low serum levels of estrogen, as in menopause, breast carcinomas frequently have high estrogen concentrations due to local synthesis and upregulation of intratumoral aromatase (232).

Estrogen signals through the two classical nuclear receptors, ERα and ERβ. In addition, estrogen also signals through the seven-transmembrane G-protein-coupled receptor 30 (GPR30), which is mainly responsible for the rapid non-genomic estrogen receptor signaling (233). Estrogen plays a role in maturation, proliferation, differentiation, and breast cancer development (234).

1.4.2. Estrogen receptors

ERα/β are members of the nuclear receptor (NR) superfamily, which become activated on ligand binding and function as transcription factors (235). ERα was discovered in the early 1960s, and was later located on the long arm of chromosome 6 (236). ERα was assumed to be the sole receptor mediating E_2 responses, but three decades later ERβ was cloned and located on chromosome 14 (237). Full length *ESR1* encodes a protein of 595 amino acids with a molecular weight of about 66 kDA, while the full length of ERβ gene (ESR2) encodes a protein of 530 amino acids and molecular weight of about 54 kDa (238).

The ER protein is composed of multiple domains, which form six functional regions, the A/B, C, D, E and F domains (Figure 1.12). The N-terminal A/B domain is the most variable with approximately 20% amino acid homology between the ERα and ERβ, suggesting that this domain is responsible for ER subtype-specific actions on target genes. Moreover, the ligand-independent activation function 1 (AF-1) is comprised within this region and shows cell-specific activity (239). The central C-domain, which is the most conserved domain with 95% structure homology between the two ER subtypes, contributes in receptor dimerization and DNA binding, thus, termed the DNA binding domain DBD. The D domain or the hinge domain shares 30% structural homology between ERα and ERβ. It connects the DBD and the E domain or ligand-binding domain (LBD) and because it contains a nuclear localization signal, it facilitates nuclear translocation of the ER (240). The LBD shares 55% structural homology between ERα and ERβ and harbors a hormone-dependent activation function 2 (AF-2), which is important for ligand binding and receptor dimerization (239). The ligand-binding cavity

of ERβ is approximately 20% smaller than that of ERα, which may explain why the two receptors have different affinities for estrogen (241). Finally, the F-domain, which has an unclear function, shares 20% structural homology between the two ER subtypes (238).

1.4.2.1. Estrogen receptor alpha isoforms

ESR1 is composed of eight exons and seven promoters and as a result of alternative promoter usage, results in three mRNA variants that differ in their 5' untranslated region (UTR) (Figure 1.12). The full length ERα is formed by splicing of the first non-coding exon to a common splice acceptor site upstream of the initiation codon of ERα, which results in expression of the full-length ERα receptor protein (66-kDa) (235). ERα is differentially expressed in cell lines, with 200 fold more in the MCF-7 breast cancer cell line, compared to osteoblast cells due to different promoter utilization (242). Human ERα-36 (hERα-36 kDa) and hERα-46 kDa are short ERα isoforms that were described more recently in MCF-7 cells (243, 244). hERα-36 lacks both transcriptional activation domains (AF1&2) and contains an exon coding for myristoylation sites, targeting the receptor to interact with the plasma membrane (244). The hERα-46 kDa also lacks AF1 and plays an important role in inhibiting the proliferative action of the hERa-66 isoform in MCF-7 cells (245).

1.4.2.2. Estrogen receptor beta isoforms

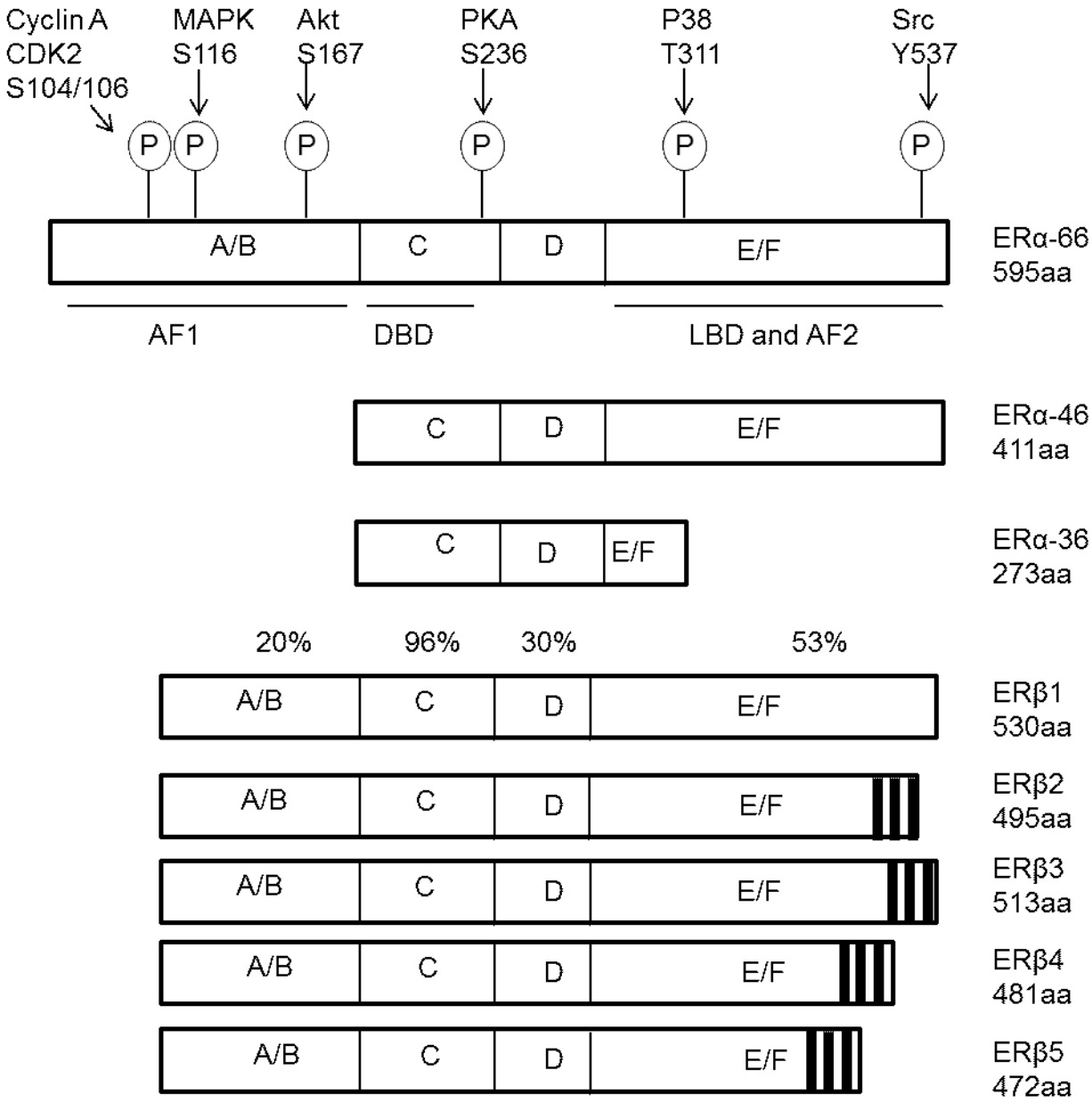
The ERβ transcripts are produced from eight exons as a result of alternative splicing, deletion, or alternative usage of untranslated exons in the 5′ end. As a result, five ERβ isoforms may be present, depending on the cell type. These are labeled ERβ1-5

(246) (Figure 1.12). ERβ1 codes for the full-length ERβ, which is translated from eight exons (247). ERβ2 has a unique C-terminus, due to alternative splicing, which results in loss of the AF-2 domain and, thus, it has no affinity for E_2. ERβ2 can inhibit ERα signaling through different mechanisms. First, ERβ2 inhibits binding of ERα to the estrogen response element (ERE), leading to downregulation of ERα-dependent genes (248). Moreover, ERβ2 targets ERα for proteasomal degradation, primarily through the formation of heterodimers with ERα (249). ERβ4 and β5 can heterodimerize with ERβ1 and increase its activation in a ligand-dependent manner. ERβ3 is only expressed in the testis and its function remains unknown (247).

1.4.2.3. G-protein-coupled receptor 30

The G-protein-coupled receptor was cloned and reported in the late 1990s (250) and shortly thereafter was assigned, *GPR30*, according to its HGMW-approved symbol (251). Despite considerable effort using several peptides, no ligands for GPR30 are identified; hence, it was called an orphan receptor (250, 252). However, recent studies have shown that GPR30 directly binds estradiol and acts as a membrane-bound estrogen receptor; thus, the orphan name of GPR30 is no longer applicable (253, 254). Interestingly, ERα antagonists such as ICI 182,780 and tamoxifen were reported to act as GPR30 agonists (253, 254), suggesting that breast cancer cells which are resistant to ER antagonist may be due to cross talk with GPR30.

Figure 1.12 Structure and function domains of estrogen receptor alpha and beta isoforms. The A-F regions are indicated as boxes with the underlined of domains DNA binding domain (DBD), ligand binding domain (LBD), two transcriptional activation functional domains, AF-1 and AF-2 and the phosphorylation sites. Estrogen receptor alpha (ERα) is presented in three isoforms (66, 46, 36 kDa) with the corresponding amino acid, while Estrogen receptor beta (ERβ) is presented in five isoforms (ERβ1, ERβ2, ERβ3, ERβ4, ERβ5) with the corresponding amino acids. Percentage of sequence similarity between ERα and ERβ as indicated in wild type ERβ. Adapted from (255).


Cyclin A
CDK2
S104/106
MAPK
S116
Akt
S167
PKA
S236
P38
T311
Src
Y537
P
A/B
C
D
E/F
ERα-66
595aa
AF1
DBD
LBD and AF2
ERα-46
411aa
ERα-36
273aa
20%
96%
30%
53%
ERβ1
530aa
ERβ2
495aa
ERβ3
513aa
ERβ4
481aa
ERβ5
472aa

Estrogen mediated a rapid phosphorylation of ERK 1/2 in MCF-7 cells, which express ERα, ERβ, and GPR30. By contrast, no effect was observed in MDA-MB-231 cells, which express ERβ but not ERα or GPR30, suggesting a possible role for GPR30 in mediating the non-genomic effect of estrogen in MCF-7 (256). Moreover, transfection of GPR30 into MDA-MB-231 cells restored responsiveness to estrogen. The same response occurred in SK-BR-3 cells, which express GPR30 and not ERα or ERβ. Together, these results strongly suggest GPR30 is implicated in mediating cellular responses to estrogen (256). Estrogen-mediated increase in Bcl-2 expression in keratinocytes was through GPR30 only because silencing the GPR30 in these cells prevented the estrogen-dependent response (257). Additionally, GPR30 was involved in activation of the proto-oncogene c-fos in breast cancer cells by estrogen through ERK 1/2 activation (258). Thus, although classical ER signaling is usually associated with gene regulation, this sequence of data exposed the ability of GPR30 to modify gene expression in response to estrogen.

1.4.3. Estrogen receptor signaling

1.4.3.1. Classical estrogen receptor signaling (ligand dependent)

Unbound ER is present in the form of a monomer bound by HSP in the cytosol (259, 260) (Figure 1.13). Binding of estrogen to ER results in receptor activation followed by conformational change and subsequent dissociation from HSPs. Estrogen-bound ERs dimerizes and translocate to the nucleus where they function as a transcription factor. ER binds to ERE located in the promoter or enhancer regions of target genes (261). The classical ERE is a palindromic sequence composed of two-inverted hexanucleotide

repeats which allow two ER dimers to bind (259). Binding of ER to the DNA recruits co-activators, such as SRC1 and p300/CBP, and interacts with basal transcription machinery to either help unwind or repress the chromatin structure (262). Ligand-bound ERα and/or ERβ may bind to EREs as homo- or heterodimers to alter gene transcription (259, 260). In addition to that, some ERα is activated in the absence of ligand and bound to ERE sites of target genes in the nucleus (263).

Alternatively, E_2-ER can interact first with other proteins or transcription factors, such as AP1, SP1 and NF-κβ, and then bind indirectly to DNA through protein-protein interaction. This type of interaction is important as it can regulate genes which do not have an ERE binding site and thus increase the efficiency of ER (264).

1.4.3.2. Ligand independent estrogen receptor signaling

It is well recognised now that ER is activated by extracellular signals in absence of E_2 (265). Epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) can activate ER and result in increase ER target genes expression (266). Both EGF and IGF-1 activates various kinases, including MAPK and PI3K/AKT, which act on the multiple phosphorylation sites located in the ER (Figure 1.12). S104/106, S118 and S167, which are located in the A/B domain, are the most common phosphorylation sites and, therefore, contribute in the AF-1 transcription activity. MAPK is constitutively activated in some breast cancer cells and contributes for cellular proliferation and progress of cancer (267). Furthermore, MAPK activation has been correlated with the loss of ER expression (226).

Prolactin plays a role in mammary gland development and its role in breast cancer has now been noticeably recognized (268). The hormone and its receptor are both expressed by the breast cancer cells, suggesting it can act in an autocrine fashion (269). Recently it was shown that prolactin could activate ER in a ligand independent manner through phosphorylation of S118 and activation ERE reporter plasmid in MCF-7 and T47D (270). Moreover, ICI antagonise the proliferative effect of prolactin by inhibiting ERE stimulation, suggesting that ligand independent ER activation by prolactin is significant in development of breast cancer by increasing proliferation (270). Other hormones such as dehydroepiandrosterone (DHEA), which is an androgen precursor, have been shown to experimentally increase cell proliferation in MCF-7 cells. The effect of DHEA on cell proliferation was through competing with estradiol to ER binding (271).

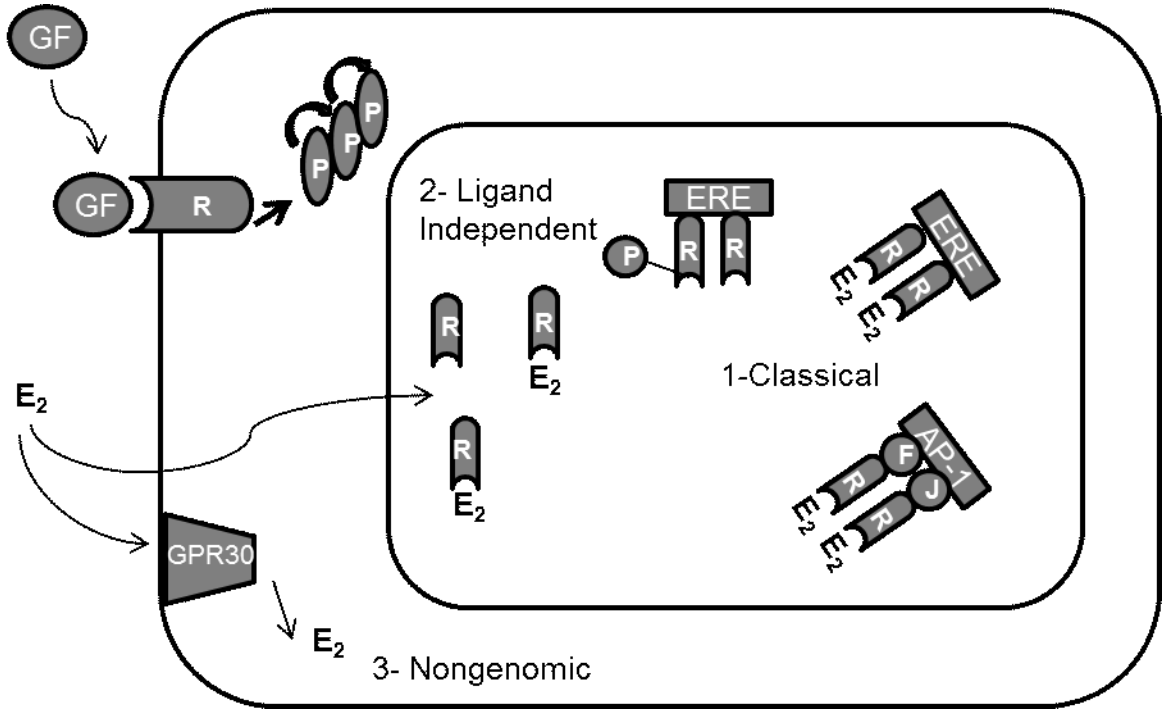
Transcription factor like X box-binding protein 1 (XBP-1), which is highly elevated in breast cancer, has been reported to enhance ER transcriptional activity in a ligand independent manner (272). Additional signaling pathways have been shown to stimulate ERα transcription activity through co-activators, such as AIB1, a member of the p160 family (273, 274). ERα activation, arising from the cross talk of ER signaling with other signal transduction pathways, is of clinical significance and may alter the response of breast cancer to hormonal therapy (275). Based on which signaling pathway is activated, a combination of hormonal and protein-kinase inhibitors may be ideal for treatment of these resistant cases.

1.4.3.3. Non-genomic estrogen receptor signaling

The rapid biological effect of estrogen in several tissues indicates that estrogen may provoke non-genomic effects, possibly through plasma membrane ER (Figure 1.13). Many growth factor receptors, such as IGF-1R, EGFR, and HER2 have been shown to associate with membrane ER to form a complex (276-278). According to the cell type, this complex may result in downstream activation of other signaling pathways such as MAPK and PI3K/AKT (276-278). Considerable controversy surrounds the source of membrane ER. It has been suggested that membrane ER originates from nuclear ER for two reasons: membrane ER is detected by the same antibody used to detect the nuclear ER, suggesting that the two receptors share the same epitiope. Membrane ER was detected after transfecting the cells with cDNA encoding the nuclear receptor. An alternative theory is that ER shares in the formation of caveolae by binding with caveolin-1 protein (279). There are many ongoing studies about non-genomic ER signaling and no established mechanism has been recognized to date. Furthermore, as a result of technical difficulties, the clinical significance of this action has not been evaluated in breast cancer (280).

Figure 1.13 Mechanisms of estradiol and estrogen receptor signaling.

Three different ER signaling pathways are indicated: 1, classical ligand-dependent, estradiol-estrogen receptor (E_2-ER) complexes bind to estrogen response element (ERE) in target genes or indirectly through protein–protein interaction. 2, Growth factors activate intracellular kinase pathways, leading to phosphorylation and activation of ER. 3, Non-genomic ER signaling through membrane ER or more recently through GPR30. Adapted from (265).


GF
GF
R
P
P
P
2- Ligand
Independent
ERE
P
R
R
ERE
R
R
E2
E2
R
R
E2
1-Classical
E2
R
E2
AP-1
F
J
R
R
E2
E2
GPR30
E2
3- Nongenomic

1.5. Rationale and Hypothesis

Previous work in our laboratory, arising from Sharon Oldford's study on breast cancer tissues, showed that HLA-DR expression on breast carcinoma *in situ* is significantly associated with reduced ERα, lower age at diagnosis and increased levels of IFN-γ mRNA (106). Taken together, these observations suggest that the estrogen receptor, hormones and cytokines modulate HLA class II expression on breast tumor cells. Moreover, estrogen treatment prior to abdominal aorta allograft transplantation in rodents results in significant reduction of MHC class II, which is associated with better graft survival (281-283). Additionally, it was shown that ovariectomy results in upregulation of IFN-γ induced MHC class II expression on macrophages in mice (284), and, E_2 downregualtes IFN-γ induced MHC class II expression on astrocytes, fibrosarcoma cells, macrophages and brain endothelial cells. All these reports suggest that E_2 modulates the IFN-γ induced activation of MHC class II expression. Moreover, signaling pathways such as MAPK that are activated in breast cancer cells modulate MHC class II expression (267). Adamski and Benveniste showed that, through the non-genomic signaling pathway E_2 activates the MAPK-JNK signaling pathway, which correlates with MHC class II downregulation. (285). In 2007 Martins and her colleagues, demonstrated that MAPK-ERK and MAPK-JNK are required for the regulation of CIITA and MHC II expression in melanoma cells through an AP1-responsive motif in pIII CIITA (203). These reports suggested that MAPK plays a significant role in HLA class II regulation.

Based on these previous reports, I hypothesised that hormones, their receptors and

cross talk between signaling pathways such as ER, IFN-γ and MAPK play a differential role in HLA class II regulation in $ER\alpha^+$ and $ER\alpha^-$ breast cancer cells.

1.6. Objectives

1- To compare HLA class II expression in $ER\alpha^+$ and $ER\alpha^-$ breast cancer cell lines with or without IFN-γ- treatment.

2- To determine if HLA class II is modulated by physiological concentrations of hormone treatment in $ER\alpha^+$ and $ER\alpha^-$ breast cancer cell lines.

3- Identify the possible mechanisms involved in HLA class II modulation by the E_2-ER pathway.

4- To investigate the possible role of MAPK on HLA class II regulation in breast cancer cell lines.

1.7. Co-authorship statement

I, Ahmed Mostafa, am the first author for all the chapters in this thesis. All experimental design, development of research methodology, data collection and analysis and preparation of manuscripts were conducted under the supervision of Dr. Sheila Drover. The author appreciates and recognises those contribution made by others as follows: the deletion constructs in promoter IV of CIITA luciferase plasmid (Chapter 5) was conducted by Yumiko Komatsu, PhD candidate, under the supervision of Dr. Kensuke Hirasawa. Jillian Green, a medical student, conducted the experimental effect of P38i and JNKi on HLA class II expression (chapter 6). Dr Sherri Christian provided extensive technical assistance with reporter gene assays and plasmids preparation. Dianne Codner served as a second reader for immunocytochemistry (Chapter 3).

Chapter 2: Materials and methods

2.1. Cell culture

A panel of five established breast cancer cell lines (BCCL) were used in this study: ERα$^+$ (MCF-7, T47D, and BT-474); and ERα$^-$ (SK-BR-3, MDA-MB-231 (Table 2.1). Cells cultured in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Hyclone), 2 mM L-glutamine (Invitrogen), antibiotic-antimycotic mixture (Invitrogen) (100 Units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B as Fungizone®). Cultures were incubated at 37^{o}C in a 5% CO_2 atmosphere in 10 cm^2 culture plates (VWR International). The medium was refreshed every 3 days and cells were harvested when 80-100% confluent.

MDA-MB-231 clone 10A and the two stable transfectants, MC2 (MDA-MB-231 transfected with the wild type *ESR1* (NM_000125) and VC5 (MDA-MB-231 transfected with an empty pCMV-Neo vector) were generous gifts from Dr. Craig Jordan (286, 287). Cells were maintained as adherent culture in estrogen-depleted medium consisting of phenol red-free (PRF) minimum essential medium (MEM) supplemented with 5% heat inactivated charcoal/dextran fetal bovine serum (CDFBS) (Hyclone), recombinant human insulin 6 ng/ml (Invitrogen), 2 mM L-glutamine, antibiotic-antimycotic mixture. VC5 and MC2 were maintained under selective condition by using 50 µg/ml G418 (Geneticin) (Invitrogen). Cell lines had been validated by Allison D. Edgecombe (MSc. Thesis, 2002).

Table 2.1 Description of human breast cancer cell lines used in this study.
*American type Culture Collection (Manassas,VA). Adapted from (293, 294).

Breast Cancer Cell Line	**Origin**	**Age Years**	**ATCC* Identification**	**Type of Cancer**	**Classification**	**Immunoprofile**	**Reference**
MCF-7	Pleural effusion	69	HTB-22	Adenocarcinoma	Luminal A	ER^+, $PR^{+/-}$, $HER2^-$	(288)
T47D	Pleural effusion	54	HTB-133	Ductal Carcinoma	Luminal A	ER^+, $PR^{+/-}$, $HER2^-$	(289)
BT-474	Breast	74	HTB-20	Invasive Duct Carcinoma	Luminal B	ER^+, $PR^{+/-}$, $HER2^+$	(290)
MDA-MB-231	Pleural effusion	51	HTB-26	Medullary Carcinoma	Basal	ER^-, PR^-, $HER2^-$	(291)
SK-BR-3	Pleural effusion	43	HTB-30	Adenocarcinoma	HER2	ER^-, PR^-, $HER2^+$	(292)

Non-adherent B cell lines RAJI and SAVC, which constitutively express HLA class II, were used as positive controls. Cells were grown as suspension culture in 25 cm^2 cell cultures flasks (Corning) and maintained in Roswell Park Memorial Institute (RPMI) medium (Invitrogen) supplemented with 2 mM L-glutamine, antibiotic-antimycotic mixture and 10% heat inactivated FBS.

2.2. Estrogen-depleted medium

For some experiments, it was necessary to exclude exogenous steroids and estrogen-like compounds from FBS and media, respectively (290, 291). The use of dextran-coated charcoal has been used to reduce estrogen levels in FBS. Moreover, medium that contains phenol red as pH indicator has been shown to stimulate estrogenic activity in some $ER\alpha^+$ breast cancer (292). Therefore, it was necessary to switch our standard cell culture medium to PRF-MEM supplemented with 5% CDFBS at least 24 hr prior the experiment and maintain them in this medium for the duration of the experiment.

2.3. Breast cancer cell line harvesting

Phase contrast microscopy was used to determine when cells were 80-100% confluent and ready for harvesting. The medium was aspirated from the adherent cells and the cells were washed once with phosphate buffered saline (PBS) and incubated with 0.25% trypsin (Invitrogen) in PBS for 3-4 min or until cells started to detach from the plate. An equivalent amount of medium was added to quench trypsin activity, mixed and transferred to a 15 ml centrifuge tube. Cells were centrifuged at 500 x g for 7 min at 8^oC,

followed by discarding the supernatant and washing the cells again in medium as before. After the last wash the cells were re-suspended in 5 ml medium, mixed and counted using a haemocytometer and phase contrast microscopy. To maintain healthy growing cells, a volume of 5 x 10^5 cells was re-plated into 10 cm^2 cell culture plates in a final volume of 10 ml medium.

2.4. Hormonal treatment and interferon gamma stimulation of breast cancer cell line

To determine the effects of hormone treatment on HLA class II expression, BCCL were treated with either E_2 (10^{-9}M), Fulverstrant (ICI 180, 720) (ICI) (10^{-6}M), Tamoxifen (TAM) (10^{-6}M) (Sigma) combinations of the above treatment or vehicle control (ethanol). They were then left un-stimulated or stimulated with IFN-γ (BD Biosciences) (100 Units/ml) for the indicated time: 24 and 96 hr for CIITA and HLA class II protein expression, respectively; 4 and 12 hr for CIITA and HLA class II mRNA expression, respectively; and 6-24 hr for reporter gene assay (depending on optimal time point for each promoter activity).

At the time of the experiment, each cell line was harvested using 0.25% trypsin and seeded into 6-well plates (VWR International) at 3 x 10^5 cells/well or seeded into 96-well plates (VWR International) at 2 x 10^4 cells/well. After 24-hr incubation, the medium was aspirated and replaced with fresh medium containing the hormone of interest or vehicle control (ethanol) for 1 hr. The cells either left un-stimulated or stimulated with IFN-γ (100 Units/ml) for the indicated time depending on the experiment.

2.5. Mitogen activated protein kinase inhibitors

A panel of MAPK inhibitors was used in the study described in Chapter 6. The MDA-MB-231 cell line was either treated with 10 μM U0126 (MEK inhibitor) (Calbiochem), 25 μM SP 600125 (JNK inhibitor) (Sigma), 25 μM SB 202190 (p38 inhibitor) (Sigma) or DMSO (Sigma) as a diluent control for 1 hr. The cells either left un-stimulated or stimulated with IFN-γ (100 Units/ml) for the indicated time depending on the experiment.

2.6.Flow cytometry

2.6.1. Cell surface protein expression

HLA class I, HLA-DR, CLIP and CD119 surface expression was detected by flow cytometry. Adherent BCCL were harvested as previously described and a B cell line was used as positive control. Cells were re-suspended in FACS buffer (0.2% CDFBS, 0.02% sodium azide (BDH chemicals, Poole, England) in PBS to give a final concentration of 3 x 10^6 cells/ml. Two hundred thousand cells (50 μl of cell suspension) were incubated with 25 μl of the appropriate antibody (diluted in FACS buffer) (Table 2.2) in 5 ml polystyrene round-bottom tubes (Falcon), for 30 min on ice.

After this incubation, the cells were washed twice using 2 ml FACS buffer and centrifuged at 600 x g for 5 min at 8^{o}C. The supernatant was discarded after the second wash and 25 μl of secondary antibody (1/40 dilution in FACS buffer), goat anti-mouse immunoglobulin-G labeled with phycoerythrin fluorochrome (Jackson ImmunoResearch) were added to each tube. The cells were incubated for 30 min on ice in the dark to avoid

fluorescence bleaching. Following this incubation, cells were washed twice and fixed by adding 200 µl of 1.0% paraformaldehyde (PFA) (Sigma) in PBS, and 10,000 cells were analyzed using a FACStarPlus flow cytometer (Becton Dickinson) and CellquestPro software (Becton-Dickinson).

2.6.2. Intracellular flow protein expression

CIITA, HLA-DM and Ii intracellular expression was also detected by flow cytometry. Adherent BCCL were harvested as previously described and a B cell line was used as positive control. Cells were re-suspended in PBS and fixed with 2% PFA for 1 hr on ice. Following incubation, the cells were washed once with complete MEM medium and once with PBS. Next, the cells were permeabilised with 0.02% tween 20 prior for 1 hr, and then washed once with intracellular FACS buffer (5% bovine serum albumin (Sigma), 0.02% Tween 20 (Sigma) in PBS) and re-suspended in intracellular FACS buffer at the same concentration as above. The rest of the steps were similar to surface flow cytometry except the antibodies were diluted in intracellular FACS buffer.

2.6.3. Interpretation of flow cytometry results

Surface and intracellular expression were determined as:

Mean Fluorescence Intensity (MFI) Test – MFI Negative control

Results were considered positive if values were at least twice the background in the three replicate experiments, data were averaged and standard errors of the mean were calculated.

Table 2.2 Primary antibodies used to detect human leukocyte class II co-chaperone protein expression by flow cytometry, immunocytochemistry and immunoblotting.

Antibody	Isotype	Specificity	Concentration			Source
			Flow	W.B	I.H.C	
W6/32 supernatant	Mouse IgG2a	Pan HLA-Class I	1/10 dilution	NT*	NT	ATCC
TAL 1B5	Mouse IgG1	HLA-DRα	NT	40 ng/ml	NT	Abcam
L243 purified supernatant	Mouse IgG2a	Pan HLA-DR	2.4 µg/ml	10 ng/ml	2.4 µg/ml	ATCC (295)
MaP.DM1	Mouse IgG1	HLA-DM	10 µg/ml	NT	10 µg/ml	BD Biosciences
TAL18.1	Mouse IgG1	HLA-DMα	NT	40 ng/ml	NT	Abcam
CD74-LN2	Mouse IgG1	Recombinant human CD74 protein	5 µg/ml	200 ng/ml	5 µg/ml	BD Biosciences
CD74-PIN-1	Mouse IgG1	12-28 aa of Ii	NT	1 µg/ml	NT	Abcam
CD-74-CerCLIP.1	Mouse IgG1	81-104 aa of Ii	Check	NT	NT	BD Biosciences
CD119	IgG2	IFNGR1	Check	NT	NT	GenScript
MOPC-21	Control	IgG1	5 µg/ml	NT	NT	BD Biosciences
NSG2a	Control	IgG2a	2.5 µg/ml	NT	NT	Local source
1D5	Mouse IgG1	ERα	NT	NT	7.5 µg/ml	Abcam

*NT=Not tested

2.7. Western blots

2.7.1. Preparation of whole cell lysates

Cell cultures and treatment were as previously described in Sections 2.1, 2.4 and 2.5. Whole cell lysates were prepared by washing the adherent cells once with ice cold PBS, followed by addition of 1 ml radioimmunoprecipitation assay (RIPA) buffer (PBS pH 7.4, NP-40 1% (Sigma), 0.1% sodium dodecyl sulfate (SDS) (Bio-Rad), 0.5% sodium deoxcycholate (Sigma)) or 1% Triton X-100 buffer/10^7 cells (20 mM Tris pH 8.0, 1% Triton X-100 (Sigma), 10% glycerol (Sigma), 2 M ethylene-diaminetetraaccetic acid (EDTA) (Sigma), 137 nM NaCl (Sigma)), containing protease inhibitor aprotinin (1 µg/ml), leupeptin (1 µg/ml), pepstatin A 100µg (1 µg/ml), phenylmethylsulfonyl fluoride (PMSF) (10 ug/ml) (Sigma) and Halt phosphatase inhibitor cocktail 10 µl/1 ml buffer (Thermo Scientific). After addition of the lysis buffer, the cells were scraped using a rubber policeman (Fisher Scientific), followed by transfer of the lysates into a 1.5 ml centrifuge tube. Lysates were mixed on a rotator at 4^{o}C for 1 hr, after which they were centrifuged at 11,000 x g for 10 min at 4°C. The supernatant was carefully collected, without disturbing the pellet and transferred to a clean tube and stored at -80^{o}C.

2.7.2. Quantification of protein in cell lysates

Proteins were quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Fifty microliters of each sample (1/10 dilution) and known amounts of BSA: 0 µg, 31.25 µg, 62.5 µg, 125 µg, 250 µg and 500 µg, were loaded in duplicate into 5 ml polystyrene round-bottom tube (Falcon, Becton Dickson Bioscience). One ml BCA protein assay working reagent (50:1, reagent A: reagent B) was added to each

sample or standard and the tubes were incubated for 30 min at 37^{o}C. Tubes were read at 562 nm using a spectrophotometer (Beckman Coulter DU® 530) and protein concentrations were calculated based on the standard curve.

2.7.3. Co-immunoprecipitation

Protein lysate was pre-cleared by mixing 30 µl of protein A agarose beads (Thermo Scientific) with 200 µg protein lysate in a total volume of 500 µl Triton X-100 buffer and rocking for 1 hr at 4^{o}C. Following the incubation period, the lysate was centrifuged at 13,000 x g for 1 min at 4°C and the pre-cleared lysate was transferred to a new centrifuge tubes with care not to touch the beads. Cross linking the antibody with agarose beads was done by incubating 1 µg of the specific antibody in each tube, and left rocking overnight at 4^{o}C. Co-immunoprecipitation (CO-IP) was achieved by adding 30 µl protein A agarose beads to each tube and mixing for 4 hr at 4^{o}C. The lysate was centrifuged at maximum for 1 min, followed by discarding the supernatant and washing the beads three times with 500 µl of cold Triton X-100 lysis buffer. For the final wash, all the supernatant was discarded. The beads were eluted by re-suspending in 50 µl of reducing buffer (2-Mercaptoethanol (ME), 5% SDS (Bio-Rad), 10% glycerol (Sigma), bromophenyl blue (Sigma) to color) and boiling for 7 min, followed by centrifugation at 13,000 x g for 1 min at 4°C. The supernatant was transferred to a fresh centrifuge tube, and 20 µl were loaded onto sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) for immunoblotting.

2.7.4. Electrophoresis of cell lysates

Equal amounts of protein (typically 10-20 µg per lane) were analyzed by SDS-PAGE. The SDS-PAGE was formed of two parts: stacking gel (250 µl acrylamide (Bio-Rad), 380 µl stacking buffer (0.5M Tris HCL buffer (Bio-Rad)) pH 6.8, 15µl 10% SDS, 860 µl H_2O), 1.5 µl tetramethylethylenediamine (TEMED) (Bio-Rad), and 15 µl 10% ammonium persulfate (APS) (Bio-Rad)) and 8% running gel (1 ml acrylamide, 1 ml running buffer (1.5M Tris HCL buffer (Bio-Rad)) pH 8.8, 40 µl 10% SDS, 1.25 ml H_20), 2 µl TEMED, and 40 µl 10% ammonium persulfate (APS)).

An appropriate volume of lysate was removed from each sample to give the indicated protein concentration and denatured by boiling the sample at 100^oC after adding reduced sample buffer (Section 2.7.3). To estimate protein product size, gels were simultaneously loaded with 4 µl PageRuler^{TM} pre-stained protein ladder (Fermantas). Mini-PROTEIN® Cell electrophoresis chamber (Bio-Rad) containing running buffer (25 mM Tris HCL pH 8.8, 0.1% SDS, 190 mM glycine (Sigma)) was used to separate the proteins at 100 volt (V) for 2 hr.

2.7.5. Electrophoretic protein transfer

Following electrophoresis, the proteins were transferred onto nitrocellulose membrane. Nitrocellulose membranes were first activated by soaking the membranes in transfer buffer (25 mM Tris HCL pH 8.8, 190 mM glycine, 20% methanol) for 15 min together with the filter papers and foam pads. Gels were removed from electrophoresis apparatus. The transfer sandwich cassette was assembled as follows: plastic base, foam pad, two filter papers, gel, nitro cellulose membrane, two filter papers, foam pad and

plastic cover. The sandwich cassette was placed in the Trans-blot cell (Bio-Rad) and transfer was carried out at 100 V for 1 hr.

2.7.6. Immunodetection of proteins

Following transfer, the membranes were incubated in blocking solution (5% milk in Tris-buffered saline (TBS-T)-Tween 20 (0.15 M NaCl, 0.05 M Tris pH 7.4, 0.05% Tween 20) for 1 hr. The appropriate concentration of the primary antibodies (Table 2.2 & 2.3) was added to 4 ml of blocking solution for each blot in a small sealed plastic box and left rocking at 4^{o}C overnight. Membranes were washed three times, 5 min each with TBS-T while rocking, and an appropriate concentration of horseradish peroxidase conjugated secondary antibodies (Table 2.4) was added for 1 hr with rocking at room temperature. The membranes were washed extensively to remove unbound secondary antibodies, and then signal detection was achieved using Immobilon Western Chemiluminescent HRP Substrate (Millipore). Blots were imaged using the ImageQuant LAS 4000 Station (GE health care), and protein band densities were analyzed using the spot density analysis software (ImageQuant TL8.1) provided with the image station.

2.7.7. Reprobing membranes

Membranes were stripped by immersion in stripping buffer (TBS pH 2.0) for 10 min three times and then washed with TBS-T three times, 10 min each.

Table 2.3 Primary antibodies used in immunoblotting.

Antibody	Isotype	Specificity	Concentration	Source
Antiserum # 21	Rabbit IgG	CIITA	1/4000	Viktor Steimle's lab (296)
HC-20	Rabbit IgG	ER	500 ng/ml	Santa Cruz Biotechnology
Phospho-Ser^{118}	Rabbit IgG	ER	1 µg/ml	GenScript
#06-501	Rabbit IgG	STAT1-T	200 ng/ml	Upstate Biotechnology
Phospho-Tyr^{701}	Rabbit IgG	STAT1-P	200 ng/ml	GenScript
Phospho-Ser^{727}	Rabbit IgG	STAT1-P	200 ng/ml	GenScript
BD-20	Mouse IgG1	IRF1	125 ng/ml	BD Biosciences PharMingen
C-20	Rabbit IgG	ISGF-3	400 ng/ml	Santa Cruz Biotechnology
T-18	Goat IgG	GILT	125 ng/ml	Santa Cruz Biotechnology
Phospho-Tyr^{1022}	Rabbit IgG	JAK1-P	200 ng/ml	GenScript
Phospho-Tyr^{1007}	Rabbit IgG	JAK2-P	200 ng/ml	GenScript
Ab-1022	Rabbit IgG	JAK1-T	200 ng/ml	GenScript
Ab-1007	Rabbit IgG	JAK2-T	200 ng/ml	GenScript
Thr^{202}/Tyr^{204}	Rabbit IgG	ERK p44/42	5 µg/ml	Cell Signaling
K-23	Rabbit IgG	ERK-1 Total	200 ng/ml	Santa Cruz Biotechnology
5E10	Mouse IgG2b	P84	1 µg/ml	Abcam
Ab8245	Mouse IgG1	GAPDH	1 ng/ml	Abcam
B-7	Mouse IgG2a	αTubulin	250 ng/ml	Santa Cruz Biotechnology

Table 2.4 Secondary antibodies used in immunoblotting

Antibody	**Isotype**	**Specificity**	**Concentration**	**Source**
HRP-conjugated affiniPure f(ab)$_2$ fragment goat anti-mouse (GAM) Fc specific	Goat IgG	Mouse IgG	1/10000	Jackson Immunoresearch
HRP-conjugated affiniPure f(ab)$_2$ fragment goat anti-rabbit (GAR) Fc specific	Goat IgG	Rabbit IgG	1/10000	Jackson Immunoresearch
Donkey anti-goat IgG-HRP	Donkey IgG	Goat IgG	1/5000	Santa Cruz

2.8. Immunocytochemistry and confocal microscopy

2.8.1. Chamber slide set-up

BCCL were grown in 8-well chamber slides at 2.5 x 10^4 cells/well, with the indicated treatment. On the day of the assay the medium was aspirated and cells washed once with PBS and left to dry overnight followed by fixation in acetone for 15 min at -20°C, and then left to air dry for at least 1 hr.

2.8.2. Cytocentrifuge preparations

B cell lines, used as positive controls, were counted using haemocytometer and an aliquot containing 6 x 10^5 cells was added to a 15 ml centrifuge tube and centrifuged at 500 x g for 7 min at 8^{o}C. Following centrifugation, medium was removed and the cells were washed once with PBS and re-suspended in 6 ml PBS (1 x 10^5 cells/ml). Cells were permanently adhered to microscope slides by adding 500 µl cells (5 x 10^4 cells) to the cytocentrifuge sample chamber, in which a filter card (Shandon Inc., Pittsburg, PA) was assembled between chamber and the microscope slide. Cells were centrifuged at 500 x g for 5 min and left to dry overnight followed by fixation with acetone as previously described in Section 2.8.1.

2.8.3. Protocol for immunocytochemistry

Acetone-fixed cells were rehydrated by adding PBS for 5 min, followed by adding 1% hydrogen peroxide in PBS to release endogenous peroxidase activity. Slides were washed once with washing buffer (WB) (PBS containing 0.5% bovine serum albumin (BSA) (Sigma), 0.05% Tween 20). Washing was done by adding 400 µl of WB to each

well of the chamber slides, left for 5 min followed by careful removal with a Pasteur pipette or by immersing slides in WB with mixing for 5 min on a magnetic stirrer. Next, the slides were blocked using 15% goat anti serum in PBS for 1 hr of blocking, followed by removal of excess blocking buffer and incubation with 100 µl of the primary antibody (Table 2.1) (optimally diluted in WB) at room temperature in a humid chamber. The slides were then washed three times for 5 min with WB and incubated with 100 µl of secondary antibody (antimouse IgG) (Vector Immpress Reagent), following by three washes for 5 min with WB. Antibody binding was detected with peroxidase substrate (Nova Red) or DAKO EnVison depending on the assay for 10-15 min at room temperature.

Cells were washed with distilled water and, counterstaining was done with Vector hematoxylin (Vector Labs) for 30 seconds. Excess dye was removed by washing cells in tap water; the slides were immersed in bluing solution (1.5 ml NH_4OH (30% stock) + 98.5 ml 70% ethanol) for 1 min. Cells were dehydrated by immersion in 70% ethanol for 1 min, 95% ethanol for 1 min, 2 changes of 100% ethanol for 1 min each and in 2 changes of xylene for 2 min each. Cells were permanently mounted in Micromount (SurgiPath) and examined by light microscopy

2.8.4. Protocol for confocal microscopy (parallel or simultaneous immunofluorescence)

Acetone fixed cells were rehydrated by adding PBS and left for 5 min followed by blocking using 15% goat serum in PBS. After 1 hr of blocking, excess blocking buffer was removed and cells incubated with 100 µl mixture of the two primary antibodies (Table 2.1) (optimally diluted in WB (0.5% BSA in PBS) for 1 hr. The slides were

washed in WB as in Section 2.8.3 and 100 µl of the mixture of the secondary antibodies goat anti-mouse (GAM) conjugate labeled with Alexaflor 555 (IgG1-specific) and Alexaflor 488 (IgG2a-specific) were incubated with the cells for 1 hr in dark. The cells were washed three times in WB, and three times in PBS in the dark, and then mounted with VECTASHIELD® mounting medium with DAPI (Vector labs). Cells were examined with a confocal laser-scanning microscope (Olympus FluoView 300).

2.8.5. Interpretation of data

Immunocytochemistry (ICC) and confocal microscopy were visualised independently by three different individuals (AM, SD and DC) blind to antibody and treatment. The percentage of positive cells was determined with a cut off value of less than 10% considered negative. Staining and subcellular localisation were also noted.

2.9. Standard reverse transcriptase polymerase chain reaction

2.9.1. RNA extraction

Medium was aspirated from adherent breast cancer cell lines growing in a 6-well plate, and cells were detached by adding 400 µl TRIZOL® reagent (Invitrogen). Detached cells were transferred to 1.5 ml centrifuge tube and incubated for 5 min at room temperature for cell lysis. Following the incubation period, 80 µl chloroform (Sigma) were added and mixed vigorously for 15 seconds. The mixture was centrifuged at 12,000 x g for 15 min at 4^{o}C. After centrifuging, the top aqueous phase, which contains the RNA, was carefully removed, without disturbing the other layers to avoid DNA contamination,

and placed in a new 1.5 ml centrifuge tube. An equivalent volume of isopropanol (Sigma) was added and left for 10 min at room temperature.

Precipitation of RNA was done by centrifugation at 12,000 x g for 15 min at 4^{o}C. The supernatant was discarded and the pellet was washed with 1 ml 75% ethanol and re-centrifuged at 7,500 x g. Following centrifugation, the supernatant was discarded and the pellet left to air-dry for 5 min at room temperature. RNA was re-suspendeed in 10 µl diethyl procarbonate (DEPC) water and quantified using a NanoDrop (Thermo Scientific).

2.9.2. DNase treatment of RNA

Contaminating DNA from the RNA extract was removed using TURBO DNA-free™ DNase treatment and removal reagents kit (Ambion®). One microliter of 10X DNase buffer and 1 µl DNA-free DNase were added to the RNA samples and incubated in a water bath at 37^{o}C for 30 min. Following the incubation period, 1.2 µl of DNase inactivation reagent was added to the mixture and left at room temperature for 2 min. Samples were centrifuged at 12,000 x g for 1 min and the supernatant was transferred to a clean centrifuge tube.

2.9.3. cDNA synthesis

First strand cDNA synthesis kit one (Amersham Pharmacia Biotech) was used for preparation of cDNA. One microgram of total RNA was diluted in a total volume of 8 µl DEPC water in 0.2 ml PCR tubes and placed in the cycler (Biometra T-Gradient) (Montreal Biotech Inc.) at 65^{o}C for 10 min to denature the RNA. Samples were then

placed on ice and the polymerase chain reaction (PCR) reaction mixture (5 µl Bulk First-Strand Reaction Mix, 1 µl Not I-d (T)18 primer and 1 µl of dithiothreitol (DTT)) was added. Samples were returned to the cycler for 1 hr at 37^{o}C and 10 min at 70^{o}C. cDNA was stored at -80^{o}C until use.

2.9.4. Reverse transcriptase polymerase chain reaction primers

Primers used to analyse mRNA transcription of CIITA, HLA class II expression (DRA, DRB, DMA, DMB, Ii) and GAPDH, purchased from Invitrogen, were based on published data (Table 2.5). OligoTech Analysis software (Wilsonville, OR) was used to determine the G+C content and melting temperature. The number of PCR cycles required to fall into the exponential phase of the amplification reaction was experimentally determined for each set of primers using RAJI mRNA, by removing small aliquots from a trial PCR reaction, every two cycles starting at cycle 16 and ending at cycle 36. Amplicons at each time point were compared by agarose gel electrophoresis with ethidium bromide staining and the amplicon intensities were calculated and plotted on a semi-log curve for quantification. To minimize the formation of spurious products, when a high number of amplification cycles are used, the minimal number of cycles in the exponential phase sufficient to detect the expected PCR products was selected. Preparing a 10-fold cDNA dilution series and repeating the PCR using the indicated cycle number further confirmed the correct cycle. Amplicon intensities were calculated and plotted on a semi-log curve for quantification. Evenly spaced amplification curves should produce a linear curve, which confirms the efficiency, and linearity of RT-PCR. The optimized conditions are summarized in Table 2.6.

2.9.5. Polymerase chain reaction amplification

A 49 µl aliquot of PCR master mix (200 mM Tris-HCl buffer (pH 8.4), 500 mM KCl, 10 mM (deoxyribonucleotide triphosphates) dNTP, 50 mM $MgCl_2$, 0. 2 µl Taq DNA polymerase), 1 µl of both forward and reverse primers, and UltraPure™ DNase/RNase-Free distilled water (Invitrogen)) was added to 0.2 ml thin walled polypropylene PCR tube followed by adding 1 µl of cDNA or 1 µl of H_20 as a negative control. Concentration of the primers and components of PCR reaction master mix varied depending on the gene of interest (Table 2.7). Next, PCR was amplified by placing PCR microtubes into Biomed T-Gradient cycler according to the optimized PCR protocol.

2.9.6. Electrophoresis of polymerase chain reaction products

One microliter of loading buffer was mixed with 5 µl of PCR product and loaded onto a 1.5% agarose gel. The gel was premade by adding 0.6 g agarose (Invitrogen) into 40 ml 0.5X Tris/borate/EDTA (TBE) buffer (50 mM Tris, 50 mM borate and 5 mM EDTA) and 0.5 µg/ml ethidium bromide. In order to identify the product size, gels were also loaded with 3 µl of 100 bp DNA ladder (Invitrogen) that was mixed with the loading buffer. PCR products were separated by electrophoresis for 25 min at 120 V using Mini-Sub cell DNA electrophoresis chamber (Bio-Rad) filled with 0.5X TBE buffer. Following separation, PCR products were visualised by UV light using Kodak Imager.

Table 2.5 Primers used to detect human leucocyte class II and class II co-chaperone transcription by polymerase chain reaction.

Gene	Sense	Anti-Sense	Size (bp)	Reference
DRA	cttctgctgcattgcttttgcgca	cgagttctatctgaatcctgacca	643	(297)
DRB	ccccacagcacgtttcttg	ccgctgcactgtgaagctct	274	Allison D. Edgecombe MSc. Thesis, 2002
DMA	ccaatgtggccagatgacctgc	gcgtgaacacttcagcgatag	303	Allison D. Edgecombe MSc. Thesis, 2002
DMB	gcagaagtgactatcacgtgg	ccgccagctgatcacaccaag	296	Allison D. Edgecombe MSc. Thesis, 2002
Ii	tcccaagcctgtgagcaagatg	ccagttccagtgactctttcg	340	(298)
CIITA	caagtccctgaaggatgtgga	acgtccatcacccggagggac	266	(299)
ESR1	gctgcaaggccttcttcttcaa	tcatcaggatctctagccag	550	(300)
GAPDH	catcaccatcttccaggagcg	tgaccttgcccacagccttg	443	(301)

Table 2.6 Polymerase chain reaction conditions used to amplify human leucocyte class II and co-chaperone mRNA.

Gene	Cycler Condition	Number of cycles
DRA	94-1', 55-1', 72-3'	25
DRB	94-1', 60-1', 72-1'	28
DMA	94-1', 60-1', 72-3'	28
DMB	94-1', 60-1', 72-3'	28
Ii	94-1', 55-1', 72-3'	23
CIITA	94-1', 60-1', 72-3'	32

Table 2.7 Polymerase chain reaction mixtures used in cDNA amplification.

	DRA	***DRB***	***DMA***	***DMB***	***Ii***	***CIITA***	***PIII,PIV***	***GAPDH***
pMol	20	25	10	10	20	20	20	20
10X PCR buffer (µl)	5	5	5	5	5	5	5	5
dNTp`s	1	1	1	1	1	1	1	1
MgCl (µl)	1.5	2	1.5	1.5	1.5	1.5	2	2
Primer + (µl)	1	1	1	1	1	1	1	1
Primer -(µl)	1	1	1	1	1	1	1	1
taq (µl)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Mol H_20 (µl)	39.3	38.8	39.3	39.3	39.3	39.3	38.8	38.8

2.10. Real time reverse transcriptase polymerase chain reaction

2.10.1. Overview

Real time reverse transcriptase polymerase chain reaction (RT-PCR) analysis was carried out using StepOne Real Time PCR systems and Software (Applied Biosystems, Life Technologies). RNA extraction and DNase treatment were done as previously described in Section 2.7.1 and 2.7.2.

2.10.2. cDNA synthesis

High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Life Technologies) were used for preparation of cDNA. Two micrograms of total RNA were diluted in a total volume of 10 µl DEPC water in 0.2 ml PCR tubes and 10 µl RT master mix (2 µl RT buffer, 0.8 µl dNTP's 100 mM, 2 µl RT random primers, 1 µl multiscribe RT, 1 µl RNase inhibitor, 3.2 µl molecular H_20) were added to the same tube and placed in the cycler (Biometra T-Gradient) at 25^oC for 10 min, followed by 2 hr at 37^oC and last extension step for 5 seconds at 85^oC. cDNA was stored at -80^0C until use.

2.10.3. Real time reverse transcriptase polymerase chain reaction primers

Real time PCR was performed using TaqMan® probe-based gene expression analysis kit for CIITA (Hs00172106_m1) and GAPDH (Hs99999905_m1) that include pre-designed primers and probes for optimal amplification (Applied Biosystems) along with TaqMan® universal PCR master mix (Applied Biosystem). All gene expression assays have a carboxyfluorescein (FAM) reporter dye at the 5' end of TaqMan minor groove binder (MGB) probe and non-fluorescent quencher at the 3' end of the probe.

2.10.4. Real time reverse transcriptase polymerase chain reaction primers amplification

All samples were assayed in triplicate to ensure accuracy with a reaction volume of 20 µl. Ten microliters of TaqMan gene expression master mix (Amplitaq® Gold DNA polymerase Ultra-Pure, Uracil DNA glycosylase, dNTP's , ROX^{TM} which act as a passive reference) were mixed with 1 µl of specific primer, 1 µl of cDNA and 8 µl UltraPure™ DNase/RNase-Free distilled water. The optimal concentration of cDNA was predetermined using a 10-fold serial dilution to generate a standard curve. Ideally, 10-fold dilution increases the cycle threshold (CT) value about 3.33 cycles. The amplification efficiency is calculated from the slope, which should lie between 85-100%. The thermal cycler protocol included an initial 2 min at 50^{o}C, 10 min at 95^{o}C, followed by 40 cycles of 15 seconds at 95^{o}C and 1 min at 60^{o}C

2.10.5. Real time RT-PCR interpretations

Real-time PCR amplification was performed by the comparative cycle threshold (ΔΔCT) method and normalized to GAPDH. A control sample without RNA and a reference sample (RAJI, B cell line) were included in each experiment. The ΔΔCT method is used to determine the relative quantity (RQ) in samples. The software determines the RQ of target in each sample by subtracting normalized target quantity in each sample from normalized target quantity in the reference sample. Therefore, the formulas used to calculate RQ are:

CT_{CIITA} - CT_{GAPDH} =ΔCT (both in samples and reference control)

ΔCT_{sample}- $CT_{reference\ control}$ = ΔΔCT

Relative quantity =$2^{-\Delta\Delta CT}$

2.11. Vectors and constructs

2.11.1. Experimental and control plasmid constructs

Several plasmid constructs were used in this study. Table 2.8 summarizes all the experimental and control plasmids that were used for various experiments.

2.11.2. Plasmid construct preparation

2.11.2.1. Preparing plasmid

Some of the plasmids used in this study were commercially available and were purchased from different vendors, while others were custom made and gifts from different laboratories (Table 2.8). These plasmids were sent to us either pre-diluted in Tris/EDTA (TE) buffer or spotted on a thick filter paper with 1 cm circle traced around the plasmid. In order to extract the plasmid, half of the circle was cut and placed with clean forceps in a 1.5 ml microcentrifuge tube. Fifty microliters of TE buffer pH 8.0 (Invitrogen) were pipetted over the filter paper in the microcentrifuge tube, vortexed for 5 min and left incubating for another 5 min at room temperature. Following the incubation period, the centrifuge tube was spun for 5 min at 12,000 x g at room temperature and the supernatant was transferred into a clean microcentrifuge tube and the DNA was measured using NanoDrop.

Table 2.8 Summary of the plasmid constructs used in this study.

Plasmid Construct	Plasmid Backbone	Promoter Insertion
pIII CIITA	PGL2-BASIC	7Kb pIII CIITA Luciferase
pIV CIITA	PGL2-BASIC	-346-+50 bp pIV CIITA Luciferase
Ii	PGL4.11	CD74 promoter Luciferase
DRA	PGL3-Basic	DRA promoter Luciferase
SV40	PRL-SV40	SV40-Luciferase
PGL2	PGL2-BASIC	N/A
GFP	pEGFP-C3	CMV promoter-GFP
Wild Type ESR1	pcDNA3	WT ESR1 ORF isoform 1
Mutant ESR1	pcDNA3	V>G ESR1 ORF isoform 1

2.11.2.2. Bacterial transformation

In order to replenish laboratory stocks of the various reporter genes and plasmid constructs, subcloning efficiency DH5α™ competent *Escherichia coli (E.coli)* cells (Invitrogen) were transformed with respective reporter genes or plasmid constructs. One tube of DH5α cells (500 µl) was thawed on ice, gently mixed and 50 µl of were dispensed into 1.5 ml microcentrifuge tubes on wet ice. Any unused cells were frozen in the dry ice/ethanol bath for 5 min before returning to the -80°C freezer. Cells were transfected with 1 to 5 µl (1-10 ng) of DNA, mixed gently and the tube was incubated on ice for 30 min. Cells were then heat-shocked for 20 seconds in a 42°C water bath without shaking then placed on ice for 2 min. Nine hundred and fifty microliters of pre-warmed lysogeny broth (LB) broth (1% Bacto-tryptone (Sigma), 0.5% yeast extract (Sigma), 1% $NaCl_2$ (Sigma)) were then added, followed by incubation at 37°C for 1 hr and shaking at 225 x g. One hundred microliters of the transformed cells were then spread on pre-warmed selective plates (LB broth, 1.5% agar, 100 µg ampicillin (Sigma)) and incubated overnight at 37°C. The remaining transformation reaction was stored at 4°C.

2.11.2.3. Preparation of miniprep

Miniprep was prepared using QIAprep® Miniprep (Qiagen) according to the manufacturer's instruction. Four different colonies were picked from the transformed bacteria growing in LB amp plate and inoculated into 5 ml of LB broth containing 100 µg /ml ampicillin and incubated at 37^{o}C shaking at 225 rpm overnight. Cells were then harvested by centrifugation of 1.5 mL cells in a centrifuge tube at 8000 x g for 3 min at room temperature. All supernatant was aspirated and an extra 1.5 ml cells were added to

the same tube and cells were harvested as before. All traces of supernatant were removed by inverting the open centrifuge tube until all medium has been drained. Pelleted bacterial cells were re-suspended in 250 µl buffer P1 in which RNase and 0.25 µl of lyse blue was added and vortexed until no cell clumps were visible. Two hundred and fifty microliters of buffer P2 were added and mixed thoroughly by inverting the tube 4–6 times until the cell suspension turned homogeneous blue without localised colorless regions. Immediately after, 350 µl buffer N3 were added and mixed by inverting the tube 4-6 times, or until all trace of blue was gone and the suspension turned a cloudy white in color. Genomic DNA is precipitated by centrifuging for 10 min at 12,000 x g, after which a white compact pellet forms. Next, the supernatant was pipetted to the QIAprep spin column and spun for 1 min to discard the flow-through, followed by washing the QIAprep spin column by adding 0.5 ml buffer PB and spun for 30 seconds at 12,000 x g. After discarding the flow-through the QIAprep spin column was centrifuged again for an additional min to remove residual wash buffer. The QIAprep column was next placed in a clean 1.5 ml microcentrifuge tube and in order to elute the DNA, 50 µl buffer EB (10 mM Tris·Cl, pH 8.5) were added to the center of the QIAprep spin column, left for 1 min, and then centrifuged for 1 min. Miniprep was collected and DNA quantified using a NanoDrop.

2.11.2.4. Restriction analysis of purified plasmid recombinants

The conventional method of selecting the desired recombinant plasmid is to characterize plasmid clones by restriction enzyme digestion. In this study, various types of reporter gene constructs with different plasmid backbones were used, thus, a set of

different combinations of restriction enzyme(s) and reaction buffers were used to identify these plasmids as summarized in Table 2.9. One microgram of plasmid DNA was digested with 1 unit of each enzyme in the presence of 1 µl of the appropriate buffer and UltraPure™ DNase/RNase-Free distilled water was added to complete a total 10µl reaction volume of. All reactions were incubated in a water bath at 37^{o}C for 90 min and the whole reaction products were resolved in 1% agarose gel together with a control un-digested plasmid construct. The final product was identified by the correct expected molecular weight based on loading 3 µl 1 Kbp DNA ladder (Invitrogen). The remaining transformed cells in LB broth from the selected clone were frozen by pipetting 500 µl of transformed cells with 500 µl glycerol and stored in -80^{o}C for future use as glycerol stock.

2.11.2.5. Preparation of midiprep

Midiprep was prepared using QIAprep® Midiprep (Qiagen) according to the manufacturer's instruction. Starter cultures were prepared by diluting 50 µl from the transformed bacterial glycerol stock with 2.5 ml LB broth amp in a 15 ml conical tube with loose cover and incubating for 7 hr with shaking at 225 rpm at 37^{o}C. Following the incubation period, 1 ml of the starter culture was further diluted in 100 ml LB broth amp medium and left shaking over night at 225 rpm at 37^{o}C. Bacteria were harvested by centrifugation at 6000 x g for 15 min at 4°C. The supernatant was discarded and the pelleted cells re-suspended in 4 ml buffer P1 in which RNase and 4 µl of lyse blue were added and vortexed until no cell clumps were visible. Four millilitres of buffer P2 were added and mixed thoroughly by inverting the tube 4-6 times until the cell suspension

Table 2.9 Restriction enzymes used to digest the plasmid constructs.

Construct	Restriction Enzymes	Reaction Buffer
pIII CIITA	HindIII/Kpn	NEB2, BSA (NewEngland)
pIV CIITA	HindIII/Kpn	NEB2, BSA (NewEngland)
Ii	BamHI	NEB1 (NewEngland)
DRA	HindIII/XbaI	NEB2, BSA (NewEngland)
SV40	HindIII/XbaI	NEB2, BSA (NewEngland)
PGL2	HindIII/Kpn	NEB2, BSA (NewEngland)
GFP	NheI/KpnI	NEB4 (NewEngland)
Wild Type ESR1	BgIII/BamHI	NEB2, BSA (NewEngland)
Mutant ESR1	BgIII/BamHI	NEB2, BSA (NewEngland)

turned homogeneous blue without localised colorless regions and incubated at room temperature for 5 min. Following the incubation period, 4 ml buffer P3 were added and mixed by inverting the tube 4-6 times, or until all trace of blue was gone and the suspension turned a cloudy white color, at which point it was incubated on ice for a further 5 min. Genomic DNA was precipitated by centrifuging for 30 min at 12,000 x g, which produced a white compact pellet. The supernatant was transformed into a clean tube and centrifuged for another 15 min at 12,000 x g. Following this, the supernatant was applied to QIAGEN-TIP 100 column, pre-equilibrated with 4 ml of buffer QBT. The column was then washed twice with 10 ml buffer QC and the DNA was eluted with 5 ml buffer QF. DNA was precipitated by adding 3.5 ml isopropanol, mixed by inverting and incubated for 10 min, followed by centrifugation at 12,000 x g for 30 min at 4^{o}C. Precipitated DNA was washed with 500 µl 75% ethanol, centrifuged at 12,000 x g for 10 min at 4^{o}C and the supernatant discarded. The pellet was left to air dry for 5-10 min, reconstituted in 100 µl TE buffer pH 8.0 and quantified by a NanoDrop.

2.12. Reporter gene assays

2.12.1. Day 1: Seeding

BCCL were seeded in a 96-well tissue culture plate at a density of 2 x 10^4 cells/well in 100 µl culture medium. Five replicate wells were prepared for each experimental each condition to control for variation in transfection efficiency.

2.12.2. Day 2: Transfection

All transfection reagent and plasmids were brought to room temperature. Optimal transfection conditions were established using Fugene HD (Roche) transfection reagent according to the manufacturer's protocol. Briefly, the master mix was prepared by diluting the appropriate plasmid with Opti-MEM (Invitrogen) to a concentration of 0.02 µg/µl; Fugene HD (Roche) was added to the same mixture in the ratio of 7:2 (Fugene HD in µl:plasmid DNA in µg), pulse vortexed for 2 seconds and left for 20 min at room temperature.

In case of dual transfection, the test plasmid (expressing firefly luciferase) was mixed with control plasmid (green fluorescence protein (GFP) reporter or SV40-Renilla luciferase constructs) at a ratio of 9:1 in Opti-MEM for a final concentration of 0.02 µg/µl. Following incubation, 5 µl of this mixture were used to transfect the cells without changing the media. The wells were returned to the incubator for a further 24 hr.

2.12.3. Day 3: Treatment and interferon gamma stimulation

The medium was aspirated and replaced with fresh medium containing the appropriate treatment as in described in Section 2.4 and incubated for a further 6-24 hr depending on the predetermined optimum time for the test reporter activity response.

2.12.4. Measuring luciferase or green florescense activity

Luciferase activity was measured using Dual-Luciferase® reporter assay system (Promega). Medium was aspirated from all wells and cells were washed once with PBS followed by addition of 20 µl of passive lysis buffer (PLB). The plates were placed on an

orbital shaker with gentle shaking to ensure complete and even coverage of the cell monolayer with PLB at room temperature for 15 min. Ten microliters lysates from each well were transferred to a new 96-well opaque white plate and mixed with 50 µl luciferase assay reagent II (LARII), previously prepared by re-suspending the provided lyophilized luciferase assay substrate in 10 ml of the supplied luciferase assay buffer II. Only 5 wells were transferred at a time and LARII was added to each well at 10-second intervals to compensate for the lag time inherent by the luminometer (Fluoroskan Ascent Fl, Labsystems). Stop & Glo® Substrate (Promega) was used to measure the Renilla luciferase activity by adding 50 µl of substrate, prepared immediately before the assay by adding 1 volume of 50 x Stop & Glo® Substrate to 50 volumes of Stop & Glo® buffer in an eppendorf tube. The Stop & Glo® substrate was added to each well at 10 second intervals and luciferase activity was measured at the same wavelength. When the GFP reporter construct was used to control for transfection efficiency, the remaining 10 µl of lysates were transferred to a new 384-well black plate and fluorescence activity was measured using (Fluoroskan Ascent Fl, Labsystems) with fluorescence filter (excitation 475-495 nm, and emission 520- 560 nm).

2.12.5. Data analysis and interpretation

Background was subtracted by measuring the luciferase or GFP activity from cells, which were either non-transfected or transfected with a mock vector. Transfection efficiency was expressed as a ratio of the test plasmid over the control plasmid.

2.13. Site directed mutagenesis study

2.13.1. Generation of deletion constructs in promoter IV of class II transactivator.

The anticipated ERE binding sites in pIV CIITA were predicted using three different computer software programs; http://tfbind.hgc.jp/, http://alggen.lsi.upc.es/, http://www.cbrc.jp/index.eng.html. Different sets of deletion mutants of pIV CIITA were generated by site-directed mutagenesis using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Mutagenic primers were designed with Agilent's web-based QuikChange Primer Design Program (*www.genomics.agilent.com*.) and are listed in Table 2.10

2.13.2. Amplification of the mutant construct

PCR reaction master mix was prepared in 0.2 ml thin walled polypropylene PCR tubes as follows: 1x QuikChange lightning reaction buffer, 25 ng pIV CIITA dsDNA template; 125 ng of each oligonucleotide primer; 1 µl dNTP's, 1.5 µl QuikSolution reagent, UltraPure™ DNase/RNase-Free distilled water to a final volume of 50 µl followed by adding 1 µl of QuikChange Lightning Enzyme. Next, PCR was amplified by placing PCR microtubes into Biometra T-Gradient cycler. The thermal cycler protocol included initial 2 min at 95^{o}C, followed by 18 cycles of 20 seconds at 95^{o}C, 10 seconds at 60°C, 30 seconds at 68°C and 5 min at 68^{o}C. Following PCR amplification, 2 µl of the provided Dpn I restriction enzyme was added directly to each amplification reaction, mixed by pipetting the solution up and down several times and the reaction mixture was spun down immediately and incubated at 37°C for 5 min to digest the parental, non-mutated, supercoiled dsDNA.

Table 2.10 List of mutagenic primers designed with Agilent's web-based QuikChange Primer Design Program.

Site 1	Original template	ctcaacctctctttgtctctgggtgggtccccacccctg
Primers	Del -328/-324 Fw	5'-ttggagagaaacagcacccaggggtggg-3'
	Del -328/-324 Rv	5'-cccacccctgggtgctgtttctctccaa-3'
Site 2	Original template	gacgttgagtcctgaacgtctagtgaacgggttcaccgaggga
Primers	Del -280/-276 Fw	5'-caactcaggacttgcacttgcccaagtggctc-3'
	Del -280/-276 Rv	5'-gagccacttgggcaagtgcaagtcctgagttg-3'
Site 3	Original template	agaggggcttcaccccgaccggtgacactccttggctgacctccgtccctg
Primers	Del -209/-191 Fw	5'-ccccgaagtgggggactggaggcagg-3'
	Del -209/-191 Rv	5'-cctgcctccagtcccccacttcgggg-3'
Site 4	Original template	cttgacgcccctccgcccctccatcctactggtcgcctgctcgacggtgt
Primers	Del -33/-14 Fw	5'-ctgcggggaggcggacgagctgcc-3'
	Del -33/-14 Rv	5'-ggcagctcgtccgcctccccgcag-3'

2.13.3. Transformation of XL10-Gold ultracompetent cells

XL10-Gold ultracompetent cells were thawed on ice and 45 µl of cells was transferred to a pre-chilled 14-ml BD Falcon polypropylene round-bottom tube. Two microliters of the provided β-ME mix were added and the tube was swirled gently, followed by 2 min incubation on ice. Cells were transfected with 2 µl Dpn I-treated DNA and bacterial transformation was done as described in Section 2.10.2.2.

2.14. Silencing estrogen receptor alpha by small interfering RNA

2.14.1. Cell plating

BCCL were seeded in a 6-well tissue culture plate at a density of 3 x 10^5 cells/well in 2 ml culture medium with no antibiotic, as antibiotics decrease sensitivity of transfection, and incubated at 37°C with 5% CO_2 overnight. Each experiment included untreated cells, positive control siRNA (targeting a GAPDH gene), negative control small interfering RNA (siRNA) (non-targeting or scrambled) and *ESR1* siRNA.

2.14.2. Transfection

Transfection conditions were established using Thermo Scientific DharmaFECT transfection reagents (Thermo Scientific Dharmacon RNAi technology) according to the manufacturer's protocol. On-TARGETplus SMARTpool, siRNA was supplied as a lyophilised powder at 5 nmol concentration. A stock of 20 µM siRNA was prepared by re-suspending the siRNA into 250 µl of siRNA buffer (prepared by mixing four volumes of sterile RNase-free water with one volume of 5X siRNA buffer (300 mM KCl, 30 mM HEPES-pH 7.5, 1.0 mM $MgCl_2$)) which was aliquoted and frozen at -80^{o}C. At the time of

the experiment one aliquot was thawed and pre-diluted with siRNA buffer to final concentration of 5 μM. Transfection-complex was prepared for each transfection by prediluting 10 μl of siRNA (5 μM) into 190 μl DharmaFECT cell culture reagent (DCCR). In a separate tube, 4 μl of DharmaFECT reagent was prediluted in 196 μl of DCCR tube and left incubating at room temperature for 5 min. Following the incubation period, the contents of the two tubes were mixed together in a 15 ml conical tissue culture tube and left for a further 20 min at room temperature. Next, 1.6 ml of MEM medium without antibiotic was added to transfection complex, mixed well and used to transfect the cells. The transfected cells were incubated at 37°C with 5% CO_2 for 24 hr. Following the incubation time, the medium was aspirated and replaced with fresh medium containing the appropriate treatment as in described in Section 2.4 and incubated for a further 4-24 hr depending on the type of assay.

2.15. Transient transfection of estrogen receptor alpha gene

MDA-MB-231 and SK-BR-3 were seeded in 10 cm plates at 3 x 10^6 cells/plate and left to adhere overnight. Transfection was done exactly as previously described in Section 2.11.2 and left for 72 hr.

2.16. Viability testing using crystal violet staining

BCCL were seeded in 96-well plate at a density of 2 x 10^4 cells/well and left to adhere overnight. In the next day medium was replaced with medium containing the appropriate treatment, using three replicate wells for each treatment and incubated for the indicated time, depending on the experiment. Medium was then aspirated and cells were

washed once with PBS and cell viability was established using crystal violet staining. One hundred microliters of crystal violet (0.5% crystal violet in 10% neutral buffered formalin (VWR-EMD) were added for 30 min at room temperature. At the end of incubation period, crystal violet was aspirated, the plate was washed under tap water, blotted on filter paper and left to dry for 5 min at room temperature. The remaining crystal violet, which represents viable cells, was detected by dissolving it in 100 µl of 30% acetic acid in water. The plate was shaken for 1 min and the absorbance read 595 nm on an ELISA reader (Bio-Rad model 3550).

2.17. Statistical analysis

Analysis was performed using Microsoft Excel 2010 software. Comparison within groups (different cell lines with different treatment) was analyzed using one-way analysis of variance (ANOVA) and Tukey post hoc. Student t-test was used for comparing of two different treatments. All tests were two-sided and difference between groups were considered significant if $p<0.05$. In the text the average of three experiments is reported as mean $\pm$ standard errors of the mean (SEM).

Chapter 3: Human leukocyte antigen class II is differentially expressed in estrogen receptor alpha positive and estrogen receptor alpha negative breast cancer cell lines with or without estradiol treatment.

3.1. Rationale and objectives

Human leukocyte antigen (HLA) class II molecules are not usually expressed on normal mammary epithelium; however, they are upregulated in lactating breast cells, most likely due to prolactin (302). HLA class II expression in breast cancer cell lines (BCCL) is modulated by cytokines such as interleukin 1 alpha (IL1α), tumor necrosis factor alpha (TNFα), interferon gamma (IFN-γ) (303), and by hormones such as estradiol (E_2) (304). Previous work in our laboratory, arising from Sharon Oldford's study on breast cancer tissues, showed that HLA-DR expression on breast carcinoma in situ is significantly associated with reduced estrogen receptor (ER)α, reduced progesterone receptor, reduced age at diagnosis, and increased levels of IFN-γ mRNA (106). Altogether these findings suggest a role of cytokines, hormones and their receptors in modulation of HLA class II expression in breast cancer cells.

1. To compare HLA class II expression in $ER\alpha^+$ and $ER\alpha^-$ BCCL with or without IFN-γ.
2. To determine if HLA class II is modulated by physiological concentrations of E_2 treatment in $ER\alpha^+$ and $ER\alpha^-$ BCCL.
3. To study the role of ERα on HLA class II expression in a BCCL model.

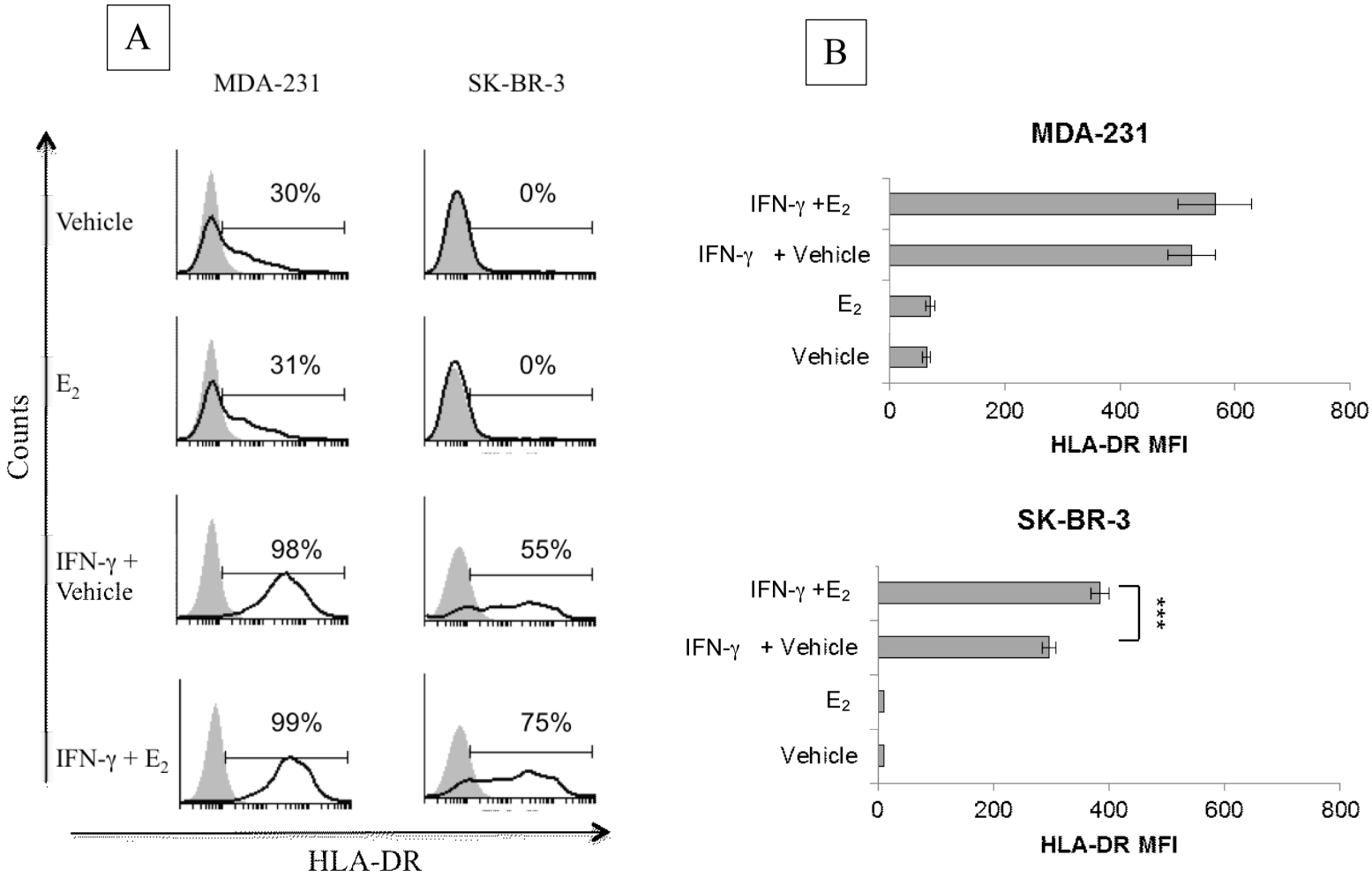
3.2. Estradiol enhances HLA-DR expression in ERα⁻ breast cancer cell lines

To determine whether estrogen, in the absence of its cognate receptor ERα modulates the HLA class II pathway, we analyzed constitutive and IFN-γ inducible HLA-DR in established ERα⁻ BCCL, MDA-MB-231, and SK-BR-3. The cells were grown in an estrogen-depleted medium and treated with E_2 and stimulated, or not, with IFN-γ. HLA-DR expression was measured using L243 antibody (pan HLA-DR) and analysed by surface flow cytometry.

The results for MDA-MB-231 differed from SK-BR-3 as MDA-MB-231 constitutively expressed, albeit weakly, HLA-DR with 30% of cells positive for L243 (Figure 3.1A) and an average mean fluorescence intensity (MFI) of 60 (Figure 3.1B). IFN-γ strongly upregulated HLA-DR in both cell lines, with 98% and 55% of the cells, respectively positive for L243 (Figure 3.1A) and an average MFI of 543 and 390 (Figure 3.1B).

To confirm flow cytometry results, immunoblots were performed on cytoplasmic extracts from the same cells treated as previously described. The relative expression was determined by measuring the density of each band in the form of pixels and expressed as a ratio of the band density for the loading control α tubulin (Figure 3.1D). No constitutive HLA-DR expression was detected in both cell lines using the HLA-DRα antibody (TAL 1B5); however, IFN-γ strongly induced HLA-DR (Figure 3.1C). E_2 significantly augmented IFN-γ-induced HLA-DR expression in SK-BR-3 ($p<0.001$), and resulted in negligible increase in MDA-MB-231 as detected by flow cytometry and immunoblotting.

Figure 3.1 Estradiol enhances HLA-DR expression in ERα$^-$ breast cancer cell lines. MDA-MB-231 and SK-BR-3 were treated with vehicle (ethanol) or E_2 (10^{-9} M) and stimulated or not with IFN-γ (100 Units/ml) for 96 hr. (A) HLA-DR surface expression (detected by L243) was analysed by flow cytometry. Shaded histogram = isotype control, black line = HLA-DR expression with the indicated % of cells (B) Bar graphs represent the average MFI for HLA-DR expression from three independent experiments. (C) HLA-DRα expression (detected by TAL 1B5) was analysed by immunoblotting from a cytoplasmic cell extract. (D) HLA-DRα levels were normalized to α tubulin and the average band intensity after normalization is presented in the bar graph. Error bars represent the ± standard errors of the mean (SEM) of three independent experiments. (***p value <0.001).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MDA-231
SK-BR-3
Counts
Vehicle
30%
0%
E2
31%
0%
IFN-γ +
Vehicle
98%
55%
IFN-γ + E2
99%
75%
HLA-DR
B
MDA-231
IFN-γ +E2
IFN-γ + Vehicle
E2
Vehicle
0
200
400
600
800
HLA-DR MFI
SK-BR-3
IFN-γ +E2
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IFN-γ + Vehicle
E2
Vehicle
0
200
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600
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HLA-DR MFI

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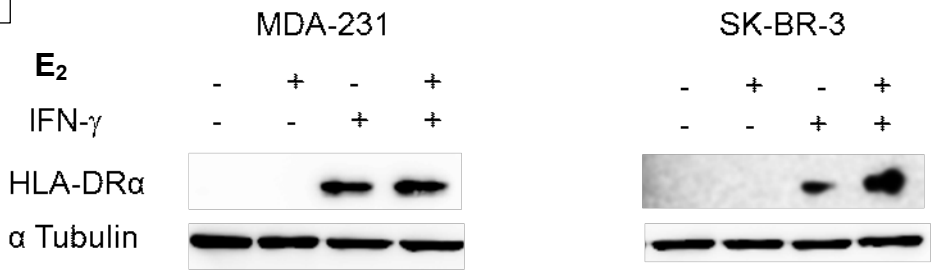
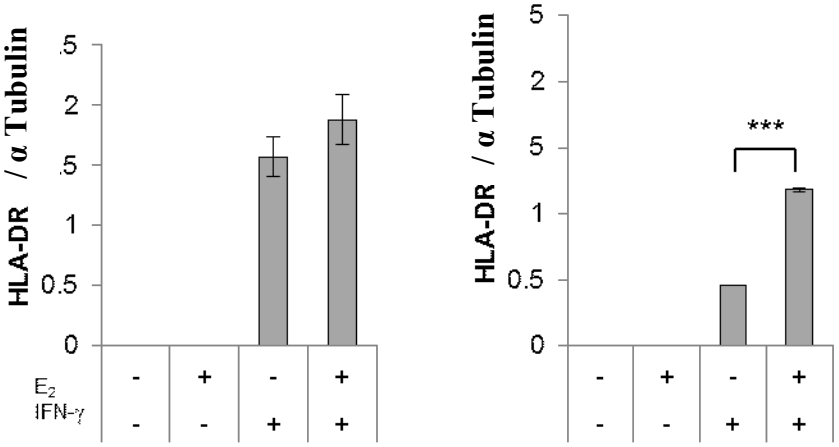

MDA-231
SK-BR-3
E2
IFN-γ
HLA-DRα
α Tubulin

D


HLA-DR / α Tubulin
5
2
1
0.5
0
E2
IFN-γ
***

3.3. Estradiol differentially modulates HLA-DR in ERα$^+$ breast cancer cell lines

Although estrogen can activate other signaling pathways (277), it acts primarily through ERα (235). Since the least HLA-DR expression in human breast carcinoma tissues was observed for ERα$^+$ tumors (106), we hypothesized that E_2-activation of the ER pathway would inhibit HLA-DR expression. To test this, we used established ERα$^+$ BCCL, MCF-7, BT-474, & T47D, which were treated as described above for the ERα$^-$ cell lines. Analysis of HLA-DR expression was done as previously described.

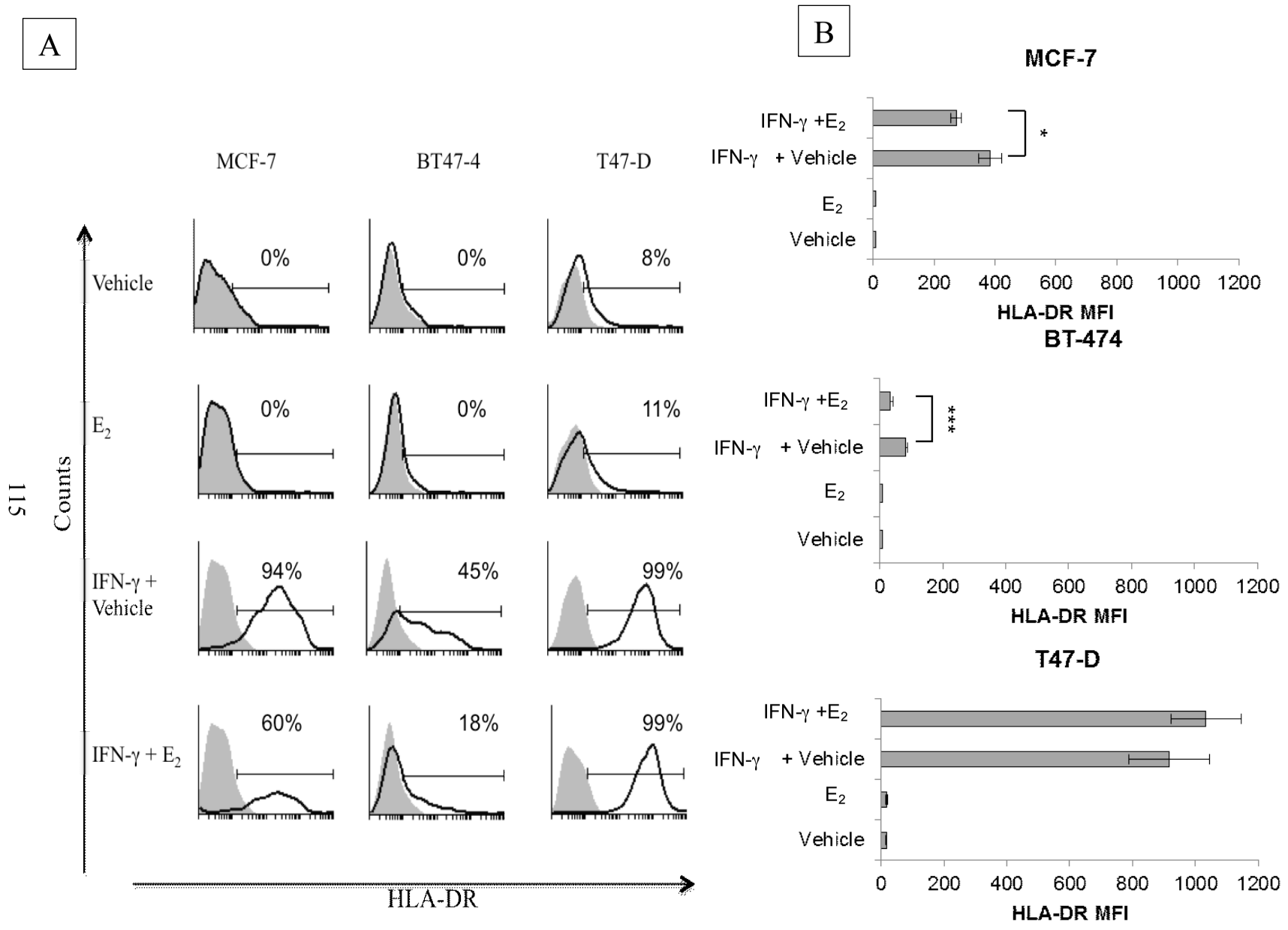
The flow cytometry results (Figure 3.2A-B) show that T47D, but not MCF-7 or BT-474 expressed very low levels of constitutive HLA-DR, with a small number of positive cells (8%), (Figure 3.2A) and an average MFI of 17 (Figure 3.2B). E_2-treatment had no detectable effect on constitutive HLA-DR expression. IFN-γ inducible HLA-DR varied substantially among the cell lines. Although more than 90% of cells were HLA-DR positive in both MCF-7 and T47D (Figure 3.2A), the MFI varied markedly with MFI of 900 in T47D compared to MFI of 380 in MCF-7 (Figure 3.2B), suggesting that T47D expresses more surface HLA-DR molecules. E_2 significantly reduced IFN-γ-induced HLA-DR expression in MCF-7 ($p<0.05$) and BT474 ($p<0.001$). On the other hand, E_2 had no significant effect on HLA-DR expression in T47D cell line, suggesting that E_2 downregulation of IFN-γ-induced HLA-DR expression may be cell specific.

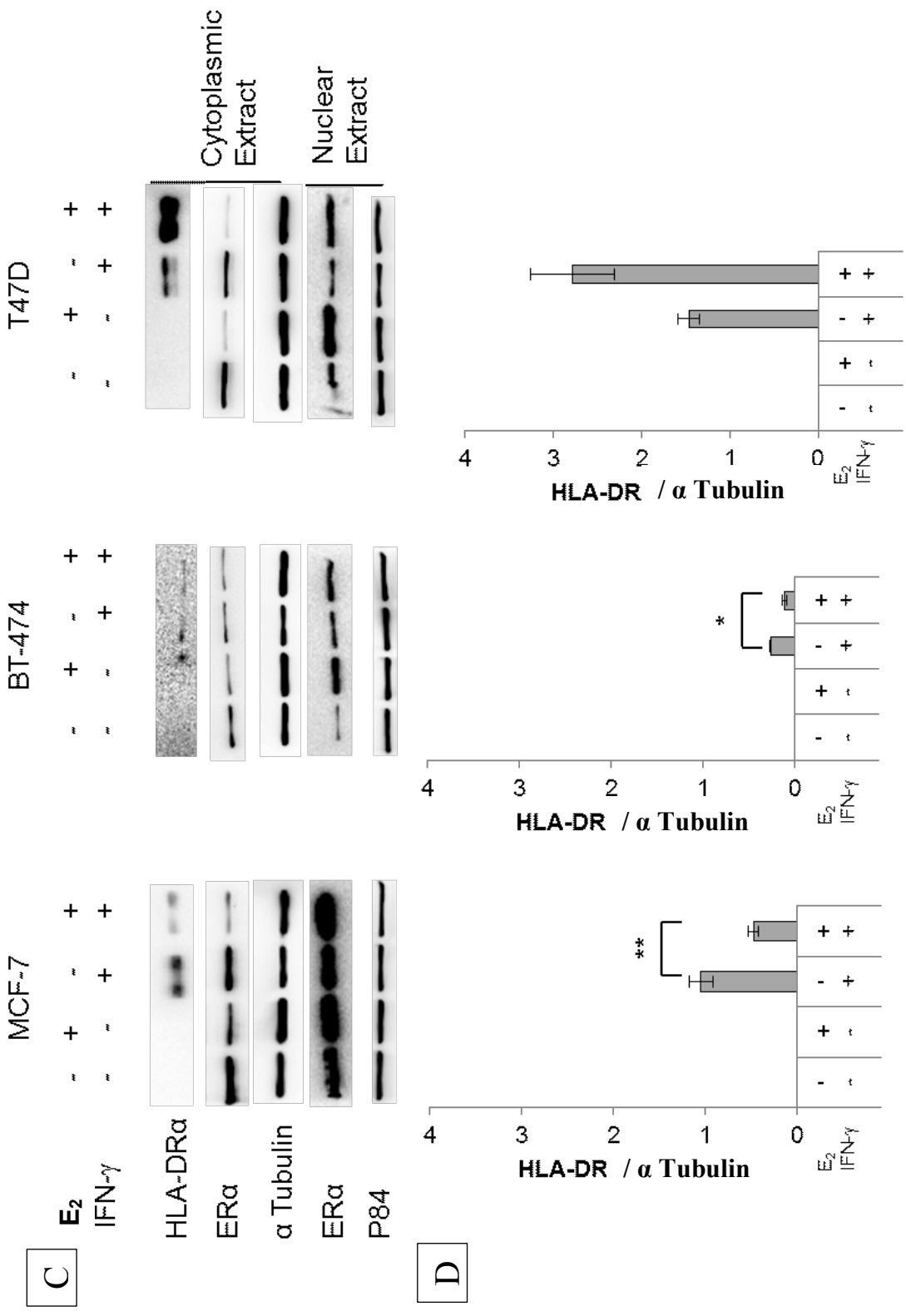
Immunoblot analysis was next performed to confirm the flow cytometry data. The three ERα$^+$ BCCL were treated exactly as described above, followed by preparation of cytoplasmic and nuclear extracts for easier ERα tracking, since hormonal treatment mobilises ERα between the nucleus and cytoplasm. The bands densities were relatively

quantified as previously described for the ERα$^-$ cell lines. HLA-DR basal expression was not detected in either of three ERα$^+$ BCCL cells; however, IFN-γ strongly induced HLA-DR (Figure 3.2C) in MCF-7 and T47D. HLA-DR was barely detected in BT-474, and thus the membrane was overexposed in order to detect the signal. Similar to the flow cytometry results, E_2-treatment inhibited HLA-DR expression in MCF-7 and BT-474, but variably increased HLA-DR in T47D. Analysis of ERα expression in cytoplasmic and nuclear extracts showed no significant effect of IFN-γ treatment on ERα levels in either cells (Figure 3.2C). As expected, E_2-treatment significantly decreased cytoplasmic, and increased nuclear ERα in the three cell lines, indicating ligand activation of the ER pathway. E_2 significantly attenuated the IFN-γ-induced HLA-DR expression in MCF-7 ($p<0.01$) and in BT474 ($p<0.05$), but no statistically significant change was seen by E_2 on IFN-γ-induced HLA-DR expression in T47D (Figure 3.2D). Thus, immunoblot analysis confirmed the results found by flow cytometry.

Overall, E_2 augmented HLA-DR expression in ERα$^-$ cell lines, while downregulating HLA-DR in two out of three ERα$^+$ cell lines. These results are consistent with the previous *in vivo* study (106), which found higher HLA-DR expression in ERα$^-$ tumors from younger women with breast cancer. This suggests that estrogen in the absence of ERα has a modulatory effect on regulation of the HLA class II pathway. Moreover, our *in vitro* data suggest that down modulatory effects of E_2 on HLA class II expression is likely dependent on ERα.

Figure 3.2 Estradiol differentially modulates HLA-DR in ERα^{+} breast cancer cell lines MCF-7, BT-474, and T47D were treated with vehicle (ethanol) or E_2 (10^{-9}M) and stimulated or not with IFN-γ (100 Units/ml) for 96 hr. (A) HLA-DR surface expression (detected by L243) was analysed by flow cytometry. Shaded histogram = isotype control, black line = HLA-DR expression with the indicated % of cells (B) Bar graphs represent the average MFI for HLA-DR expression from three independent experiments. (C) Immunoblot analysis was performed on cytoplasmic and nuclear extracts for ERα expression (HC-20) and on cytoplasmic extracts for HLA-DRα (TAL 1B5). (D) HLA-DRα levels were normalized to α tubulin and the average band intensity after normalization is presented in the bar graph. Error bars represent ± SEM of three independent experiments (*$p<0.05$, **$p<0.01$ and ***$p<0.001$).


A
MCF-7
BT47-4
T47-D
Vehicle
E2
IFN-γ + Vehicle
IFN-γ + E2
0%
0%
8%
0%
0%
11%
94%
45%
99%
60%
18%
99%
Counts
HLA-DR
B
MCF-7
IFN-γ +E2
IFN-γ + Vehicle
E2
Vehicle
*
0
200
400
600
800
1000
1200
HLA-DR MFI
BT-474
IFN-γ +E2
IFN-γ + Vehicle
E2
Vehicle
***
0
200
400
600
800
1000
1200
HLA-DR MFI
T47-D
IFN-γ +E2
IFN-γ + Vehicle
E2
Vehicle
0
200
400
600
800
1000
1200
HLA-DR MFI


C
D
E2
IFN-γ
MCF-7
BT-474
T47D
Cytoplasmic Extract
Nuclear Extract
HLA-DRα
ERα
α Tubulin
ERα
P84
HLA-DR / α Tubulin
**
*
4
3
2
1
0

3.4. Transfection of ERα gene in an ERα⁻ cell line decreases IFN-γ inducible HLA-DR proteins

To further define the role of ERα on IFN-γ inducible HLA-DR expression, two stable transfectants, derived from MDA-MB-231 clone10A were used: MC2 expresses wild type ERα and VC5 expresses the empty vector. The cells were grown in an estrogen-depleted medium and stimulated with IFN-γ or left untreated. HLA-DR expression was measured using L243 antibody and analysed by surface flow cytometry.

As shown in Figure 3.3A-B, IFN-γ upregulated HLA-DR expression in all three cells. Interestingly, inducible HLA-DR as measured by MFI was significantly decreased in VC5, compared to the parent cell line, MDA-MB-231, even though the percentage of HLA-DR positive cells was the same for both. This suggests that the empty plasmid is interfering with HLA-DR expression. Transfecting the ERα gene into the ERα⁻ BCCL resulted in drastically reduced HLA-DR expression in MC2 when compared to VC5 and MDA-MB-231 clone 10A. Taken together, these results suggest that expression of ERα, even in the absence of E_2, downregulates HLA-DR expression in breast cancer cells.

To ensure that diminished IFN-γ-induced HLA-DR expression in MC2 was reproducible by alternative techniques, immunocytochemistry (ICC) and immunoblotting were used to evaluate IFN-γ inducible HLA-DR expression in MC2, VC5 and MDA-MB-231. The advantage of ICC is that it will detect differential localisation of the HLA-DR expression in these cells. As shown in Figure 3.3C, the cells were clearly positive for cytoplasmic and membranous staining of HLA-DR. Membrane and cytoplasmic HLA-DR staining were detected in MC2, but the percentage of positive cells was only 30%,

compared to 70% in MDA and VC5. Interestingly, HLA-DR positive cells showed large granules distributed in the cytoplasm in all three cells examined, which were not present in the normal B cell control, SAVC.

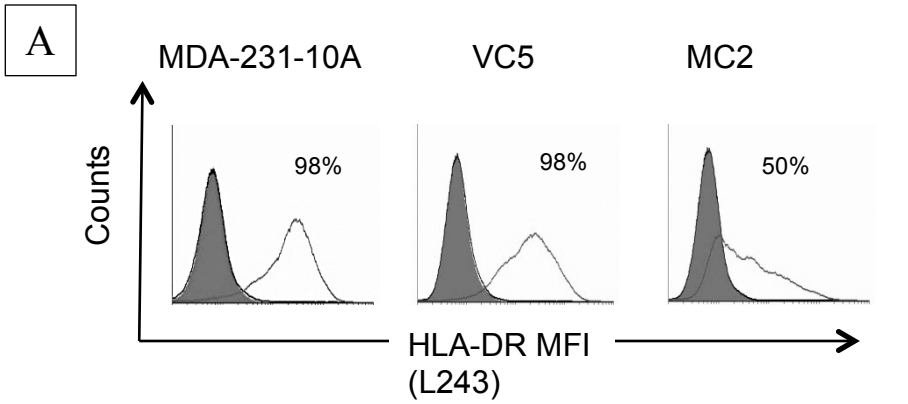
Immunoblotting of cell lysates, confirmed reduced HLA-DR in MC2 as compared to VC5 and MDA (Figure 3.3D). Since expression of ERα varies with different culture conditions (305, 306), we examined ERα in the same lysates to ensure its expression in estrogen-depleted conditions. (In this experiment, we examined ERα expression in the whole cell lysates but later (Section 4.4) cellular localisation of ERα was determined). The observation ERα was strongly expressed in MC2 while HLA-DR expression was dramatically reduced, added support to our hypothesis that ERα modulates HLA-DR expression.

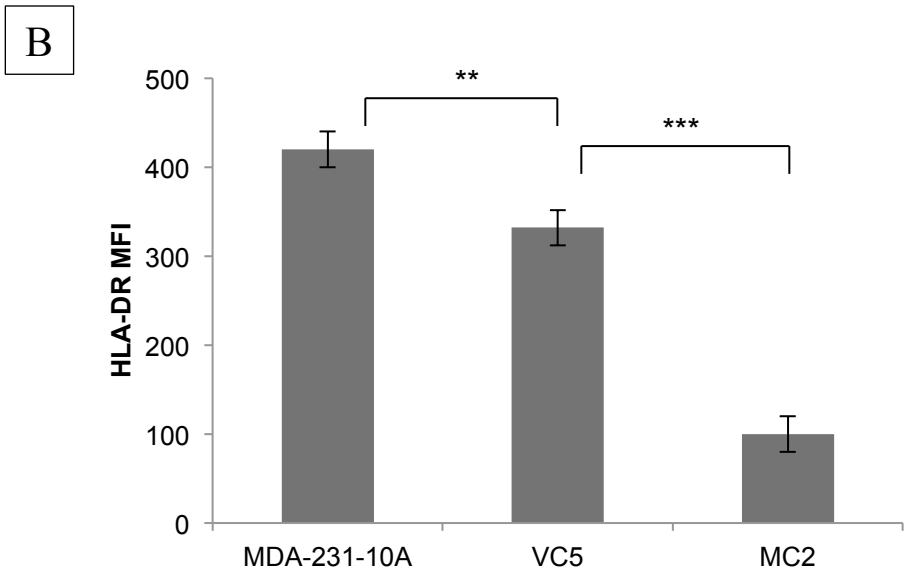
3.5.Estradiol downregulates HLA-DR expression in MC2 in a dose dependent manner

We next examined the effect of ligand activation of ERα on HLA-DR expression. The physiological level of estrogen varies during the lifetime of females (307). The highest levels of estradiol, nearly 10^{-6} M, are reached during pregnancy. Intermediate levels, ranging from 10^{-9} M to 10^{-8} M, are found during normal reproductive age and vary with the menstrual cycle. The lowest levels, ranging from 10^{-12} M to 10^{-11} M, occur during menopause, which is also variable, ranging from 40-61 years (307). To study the effect of physiological levels of estradiol on HLA-DR expression in breast cancer cells, VC5 and MC2 breast cancer cells were treated with exogenous E_2 ranging from 10^{-6}M-10^{-12}M and IFN-γ for 96 hr. HLA-DR surface expression was then examined by flow cytometry.

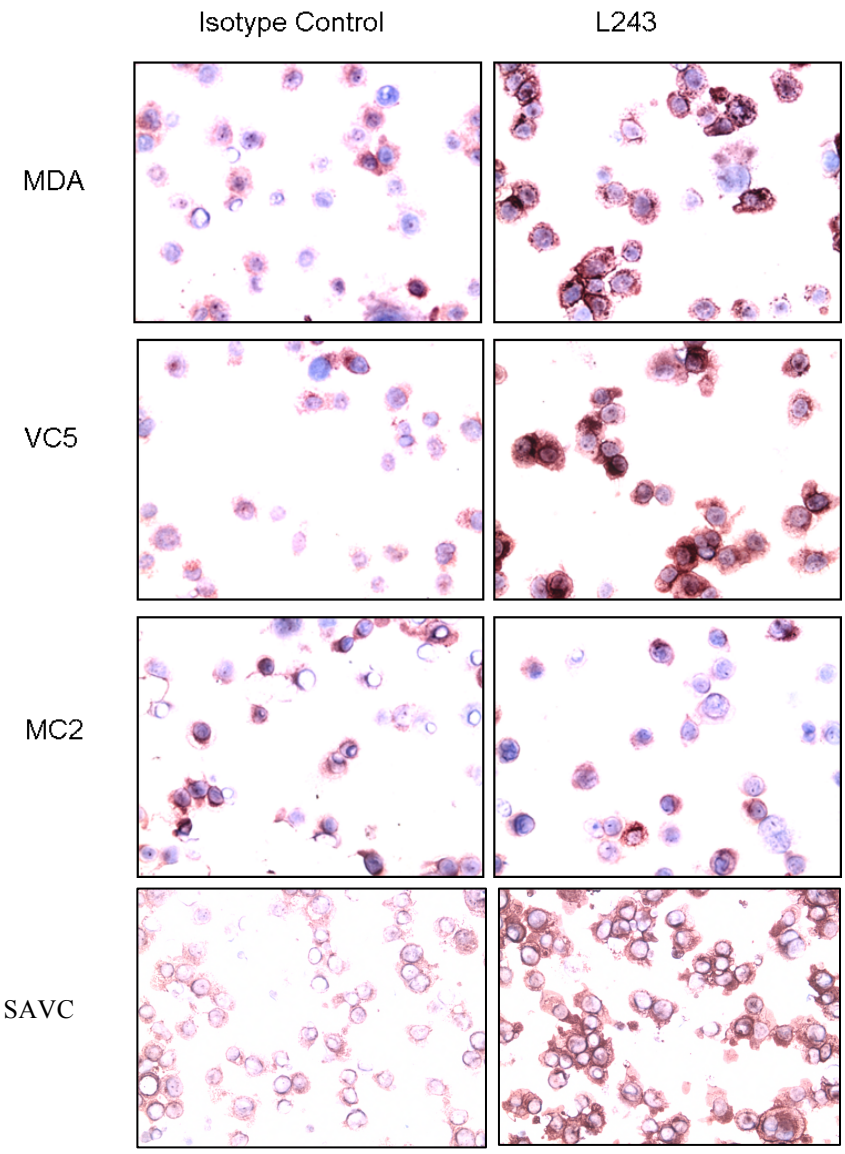
Figure 3.3 Differential expression of HLA-DR in the breast cancer cell line model.

MDA-MB-231 clone 10 A, VC5 and MC2 were cultured either in estrogen-depleted medium and stimulated with IFN-γ (100 Units/ml) for 96 hr. (A) HLA-DR cell surface expression (L243) was analysed by flow cytometry: shaded histogram, isotype control; black line, HLA-DR expression with the indicated % of cells (B) Bar graphs represent the MFI ± SEM for HLA-DR expression of three independent experiments. (**$p<0.01$, ***$p<0.001$). C) Cytopreps were prepared and acetone fixed, HLA-DR was detected using L243 and visualised by NovaRed stain, nucleus was stained with haematoxylin for counterstain. A. Mostafa, S. Drover and D. Codner read the slides in a blinded manner. Figure represents one experiment. D) Whole cell lysates were prepared using Triton X-100 1% lysis buffer and HLA-DR detected by Tal 1B5 and ER by HC-20. Figure indicates one experiment.

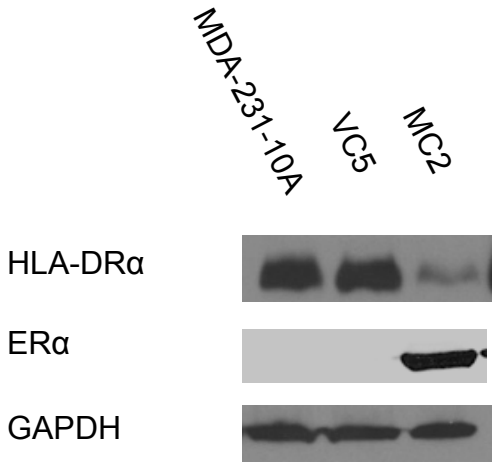

A
MDA-231-10A
VC5
MC2
Counts
98%
98%
50%
HLA-DR MFI
(L243)


B
500
400
300
200
100
0
HLA-DR MFI
**
***
MDA-231-10A
VC5
MC2

C


Isotype Control
L243
MDA
VC5
MC2
SAVC

D


MDA-231-10A
VC5
MC2
HLA-DRα
ERα
GAPDH

As shown in Figure 3.4A, HLA-DR expression in MC2 and the concentration of E_2 were inversely correlated, suggesting that E_2 mediated decreased HLA-DR expression through the E_2-ER signaling pathway. Higher concentrations of E_2 (10^{-6}-10^{-8} M) had no further effect on HLA-DR expression, which implies that ERs were completely saturated at 10^{-9} M E_2. On the contrary, E_2-treatment in VC5 had no significant effect on HLA-DR expression. Based on the results, 10^{-9} M E_2 was selected to investigate the physiological effect of E_2 on HLA class II expression in the BCCL model.

To confirm the E_2-ER-mediated downregulation on HLA-DR, immunoblotting was performed on whole cell lysates from VC5 and MC2 cells. Figure 3.4B shows abundant IFN-γ induced HLA-DR proteins in VC5. Conversely, HLA-DR protein was reduced in the ERα expressing MC2, and further diminished by E_2-treatment. As the only known difference between MC2 and VC5 is the presence of ERα (Figure 3.4B), these results implicate ERα in negatively regulating HLA-DR expression.

Overall, these results are consistent with reduced HLA-DR expression by E_2 in wild type ERα^+ lines, MCF-7 and BT-474 (Figure 3.2).

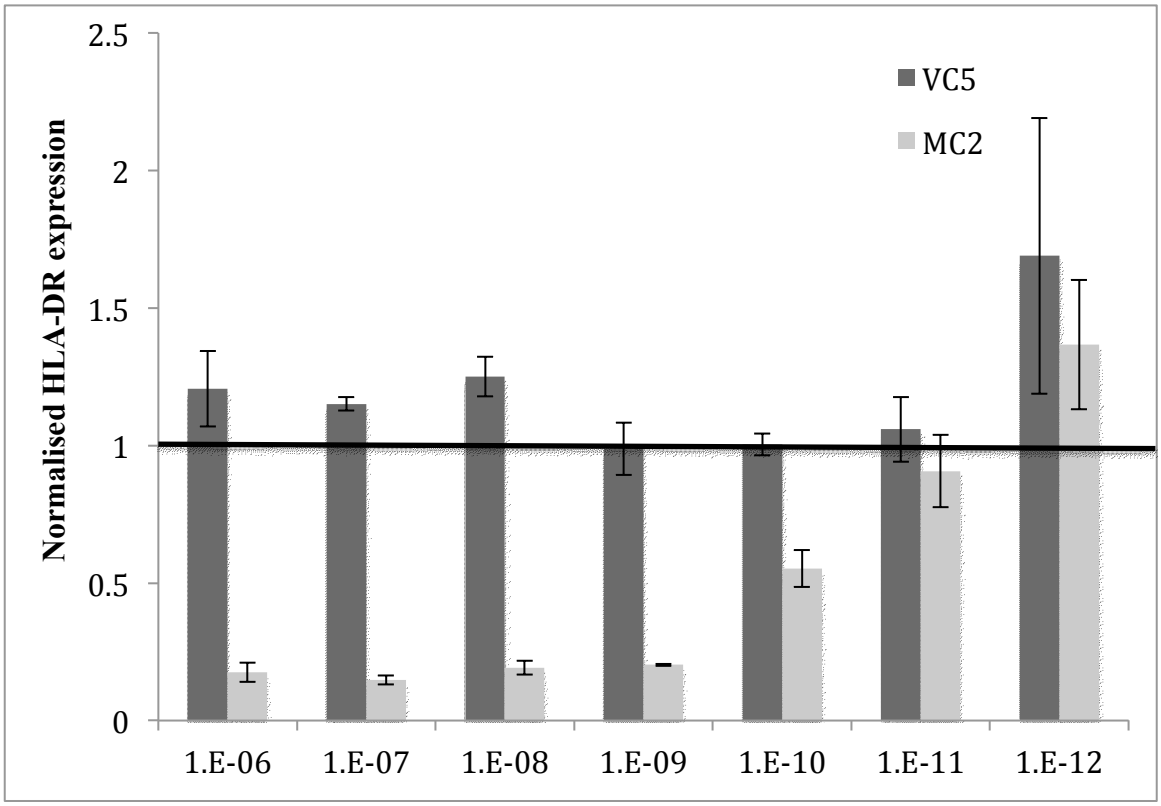
3.6. The classical E_2-ER activation resulted in downregulation of HLA class II proteins

Since HLA class II genes are coordinately regulated, we next tested whether the inhibitory effects of ERα and E_2 on HLA-DR expression extended to the co-chaperones, HLA-DM and Ii. As shown in Figures 3.5A-F, only MDA-MB-231 clone 10A cells constitutively expressed small amounts of HLA-DR, Ii and HLA-DM. Ethanol and E_2 had no effect on expression. IFN-γ treatment upregulated HLA-DR, Ii and HLA-DM in all

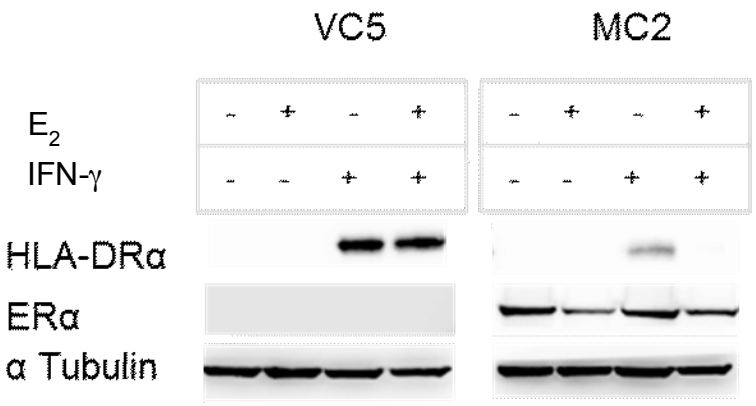
Figure 3.4 E_2-ER signaling mediated downregulation on HLA-DR expression.

(A) MC2 and VC5 were treated with log fold dilutions of E_2 (10^{-6}-10^{-12} M) and stimulated with IFN-γ (100 Units/ml) for 96 hr. HLA-DR surface expression (detected by L243) was analysed by flow cytometry. The data are expressed as the ratio of MFI of E_2-treated cells/MFI ethanol-treated cells ± SEM of three independent experiments. (B) HLA-DRα (detected by TAL 1B5) and ERα (HC-20) expression was analysed by immunoblotting from a whole cell extract. Figure indicates one experiment.

A


Normalised HLA-DR expression
2.5
2
1.5
1
0.5
0
VC5
MC2
1.E-06
1.E-07
1.E-08
1.E-09
1.E-10
1.E-11
1.E-12

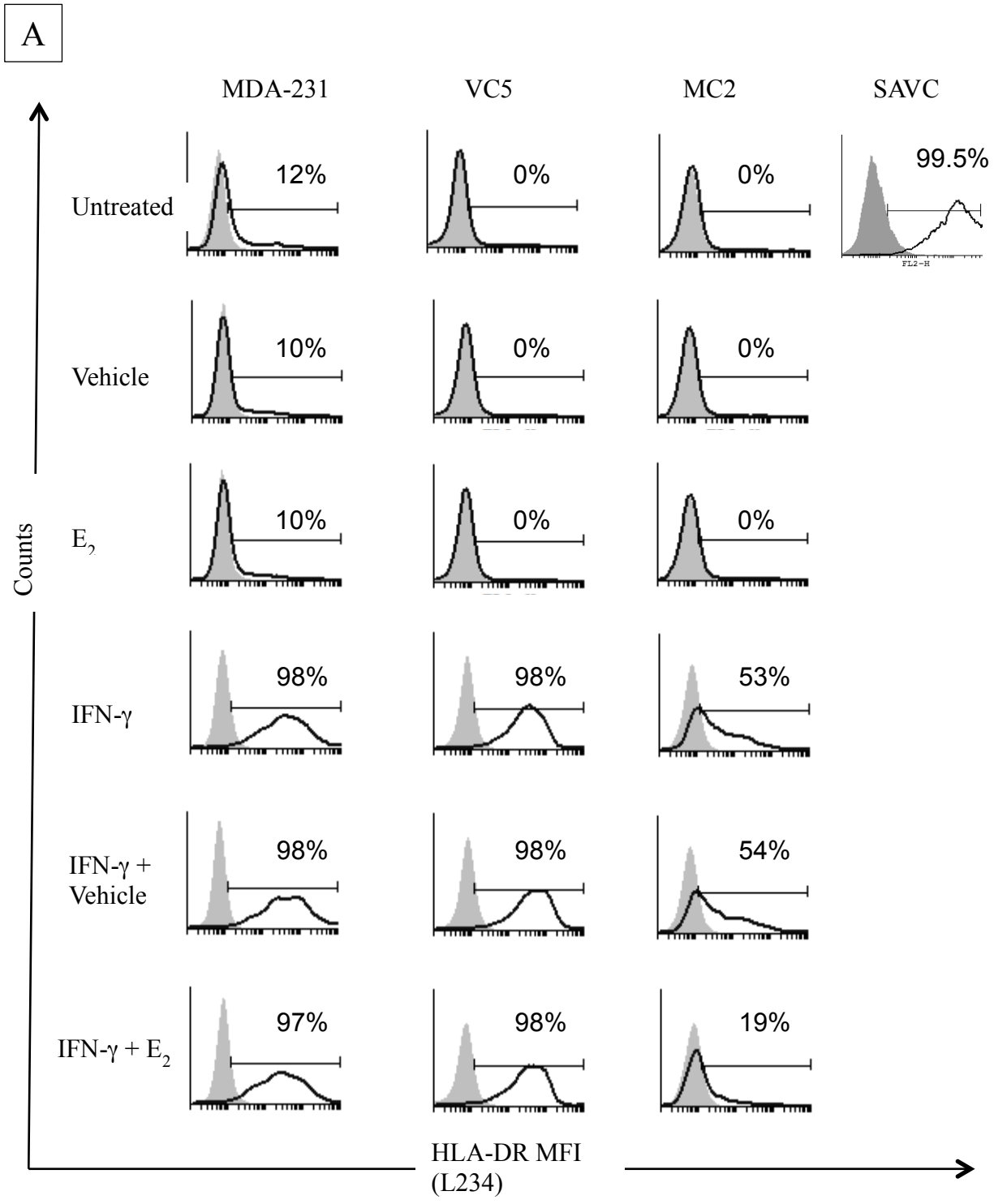
B


VC5
MC2
E2
IFN-γ
HLA-DRα
ERα
α Tubulin

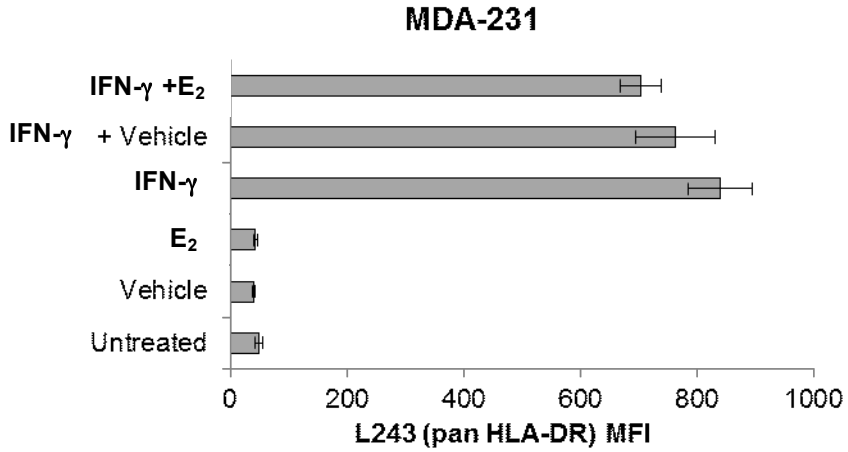
cell lines as indicated by increased percentage of positive cells for HLA-DR, Ii and HLA-DM (Figure 3.5A, C, E) and increased MFI (Figure 3.5B, D, F). E_2 or ethanol treatment had no significant effect on IFN-γ inducible HLA class II expression in either VC5 or MDA MB 231 clone 10A. MC2 significantly expressed less HLA-DR, Ii and HLA-DM ($p<0.001$) compared to VC5 and MDA-MB-231 clone 10A, as detected by low number of positive cells (Figure 3.5A, C, E) and low MFI (Figure 3.5B, D, F). Furthermore, addition of E_2 significantly reduced expression of HLA class II molecules ($p<0.001$) compared to the vehicle control (Figure 3.5A-F), suggesting that E_2-activation of ER signaling further potentiated the downregulation of HLA class II expression and co-chaperones.

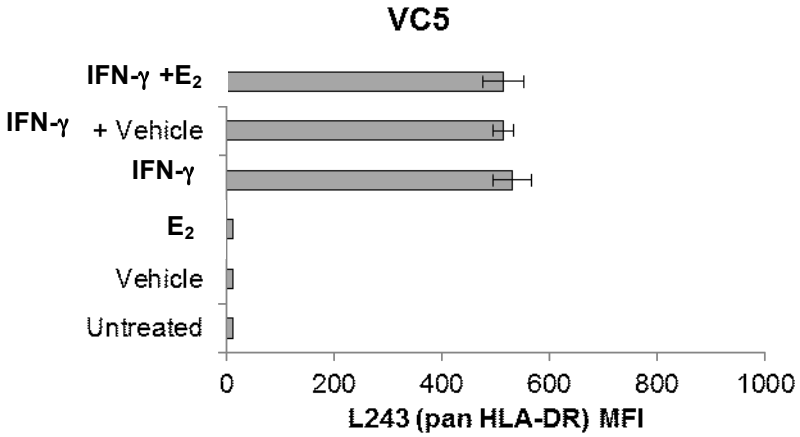
In the absence of HLA-DM expression, most HLA-DR peptide complexes on the cell surface are CLIP peptides with very few antigenic peptides derived from the endocytic pathway (308). IFN-γ induced surface CLIP expression was analyzed by flow cytomtery in the BCCL model. Two B cell lines were used as positive control, SAVC, which expresses the wild type form of HLA-DM, and 9.5.3, which does not express HLA-DM due to mutation of HLA-DMB gene (309). As depicted in Figure 3.6, all BCCL expressed little (if any) CLIP compared to the SAVC and 9.5.3 B cell lines in which 35% and 99% of the cells expressed CLIP, respectively. These results suggest that only a small amount of HLA-DM (Figure 3.5E-F) is required for proper antigen presentation in the non-professional APC, as the presence of low surface expression of CLIP is indicative of functional HLA-DM. On the other hand, the absence of CLIP surface expression in MC2 is probably due to low Ii or HLA-DR expression (Figure 3.5C-D).

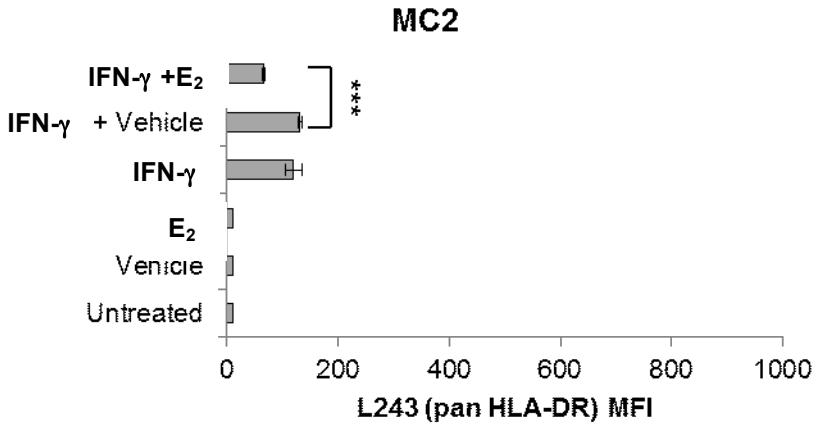
Figure 3.5 E_2-ER signaling causes coordinate downregulation of HLA class II proteins. MDA-MB-231 clone 10, VC5 and MC2 were treated with vehicle (ethanol) or E_2 (10^{-9} M) and stimulated or not with IFN-γ (100 Units/ml) for 96 hr. SAVC (B cell line) was used as positive control. (A-B) HLA-DR surface expression (L243), (C-D) Ii expression (CD74) and (E-F) HLA-DM (DM.1) were analyzed by intracellular flow cytometry. Shaded histogram = isotype control, black line = HLA class II proteins expression with the indicated % of cells. Bar graphs represent the MFI for HLA class II expression from three independent experiments. Error bars represent the ± SEM of three independent experiments. (***$p < 0.001$).


A
MDA-231
VC5
MC2
SAVC
Untreated
Vehicle
E2
IFN-γ
IFN-γ + Vehicle
IFN-γ + E2
12%
0%
0%
99.5%
10%
0%
0%
10%
0%
0%
98%
98%
53%
98%
98%
54%
97%
98%
19%
Counts
HLA-DR MFI (L234)

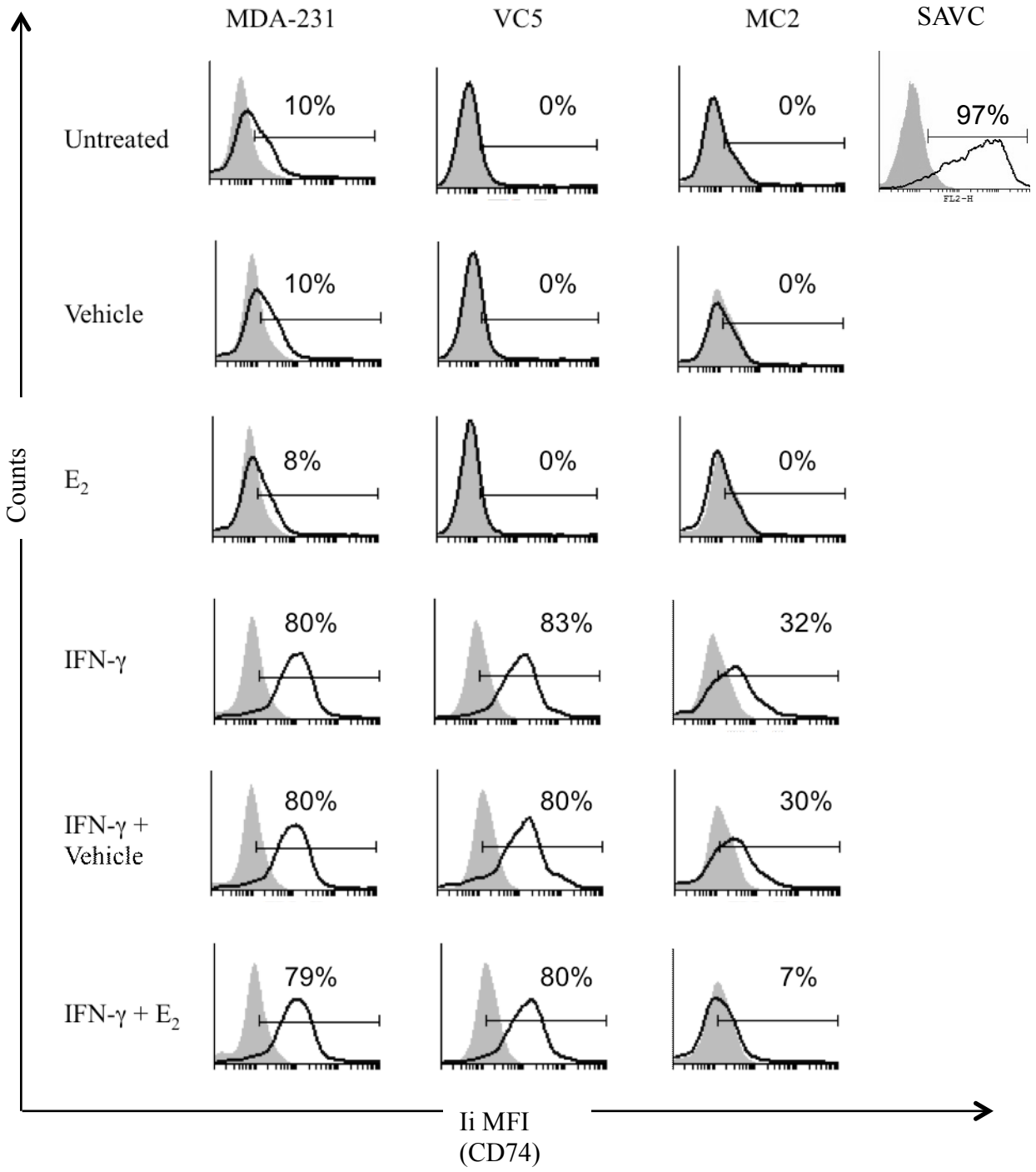
B


MDA-231
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
0
200
400
600
800
1000
L243 (pan HLA-DR) MFI

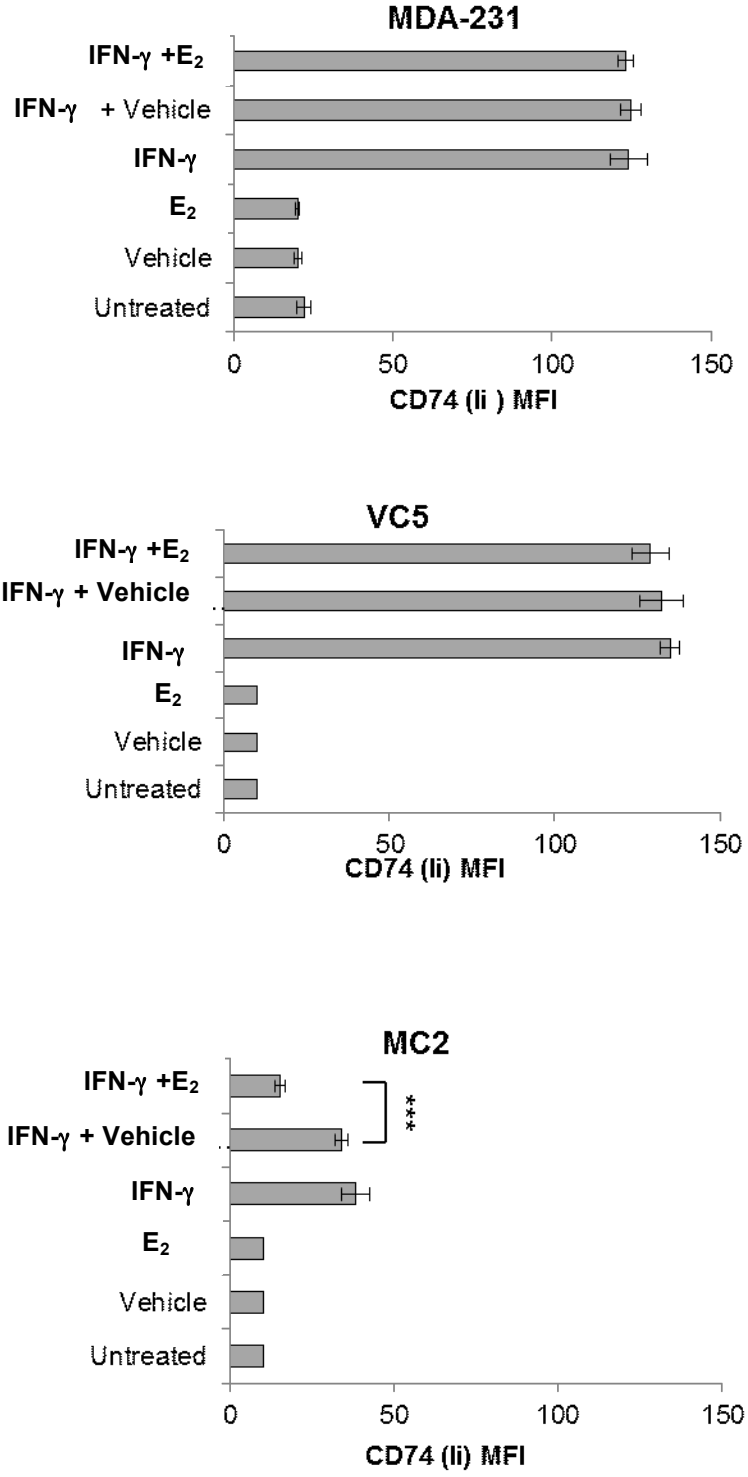

VC5
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
0
200
400
600
800
1000
L243 (pan HLA-DR) MFI

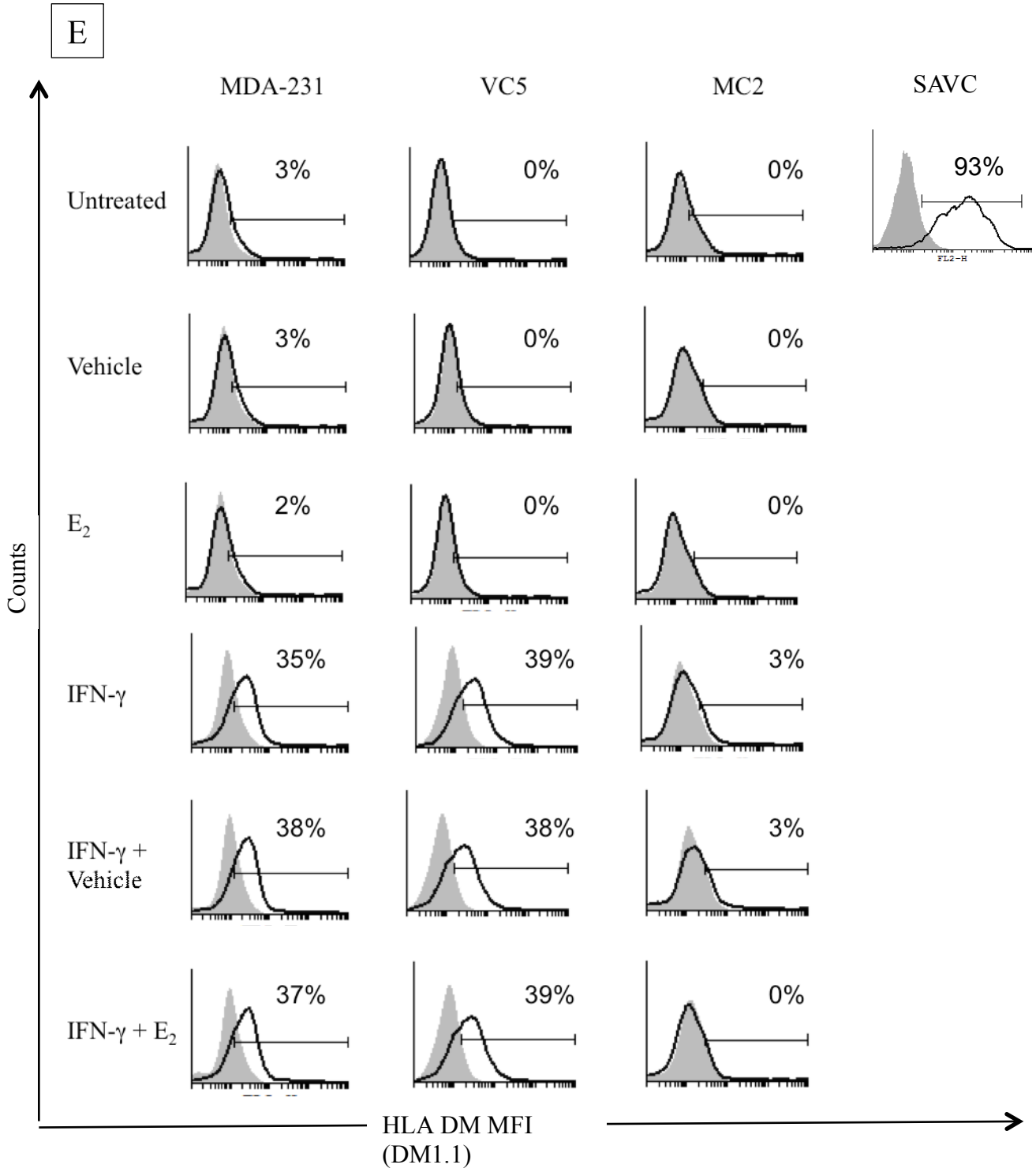

MC2
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
***
0
200
400
600
800
1000
L243 (pan HLA-DR) MFI

C


MDA-231
VC5
MC2
SAVC
Untreated
Vehicle
E2
IFN-γ
IFN-γ + Vehicle
IFN-γ + E2
10%
0%
0%
97%
10%
0%
0%
8%
0%
0%
80%
83%
32%
80%
80%
30%
79%
80%
7%
Counts
Ii MFI (CD74)

D


MDA-231
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
0
50
100
150
CD74 (Ii) MFI
VC5
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
0
50
100
150
CD74 (Ii) MFI
MC2
IFN-γ +E2
IFN-γ + Vehicle
***
IFN-γ
E2
Vehicle
Untreated
0
50
100
150
CD74 (Ii) MFI


E
MDA-231
VC5
MC2
SAVC
Untreated
Vehicle
E2
IFN-γ
IFN-γ + Vehicle
IFN-γ + E2
3%
0%
0%
93%
3%
0%
0%
2%
0%
0%
35%
39%
3%
38%
38%
3%
37%
39%
0%
Counts
HLA DM MFI (DM1.1)

F

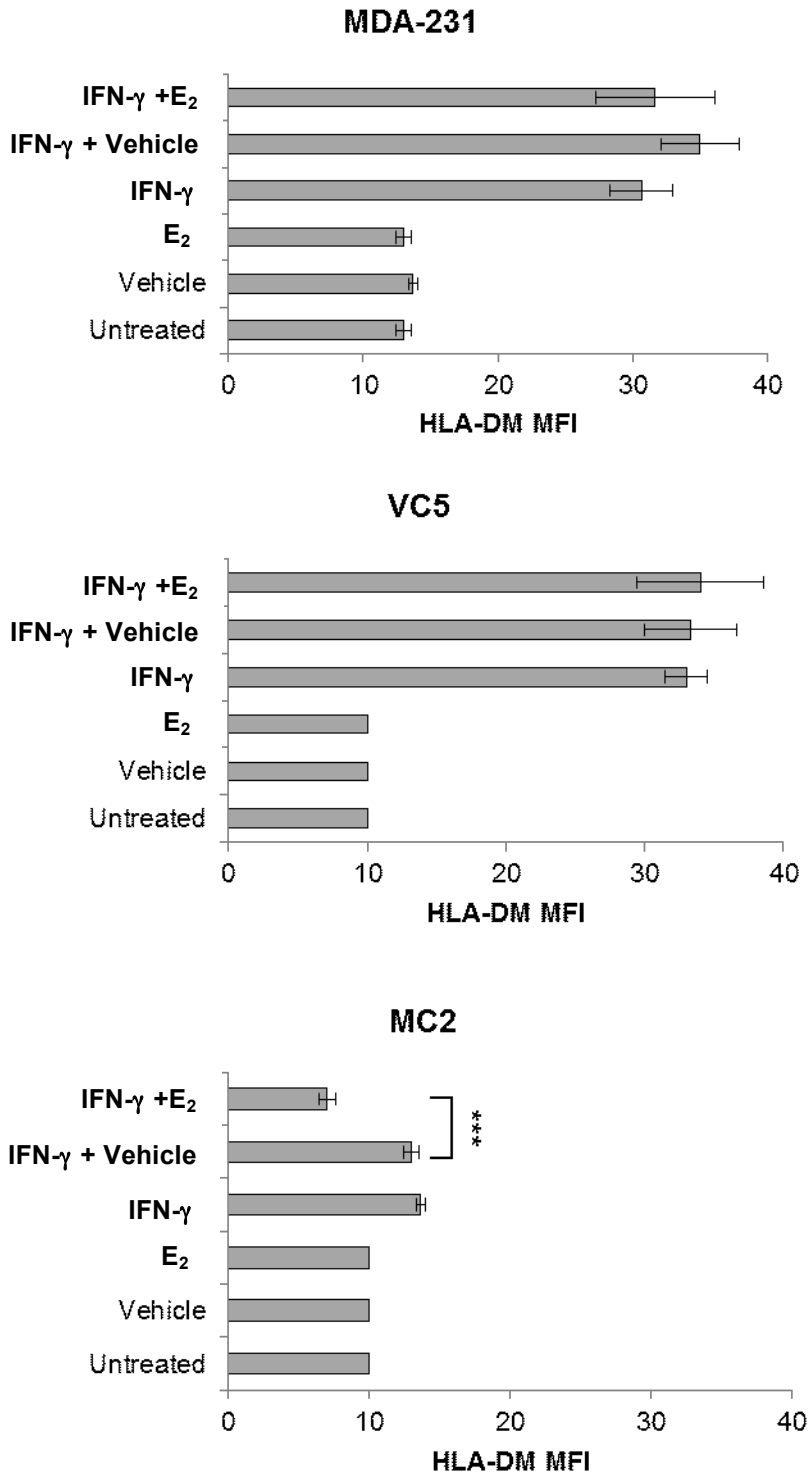
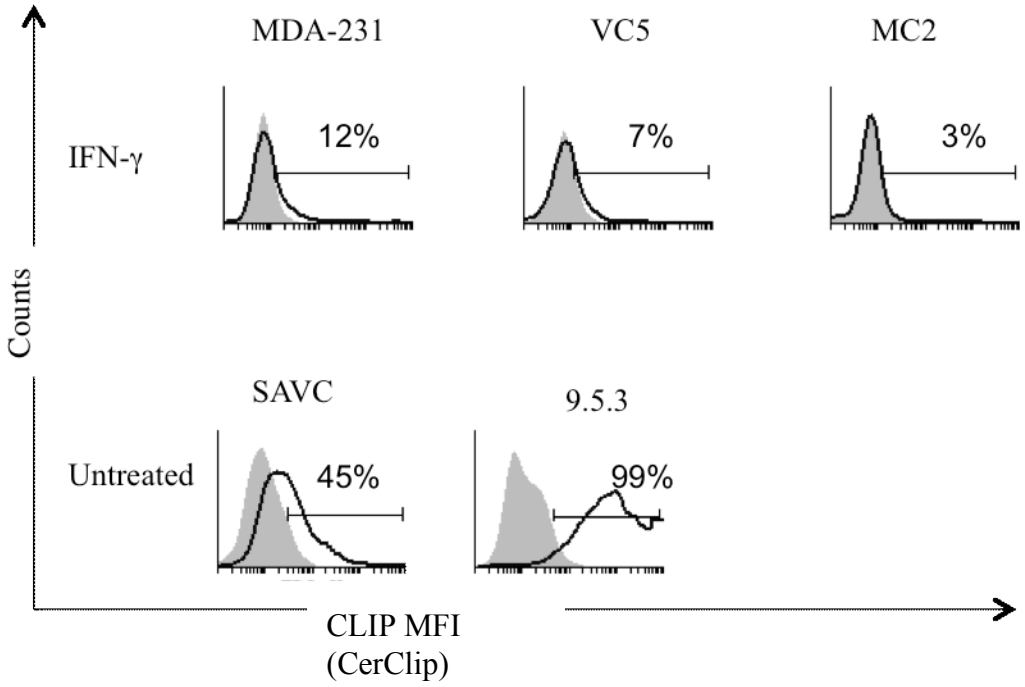

MDA-231
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
0
10
20
30
40
HLA-DM MFI
VC5
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
0
10
20
30
40
HLA-DM MFI
MC2
IFN-γ +E2
IFN-γ + Vehicle
***
IFN-γ
E2
Vehicle
Untreated
0
10
20
30
40
HLA-DM MFI

Figure 3.6 HLA-DR/CLIP complexes are poorly expressed in breast cancer cell line. MDA-MB-231 clone 10A, VC5 and MC2 were stimulated with IFN-γ (100 Units/ml) for 96 hr. CLIP surface expression (detected by Cer-CLIP) was analyzed by flow cytometry. SAVC and 9.5.3 were used as positive controls. Grey histograms represent isotype controls and black lines represent CLIP expression. Figure indicates one experiment.


MDA-231
VC5
MC2
IFN-γ
12%
7%
3%
Counts
SAVC
9.5.3
Untreated
45%
99%
CLIP MFI
(CerClip)

3.7. E_2-ER signaling does not interfere with HLA class I expression in ERα$^+$ breast cancer cell model

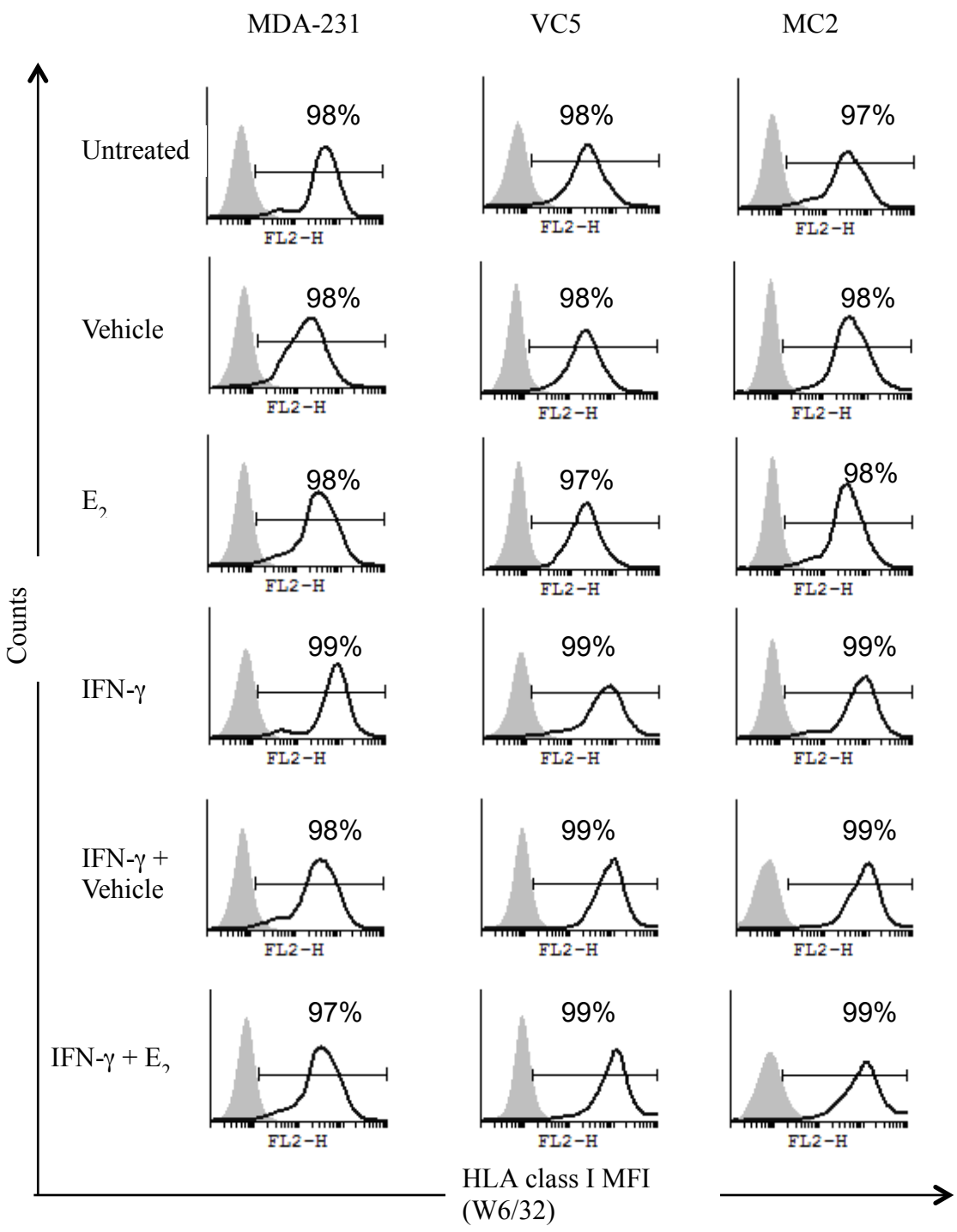
We next questioned if the downregulation of HLA class II by ER-E_2 is due to global downregulation of HLA on BCCL surface. Analysis of constitutive and IFN-γ induced HLA class I surface expression by flow cytometry showed that MDA MB 231 clone 10A, VC5 and MC2 cells expressed similar HLA class I as indicated by the percentage of positive cells (Figure 3.7A), and average MFI (Figure 3.7B). Moreover, E_2 did not have any significant effect on HLA class I expression. This suggests that ERα transgene in the presence or absence of E_2 did not interfere with HLA class I expression, nor is the downregulation of HLA class II proteins due to global interference with the IFN-γ signaling pathway because both class I and II are induced by IFN-γ.

3.8. Effect of transient transfection of wild type and mutant ERα on HLA class II expression.

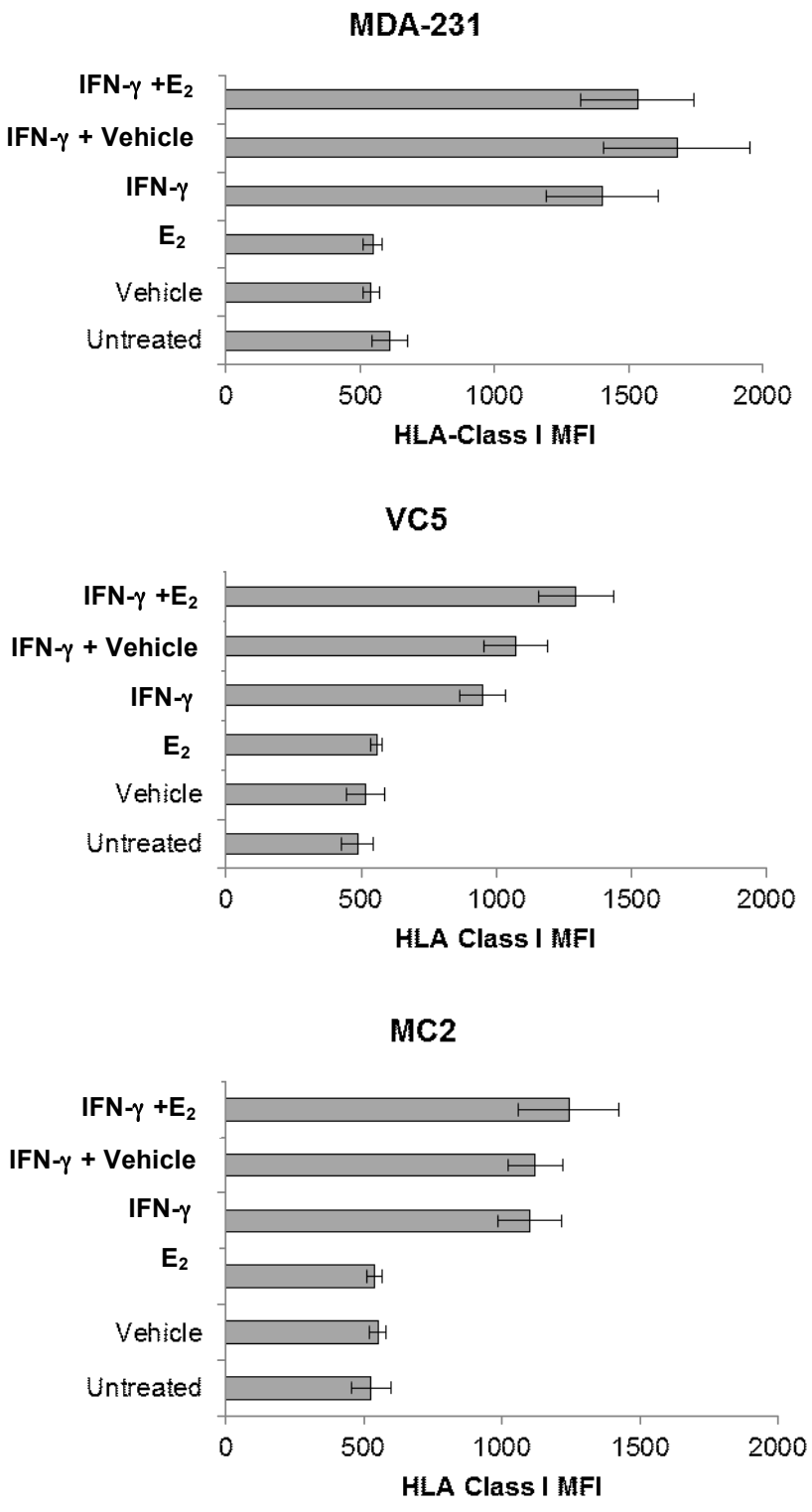
The significant correlation between HLA class II expression and ER status in our BCCL model raised the question whether this could be cell line specific. To address this, SK-BR-3, which is a luminal ER$^-$ and HER2$^+$ BCCL was transiently transfected with either ERα gene or an empty vector (pcDNA3) and stimulated with IFN-γ. Whole cell lysates were prepared and subjected to immunoblotting.

Figure 3.7 E_2-ER signaling did not modulate HLA class I proteins.

MDA-MB-231 clone 10, VC5 and MC2 were cultured in estrogen-depleted media, treated with vehicle (ethanol) or E_2 (10^{-9} M) and stimulated or not with IFN-γ (100 Units/ml) for 96 hr. (A) HLA class I expression (detected by W6/32) was analyzed by surface flow cytometry. Shaded histogram = isotype control, black line = HLA class I expression with the indicated % of cells (B) Bar graphs represent the MFI for HLA class I expression from three independent experiments. Error bars represent the mean ± SEM of three independent experiments.


MDA-231
VC5
MC2
Untreated
Vehicle
E,
IFN-γ
IFN-γ + Vehicle
IFN-γ + E,
98%
98%
97%
98%
98%
98%
98%
97%
98%
99%
99%
99%
98%
99%
99%
97%
99%
99%
FL2-H
Counts
HLA class I MFI (W6/32)

B


MDA-231
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
0
500
1000
1500
2000
HLA-Class I MFI
VC5
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
0
500
1000
1500
2000
HLA Class I MFI
MC2
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
0
500
1000
1500
2000
HLA Class I MFI

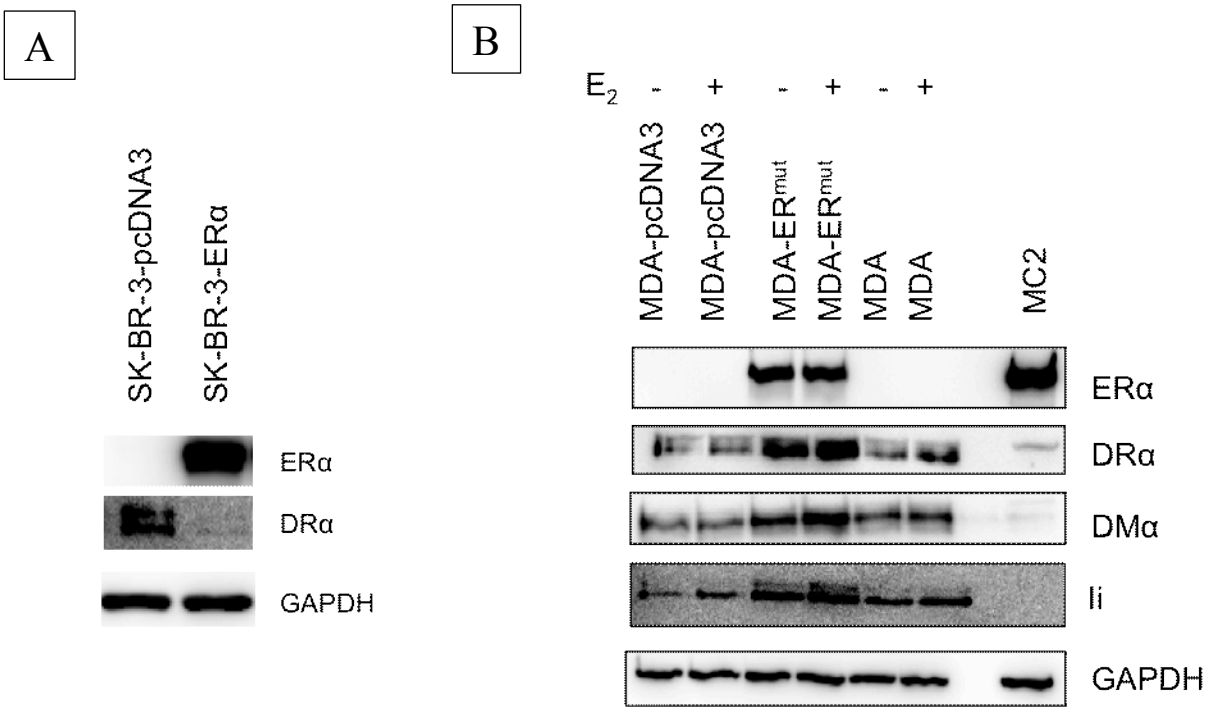
As shown in Figure 3.8A, HLA-DR was barely detected in cells transfected with ERα, compared to cells transfected with empty plasmid. This result is consistent with our findings for the ERα transfectants MC2 (Figure 3.3) and S30 cells (data not shown). Taken together, these results strongly suggest that ERα, even in the absence of E_2 downregulates IFN-γ inducible HLA-DR expression.

To further confirm that E_2 downregulates HLA class II through the classical ERα pathway, MDA-MB-231 was transfected with a mutant form of ERα that has a valine for glycine substitution (V>G) at position 400 (MDA^{mutER}). This mutation alters ligand binding (310). As a mock control, MDA-MB-231 clone 10A was transiently transfected with the empty vector (pcDNA3). The cells were treated with E_2 or vehicle control (ethanol) and stimulated with IFN-γ. As expected, E_2 treatment did not result in downregulation of the ERα (Figure 3.8B). In contrast to diminished HLA class II proteins observed for MC2 and the transiently ERα transfected SK-BR-3 (Figure 3.8A), transfection of $ERα^{mut}$ construct resulted in increased expression of HLA class II proteins, as compared to the mock transfected MDA-MB-231 cells. Moreover, there was no indication that any of HLA class II proteins are downregulated in the presence of E_2. MC2 cells were used as a positive control for ERα. These results suggest that E_2 activation of wild type ERα is responsible for the downregulation of IFN-γ inducible HLA class II expression in breast cancer cells.

Figure 3.8 Transient transfection of wild type and mutant ERα in breast cancer cell line.

(A) SK-BR3 was transiently transfected with the wild type ERα plasmid and the empty plasmid vector PCDNA3. The cells were stimulated for 24 hr with IFN-γ (100 Units/ml) and subjected to immunoblotting from whole cell lysates. Figure represents one experiment.

(B) MDA-MB-231 was left untransfected or transfected with $ER\alpha^{mut}$ or empty plasmid (PCDNA3). Cells were either treated with vehicle control (ethanol) or E_2 (10^{-9}M) and both were stimulated with IFN-γ (100 Units/ml) for 24 hr. Whole cell lysates were prepared and probed with the indicated antibodies. Figure indicates one experiment.


A
SK-BR-3-pcDNA3
SK-BR-3-ERα
ERα
DRα
GAPDH
B
E2
MDA-pcDNA3
MDA-pcDNA3
MDA-ERmut
MDA-ERmut
MDA
MDA
MC2
ERα
DRα
DMα
Ii
GAPDH

3.9. Discussion

Previous studies in our laboratory have shown that HLA-DR positive breast tumors negatively correlate with $ER\alpha^+$ tumors and age of the patients (106). These observations suggest that both ERα and hormones play a role in HLA-DR regulation. In the present work, E_2 inhibited HLA-DR expression in two established $ER\alpha^+$ BCCL, however, in established $ER\alpha^-$ BCCL, E_2 increased HLA-DR expression. In parallel, inducible HLA class II expression was significantly reduced in the ERα BCCL, MC2, compared to VC5. Additionally, E_2 resulted in further reduction of HLA class II expression in MC2. These data implicate E_2-ER signaling in HLA class II regulation and support our previous findings in breast tumors (106).

Although both MCF-7 and T47D are classified as luminal A breast cancer cells (ER^+, $PR^{+/-,}$ $HER2^-$), ERα expression is higher in MCF-7 than in T47D, as detected by enzyme immune assay (311), real time PCR (312) and as shown by immunoblotting (Figure 3.2). Differential expression of HLA-DR expression has previously been reported between MCF-7 and T47D (95). This is consistent with our data (Figure 3.2), and combined with our data on the BCCL model (Figure 3.3) suggest that cells which have higher levels of ERα such as MCF-7 and MC2 model have reduced HLA-DR expression. The effects of E_2 on gene regulation depend on the proportion between ERα and ERβ subtypes (238). E_2 binds to ERβ and ERα with high affinity (313); however, activation of ERβ results in negative regulation of ERα through inhibition of ERα recruitment to the estrogen response element (ERE) in the target genes, leading to the suppression of ERα-regulated genes (249). Moreover, ERβ2 induces proteasome-dependent degradation of

ERα, mainly through the formation of ERβ2/ERα heterodimers (249). T47D expresses the highest levels of ERβ2, compared to MCF-7 and BT-474, and addition of E_2 results in upregulation of ERβ mRNA only in T47D with no effect in MCF-7 and BT-474 (314). Thus, we speculate that increased expression of ERβ in T47D may interfere with the E_2-ERα mediated downregulation of HLA-DR expression that was observed for MCF-7 and BT-474.

The inhibitory effect of E_2 on class II expression had been studied previously by Tzortzakaki et. al. (2003), who linked it to the E_2 mediated squelching of steroid receptor co-activator (SRC1) (304). SRC1, which aids in the proper assembly and transcription of IFN-γ induced CIITA, is also essential for ER-regulated gene transcription (60, 262). On activation of ER by E_2, SRC1 binds to ER and recruits other transcription machinery factors. In the previous study, SRC1 was not shown to be the sole factor responsible for IFN-γ induced HLA-DR downregulation by E_2 because recruitment of SRC1 to the HLA-DR promoter was only partially reduced, suggesting other mechanisms are involved in this downregulation. Moreover, studies in mouse models using an immortalized brain endothelial (IBE) cell line showed that E_2-mediated inhibition of class II expression is linked to E_2 activation of JNK/MAPK. Furthermore, they showed that the class II inhibition correlated with histone 3 (H3) and H4 hypoacetylation and by treating the cells with MAPK inhibitors they reversed the hypoacetylation and restored class II expression (213, 315).

Since estrogen is known to have non-ER modulatory effects on expression of various genes (316), we examined its effects on HLA-DR expression in $ER\alpha^-$ BCCL. As

opposed to the ERα$^+$ BCCL, E_2 upregulated HLA-DR expression in both MDA-MB-231 and SK-BR-3, suggesting that non-genomic (non-classical) estrogen signaling was implicated in the IFN-γ inducible HLA class II pathway. Moreover, MDA-MB-231 and SK-BR-3 express EGFR and HER2 receptors respectively, and both receptors are associated with activation of other signaling pathways such as NFκB and MAPK pathways (317, 318). The latter pathways have been involved in HLA class regulation (203, 319) (discussed in Chapter 6). Estrogen activates other signaling pathways, like MAPK through a non-genomic pathway that does not involve the classical ER. Estrogen, through the GPR30 receptor, can activate MAPK by transactivation of EGFR (253, 256). GPR30 is constitutively expressed in both MDA-MB-231 and SK-BR-3 cell lines at the mRNA level (320), however, it is expressed in higher levels at the protein level in SK-BR-3 and has barely detectable levels in MDA-MB-231 (256). We speculate that increased expression of HLA-DR by E_2 in SK-BR-3 (Figure 3.1) could be due to activation of MAPK through GPR30, but further investigations are required to confirm its involvement. In conclusion, the interpretation of differential expression of HLA-DR upon E_2 treatment of the cell lines, which endogenously express the ERα (MCF-7, BT-474, T47D), was challenging due to cell specific signaling pathways, beside the expression of different levels of ERα, ERβ and GPR30 as previously discussed.

Comparing HLA-DR surface expression between MDA-MB-231 and VC5, we noticed that only MDA-MB-231 constitutively expressed HLA class II, albeit at low levels. Moreover, IFN-γ stimulation resulted in significantly higher MFI for HLA-DR surface expression in MDA-MB-231 compared to VC5 without affecting the percentage

of positive cells. The only difference between these cells is the presence of the vector plasmid, suggesting that the vector plasmid is somehow interfering with HLA-DR expression on a per cell basis. Vector plasmid transfection in different kinds of cells can cause them to behave differently from parent cells with no explanation for this phenomenon (321). Alternatively, differences in cell line passage numbers may have an impact on expressing certain molecules due to acquired mutations, or treatment of VC5 with the selective drug G418 may have a suppressive effect on class II surface molecules (322).

Since E_2 also supressed HLA-DR expression in MCF-7 and BT-474, we do not think that its inhibitory effects on HLA class II in MC2 is due to experimental artifacts in transfectants expressing non-physiological amounts of ERα (data not shown). This is the first report to demonstrate an inhibitory effect of E_2 on the HLA class II co-chaperone molecules, Ii and HLA-DM in BCCL. Despite the almost complete absence of HLA-DM in MC2, we did not observe increased levels of HLA-DR/Clip complexes. Certainly, in professional APC such as B-cells, HLA-DM acts as a peptide editor by removing CLIP from the peptide-binding groove and replacing it with highly stable peptides, and cells without HLA-DM express high levels of surface CLIP (323). The lack of HLA-DR/CLIP complexes in MC2 and their reduced expression in VC5 and MDA-231 may suggest that cancer cells, even when activated by IFN-γ, do not behave like professional APC. Cathepsin S is responsible for the final degradation of Ii to form CLIP, which covers the peptide-binding groove of HLA-DR molecules. Different levels of cathepsin S between cells may affect the degradation of Ii and thus, result in different level of CLIP (324).

Furthermore, the reduced levels of Ii and HLA-DR in MC2 (Figure 3.8C-D) may simply explain the absence of surface CLIP in these cells. These results agree with a previous report on thyroid epithelial cells that showed no CLIP complexes were detected with low HLA-DM expression (325). Notably, our results appear contradictory to a previous study on neuroendocrine epithelial cells, which reported CLIP complex found in DM^-, Ii^+, DR^+ but not in DM^+, Ii^+, DR^+ (326). Furthermore, Spurrell et. al. (2004), showed CLIP on synovial fibroblasts is time dependent and HLA-DR allele specific (327).

Overall, ERα in the presence or absence of E_2 plays an important role in the regulation of HLA class II in breast cancer cells. This may explain differences in the outcomes between $ER\alpha^-$ and $ER\alpha^+$ tumors and also explain how the age and the presence of hormones and IFN-γ levels in the context of ERα have an impact on the prognosis of breast tumors and tumor immunity.

Chapter 4: The effect of selective estrogen receptor modulator on human leukocyte antigen class II expression in estrogen receptor alpha positive breast cancer cell model

4.1. Rationale and objectives

In a previous study in our lab, human leukocyte antigen (HLA)-DR was differentially expressed in estrogen receptor (ER) α^+ and ERα^- breast tumors (106). Moreover, in Chapter 3 we showed that estradiol (E_2) treatment reduced HLA-DR in ERα^+ breast cancer cell line (BCCL), whereas it increased expression in ERα^- BCCL, suggesting that E_2-ER signaling is implicated in HLA class II regulation. Based on these findings, we hypothesized that different molecular mechanisms are involved in HLA-DR regulation in ERα^- and ERα^+ breast cancer. Since E_2-ER signaling is involved in the downregulation of HLA-DR, we predicted blocking ER by selective estrogen receptor modulator (SERM) would reverse the downregulatory effect of ER and E_2 on HLA-DR expression. Additionally, regulation of HLA class II (HLA-DR, invariant chain (Ii) and HLA-DM) occurs mainly at the transcription level, through interaction of transcription factors with a non-DNA binding protein class II transactivator (CIITA). This suggests that E_2-ER may be implicated in HLA class II regulation by interfering with the transactivation of CIITA.

1. To determine whether selective SERMs, Tamoxifen (TAM) and Fulvestrant (ICI 182, 780) (ICI) can reverse the inhibitory effect of E_2 on HLA class II expression in ERα^+ breast cancer cells.
2. To determine if E_2-ER signaling interferes with CIITA expression at the mRNA and protein level.
3. To study the role of silencing the ERα gene (*ESR1*) on CIITA expression.

4.2. Tamoxifen mimics E_2 inhibition on HLA class II expression

The data presented in Chapter 3 clearly showed that HLA class II expression was decreased in MC2 as compared to VC5 and was further decreased by E_2. To address whether the classical ligand-dependent ER pathway is implicated in HLA class II regulation, TAM was used to block E_2-ER signaling. MC2 and VC5 were treated with either exogenous E_2 and/or TAM. The cells were stimulated with IFN-γ for 96 hr and HLA-DR expression was measured by flow cytometry.

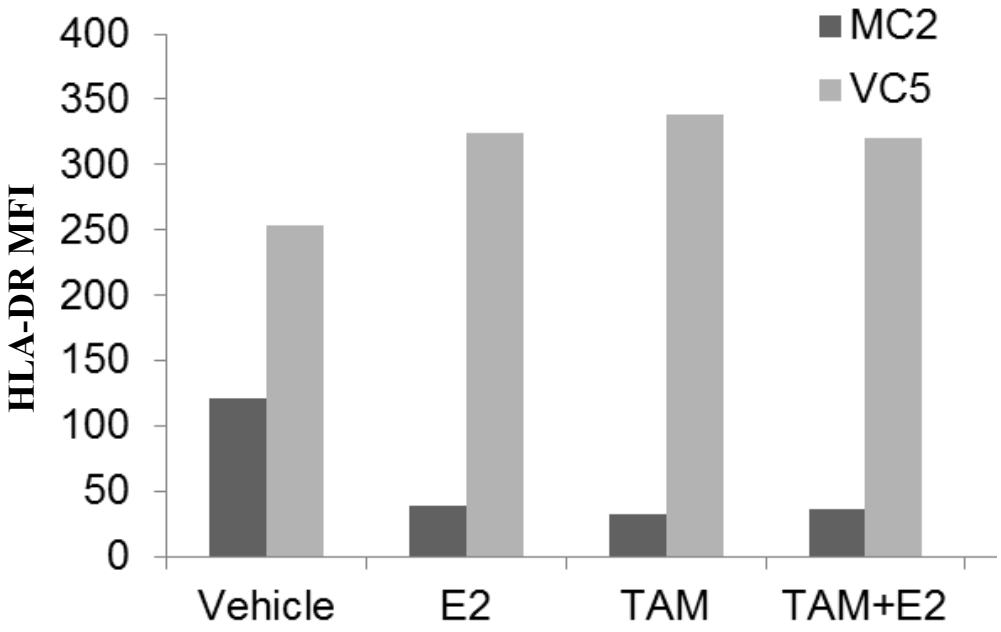
As previously shown in Chapter 3, E_2 treatment augmented the downregulatory effect of ER on HLA-DR expression in MC2 and resulted in more than 60% reduction (Figure 4.1). Although TAM is known to block E_2-ER signaling, it mimicked the E_2-mediated effect on HLA-DR expression in MC2. Surprisingly, simultaneous treatment of TAM and E_2 did not result in any further downregulation. These results suggest that both TAM and E_2 activate ERα signaling, possibly by increasing the nuclear translocation of ER from the cytoplasm with no functional ER degradation (328). Thus, TAM may not a suitable ERα blocker to address whether ER is implicated in HLA class II expression. In VC5 cells, TAM and E_2 resulted in slight upregulation of HLA-DR, which could be due to the involvement of the non-genomic E_2 signaling pathway (315), since these cells lack ER.

4.3. Degradation of ERα induced by the pure antagonist Fulvestrant

As TAM downregulated HLA-DR expression on MC2 similar to E_2, the pure anti-estrogen compound ICI was used to block ERα signaling. ICI at 10^{-6}M inhibits ER

Figure 4.1 Tamoxifen mimics the inhibitory effect of E_2 on HLA-DR expression.

VC5 and MC2 were treated with E_2 (10^{-9}M) and/or TAM (10^{-6}M) and stimulated with IFN-γ (100 Units/ml) for 96 hr. HLA-DR surface expression (detected by L243) was analyzed by flow cytometry. Figure indicates one experiment.


HLA-DR MFI
400
350
300
250
200
150
100
50
0
MC2
VC5
Vehicle
E2
TAM
TAM+E2

signaling within 1 hr of treatment (329). To test whether this is an effective concentration, MC2 was treated for 1 or 24 hr with ICI (10^{-6} M) or vehicle control (ethanol) and ERα was assayed by immunocytochemistry (ICC). As shown in Figure 4.2A, moderate to strong nuclear and cytoplasmic ERα staining was observed in ~90% of vehicle control treated MC2 cells compared to ~10% in cells, which were either treated for 1 or 24 hr with ICI. Thus, a pre-treatment of 1 hr with ICI was sufficient to knock down ERα.

We next determined the optimal concentration of ICI that knocked down ERα and was minimally toxic over a 96-hr period to MC2 cells. The cells were pre-treated for 1 hr with log fold dilutions of ICI (10^{-10}-10^{-4} M), followed by stimulation with IFN-γ for 96 hr. Immunoblotting (Figure 4.2B) revealed dramatic loss of ERα from the cytoplasmic extract at 10^{-9} M to 10^{-4} M ICI and from the nuclear extract at 10^{-6} M to 10^{-4} M ICI. There was little difference in the absorbance at all the concentrations used except for those higher than 10^{-5} M, which resulted in 25% less absorbance compared to vehicle control. (Figure 4.2C). Overall, these experiments confirmed that 1-hr pre-treatment with ICI 10^{-6} M is sufficient to degrade ERα in both the cytoplasm and nucleus and is not cytotoxic to MC2 cells.

4.4. Fulvestrant restores HLA class II expression in E_2-treated ERα$^+$ cells

Next, immunocytochemistry (ICC) on MC2 cells assessed expression of ERα, HLA-DR, Ii and HLA-DM. As expected, 90% of the cells in the vehicle control samples had nuclear staining for ERα (Figure 4.3). Treatment of the cells with ICI resulted dramatic loss of the number of labeled nuclei (~70%), (as well as a marked decrease in

Figure 4.2 Optimization of Fulvestrant (ICI 182, 780) (ICI) treatment.

MC2 was treated with increasing concentrations of ICI (A) ICC, showing ERα protein expression using ERα (1D-5) antibody. (B) Immunoblots from nuclear and cytoplasmic extracts, ERα was detected using ERα (HC-20) antibody. (C) Crystal violet staining expressed as optical density (OD) absorbance. Each figure indicates one experiment.

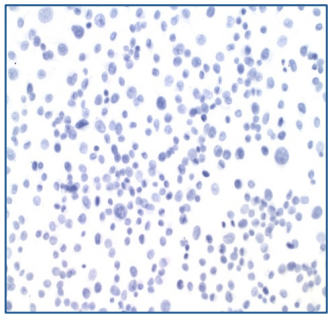
A

MC2

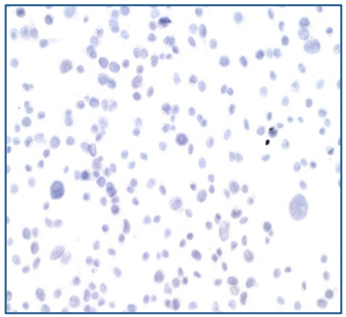
After 1 hr

After 24 hr

IgG1

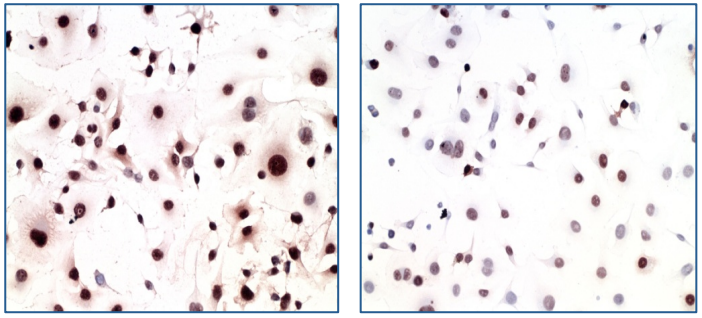


IgG1



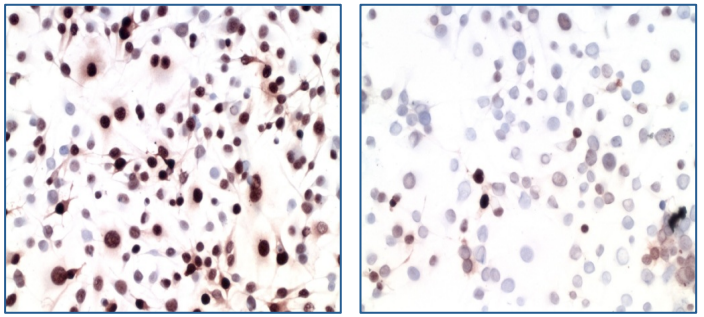
Vehicle ERα

ICI 1μM ERα

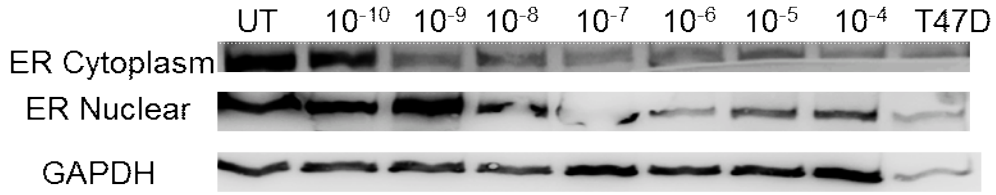


Vehicle ERα

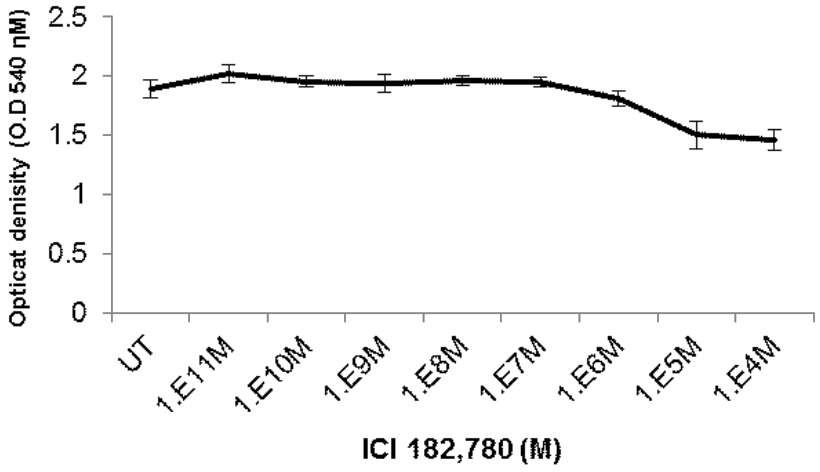
ICI 1μM ERα



B


UT
10-10
10-9
10-8
10-7
10-6
10-5
10-4
T47D
ER Cytoplasm
ER Nuclear
GAPDH

C

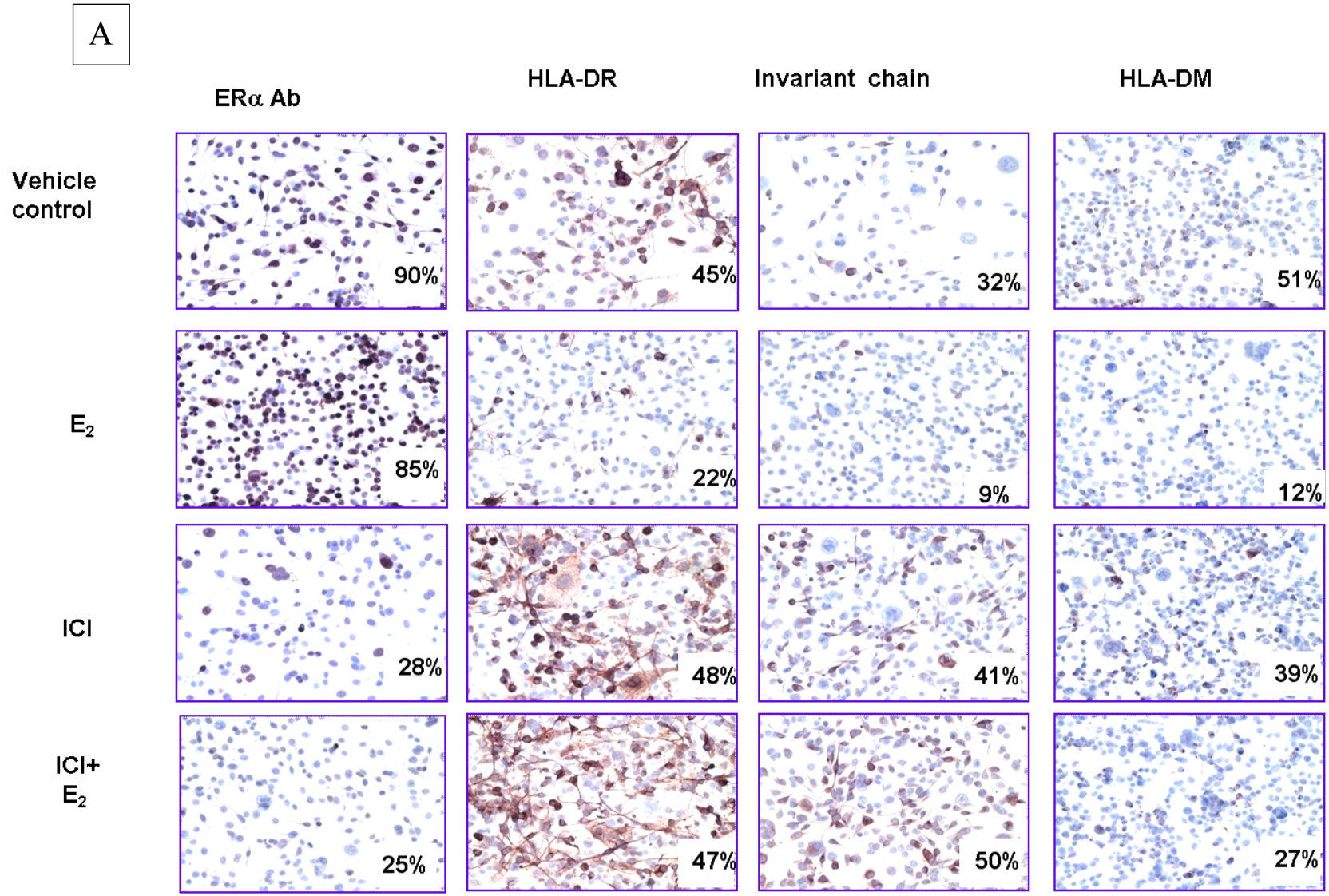

Opticat denisity (O.D 540 nM)
2.5
2
1.5
1
0.5
0
UT
1.E11M
1.E10M
1.E9M
1.E8M
1.E7M
1.E6M
1.E5M
1.E4M
ICI 182,780 (M)

the staining intensity of the nuclei when compared to vehicle control. Although the percentage of ERα positive cells in the vehicle control and E_2 cells were approximately the same, the intensity of nuclear staining was increased in the E_2 treated cells, suggesting upregulation of ERα upon E_2 treatment. Moreover, E_2 resulted in reduction of HLA-DR, Ii and HLA-DM expression by almost half, which was restored by ICI and the combination of E_2 and ICI. ICI, in the presence or absence of E_2, resulted in slight downregulation of HLA-DM as compared to vehicle control. These results suggest that downregulation of HLA class II expression correlates with the upregulation of nuclear ERα by E_2, and support our previous finding (Figure 3.2) in MCF-7 cells. Additionally, the restored HLA class II expression by simultaneous treatment of E_2 and ICI suggests involvement of classical ER signaling in downregulation of HLA class II.

Thereafter, we compared the effect of ICI on HLA class II expression in both $ERα^+$ and $ERα^-$ BCCL models. HLA class II analysis by flow cytometry confirmed that HLA-DR, Ii and HLA-DM (Figure 4.4A) were significantly reduced in vehicle-treated and E_2-treated MC2 as compared to VC5 cells. Surprisingly, ICI-mediated knockdown of ERα in MC2 resulted in no change in HLA class II compared to vehicle control and did not restore HLA class II to the levels of VC5. However, ICI combined with E_2 reversed the downregulatory effect of E_2 on HLA class II and co-chaperones in MC2. Neither ICI nor E_2 significantly modulated HLA class II in VC5 cells. Similar results were obtained by immunoblotting (Figure 4.4B-C). ICI-treatment resulted in negligible decrease of HLA-DR expression in MC2. ERα was upregulated by E_2 and was dramatically reduced by ICI (~80% reduction), compared to vehicle control.

Figure 4.3 Fulvestrant restores HLA class II expression in E_2-treated ERα^+ breast cancer cells.

MC2 cells were grown in an 8-well chamber. The cells were pre-treated, or not, for 1 hr with ICI (10^{-6}M) and/or E_2 (10^{-9}M), followed by stimulation with 100 Units/ml IFN-γ for 96 hr. Immunocytochemical analysis of ER (anti-ER, 1D5) and HLA-DR (L243), Ii (LN2) and HLA-DM (DM.1) was done using light microscopy (A) 10X magnification, (B) 40X magnification. Figure indicates one experiment.


A
ERα Ab
HLA-DR
Invariant chain
HLA-DM
Vehicle control
E2
ICI
ICI+ E2
90%
45%
32%
51%
85%
22%
9%
12%
28%
48%
41%
39%
25%
47%
50%
27%

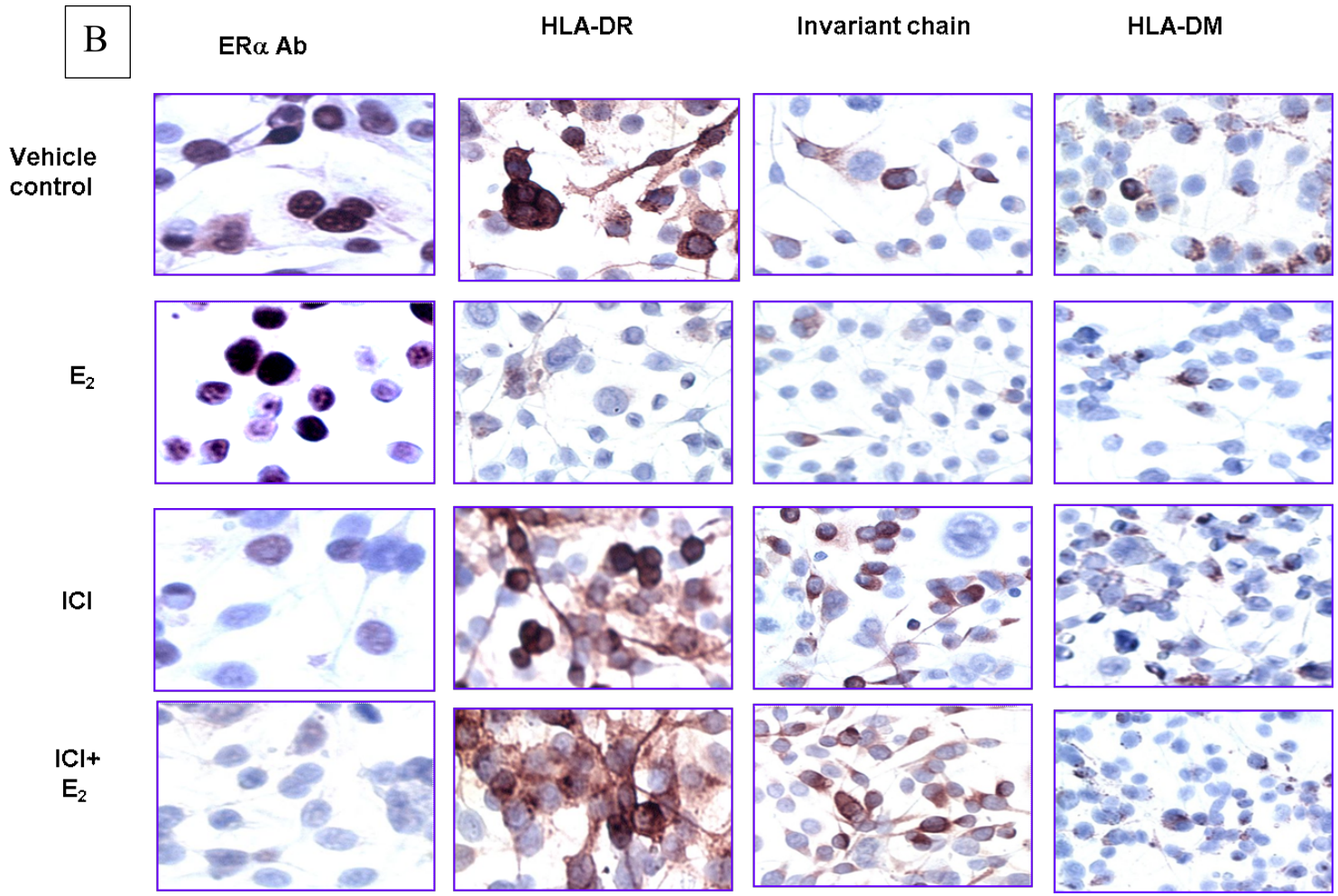
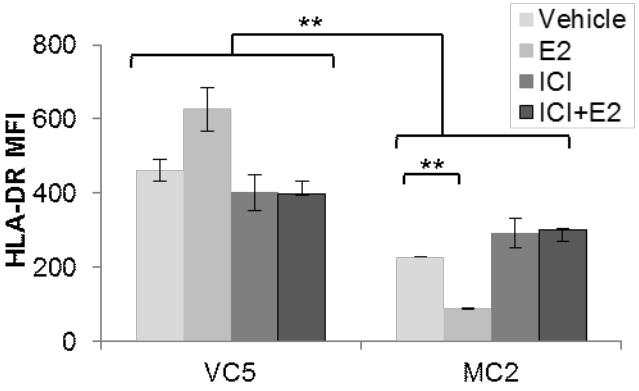
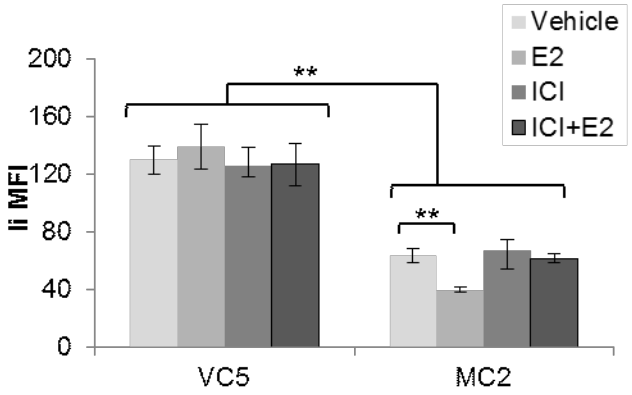

B
ERα Ab
HLA-DR
Invariant chain
HLA-DM
Vehicle control
E2
ICI
ICI+ E2

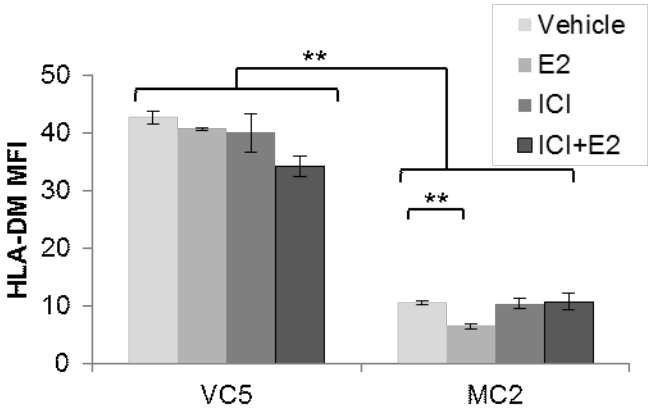
Figure 4.4 Coordinate down regulation of IFN-γ inducible HLA class II expression by E_2 is reversed by ICI-mediated degradation of ERα in MC2 cells.

VC5 and MC2 cells were cultured in estrogen-depleted media, treated with vehicle (ethanol), E_2 (10^{-9}M) or/and ICI (10^{-6} M) followed by stimulation with IFN-γ (100 Units/ml) for 96 hr. HLA class II expression was analyzed by (A) flow cytometry using anti-DR, (L243),) anti-DM (Map.DM1) and anti-Ii (LN2). Bar graphs represent the MFI ± SEM of three independent experiments. (*$p<0.05$, **$p<0.01$). (B) HLA class II expression was analyzed by immunoblotting whole cell extracts using anti-DRα (TAL 1B5), anti-DM (TAL18.1) and anti-Ii (LN2). (C) Bar graphs show the ratio of band intensities, normalized to GAPDH band intensities and represent the mean ± SEM of three independent experiments: (* $p<0.05$, ** $p<0.01$).

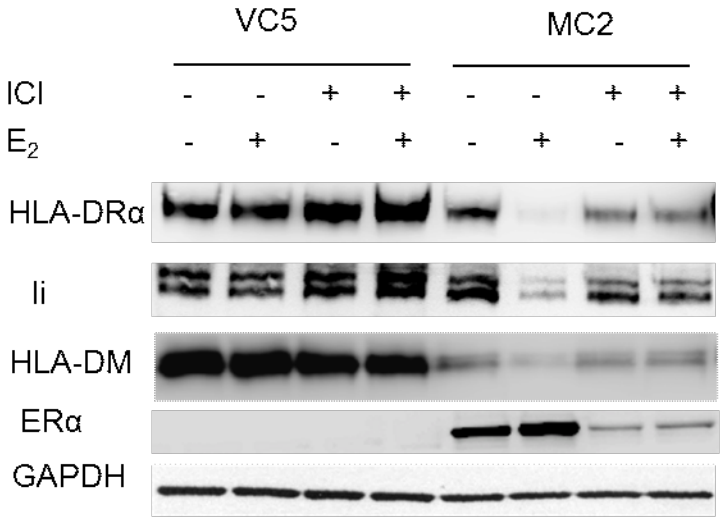
A

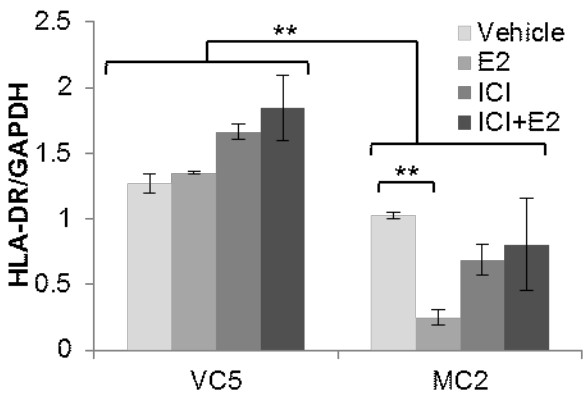

HLA-DR MFI
800
600
400
200
0
**
**
Vehicle
E2
ICI
ICI+E2
VC5
MC2


Ii MFI
200
160
120
80
40
0
**
**
Vehicle
E2
ICI
ICI+E2
VC5
MC2


HLA-DM MFI
50
40
30
20
10
0
**
**
Vehicle
E2
ICI
ICI+E2
VC5
MC2

B


VC5
MC2
ICI
- - + + - - + +
E2
- + - + - + - +
HLA-DRα
Ii
HLA-DM
ERα
GAPDH


HLA-DR/GAPDH
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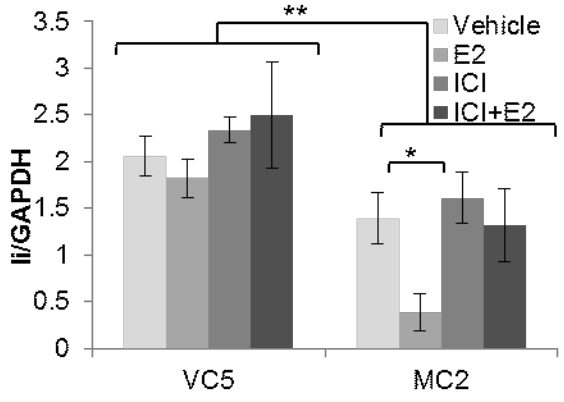
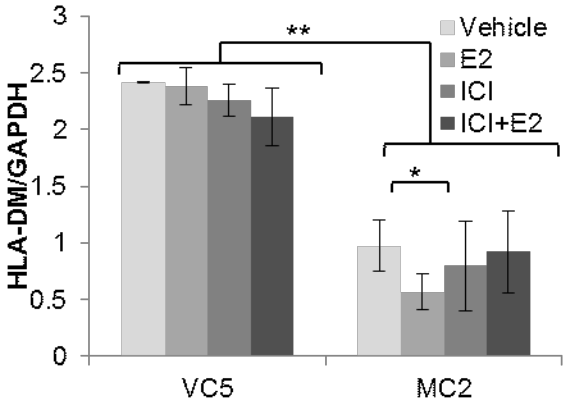
2
1.5
1
0.5
0
**
**
Vehicle
E2
ICI
ICI+E2
VC5
MC2


Ii/GAPDH
3.5
3
2.5
2
1.5
1
0.5
0
**
*
Vehicle
E2
ICI
ICI+E2
VC5
MC2


HLA-DM/GAPDH
3
2.5
2
1.5
1
0.5
0
**
*
Vehicle
E2
ICI
ICI+E2
VC5
MC2

Taken together these results strongly suggest that E_2-mediated reduction of HLA class II is due to E_2 ligand-activation of ERα. Failure of ICI, which clearly degraded ERα, to restore HLA class II to VC5 levels suggests additional mechanisms. The coordinate downregulation of HLA-DR and the co-chaperones by E_2 in the $ER\alpha^+$ BCCL implies that CIITA, the master regulator of HLA class II expression, is also negatively impacted.

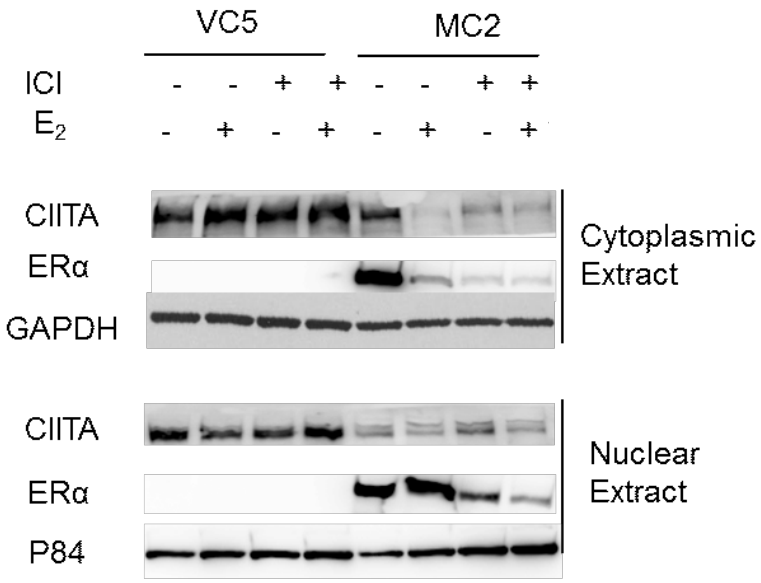
4.5. CIITA protein is down regulated by activation of the ER signaling pathway

Since HLA class II and co-chaperones expression are regulated by CIITA, we predicted that E_2-activation of ER interfered with CIITA and blocking ER signaling in MC2 would restore CIITA to VC5 levels. MC2 and VC5 were treated with E_2 and/or ICI, followed by IFN-γ for 24 hr, which is the optimum time for induced CIITA expression (Appendix 1). Immunoblots from cytoplasmic and nuclear extracts revealed significantly reduced CIITA expression in untreated and treated MC2 as compared to VC5 (Figure 4.5). Similar to our results for HLA class II, E_2 further reduced CIITA in MC2 while increasing the amount of nuclear ER; by contrast, ICI reversed the inhibitory effect of E_2 on CIITA expression, coincident with reduced ER levels. Yet, ICI alone or in combination with E_2 did not restore CIITA to VC5 levels even though ICI significantly reduced cytoplasmic and nuclear ER (Figure 4.5A, Lane 7). Although there was a decrease of CIITA in ICI-treated MC2, this decrease was insignificant. As noted previously, the failure of ICI to restore HLA class II, and now CIITA to levels observed in VC5, which differs only by the ERα transgene, suggests an alternate mechanism of HLA class II and CIITA downregulation in MC2.

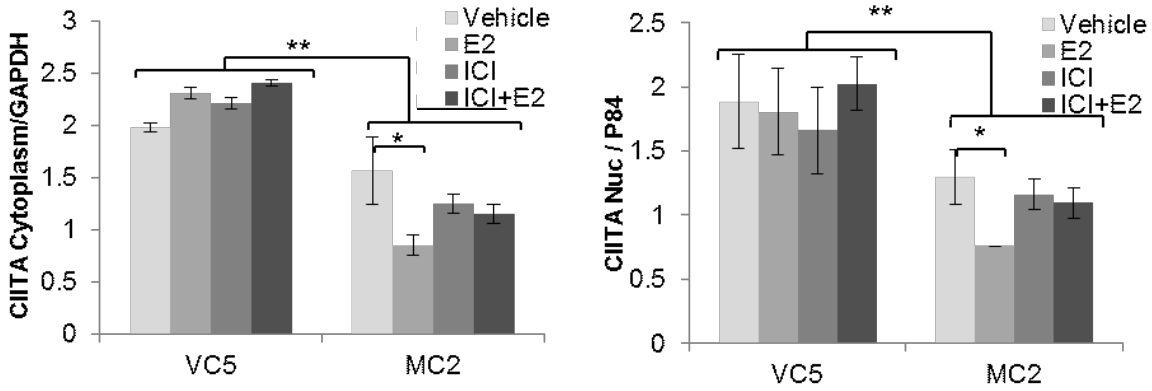
Figure 4.5 CIITA is downregulated by activation of the ERα signaling pathway.

VC5 and MC2 cells were cultured in estrogen-depleted media treated with vehicle (ethanol), E_2 (10^{-9}M) or/and ICI (10^{-6}M) and stimulated with IFN-γ (100 Units/ml) for 24 hr. (A) Immunoblot analysis was performed on cytoplasmic and nuclear extracts for CIITA (antiserum #21) and ERα (HC-20). (B) Cytoplasmic CIITA and nuclear CIITA were normalized to GAPDH and P84 respectively. Bar graphs represent the average ± SEM of three independent experiments (**$p<0.01$, * $p<0.05$).

A


VC5
MC2
ICI
E2
CIITA
ERα
GAPDH
Cytoplasmic Extract
CIITA
ERα
P84
Nuclear Extract

B


CIITA Cytoplasm/GAPDH
Vehicle
E2
ICI
ICI+E2
VC5
MC2
CIITA Nuc / P84

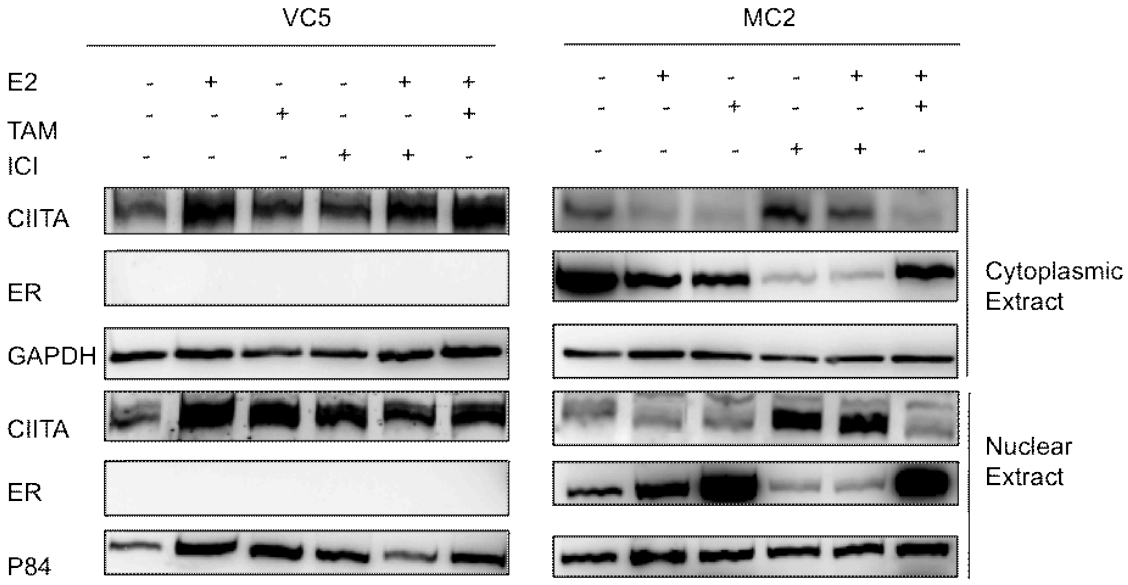
Earlier (Section 4.1), we showed that TAM mimics the inhibitory effect of E_2 on HLA-DR expression in ERα$^+$ BCCL but not in the ERα$^-$ cell line. Since E_2 (Figure 4.5A) and TAM (328) increase ERα localization in the nucleus, we predicted that their inhibitory effect on HLA class II expression was through down regulation of CIITA. To address this question, MC2 and VC5 were pre-treated with E_2, ICI, and TAM and/combination, followed by stimulation with IFN-γ for 24 hr and preparation of nuclear and cytoplasmic lysates. As shown in Figure 4.6, most of ER was localized in the nuclear fraction of MC2, treated with TAM, E_2 or in combination. This correlated with CIITA downregulation. ICI treatment resulted in ER degradation, which correlated with increased CIITA expression, thus confirming our previous observations (Figure 4.5). By contrast, TAM, E_2 and ICI treatment in VC5 resulted in a slight upregulation of CIITA. These data support the hypothesis that downregulation of HLA class II expression by either E_2 or TAM activation of ER is through CIITA.

4.6. HLA class II and CIITA expression are downregulated in MC2 at the transcriptional level

Regulation of HLA class II gene expression occurs mainly at the transcription level by a highly conserved regulatory module located in the promoter genes encoding the α-chain and β-chain of all HLA class II molecules (330). To determine whether the inhibitory effect of ERα on HLA class II expression is at the transcription level, VC5 and MC2 were stimulated with IFN-γ for 12 hr, followed by RNA extraction. As shown in Figure 4.7, the overall transcription level for CIITA and the downstream genes controlled

Figure 4.6 CIITA expression inversely correlates with expression of ERα.

VC5 and MC2 cells were cultured in estrogen-depleted media, pre-treated with the following SERM combinations: E_2 (10^{-9}M), ICI (10^{-6}M), TAM (10^{-6}M) and vehicle (ethanol), followed by stimulation with IFN-γ (100 Units/ml) for 24 hr. Immunoblot analysis was performed on cytoplasmic and nuclear extracts for CIITA (antiserum #21) and ERα (HC-20). GAPDH and P84 were used as a loading control for cytoplasmic and nuclear extracts respectively. Figure indicates one experiment.


VC5
MC2
E2
TAM
ICI
CIITA
ER
GAPDH
CIITA
ER
P84
Cytoplasmic Extract
Nuclear Extract

by CIITA [HLA-DR (A,B), HLA-DM (A,B) and Ii] were reduced in MC2 compared to VC5 suggesting that ER in the absence of E_2 has a significant role in the downregulation of inducible CIITA. Furthermore, Ii and DMA were constitutively expressed in the absence of constitutive CIITA expression by RT-PCR. This could be due to the existence of some regulatory elements in the promoters of HLA-DM not shared by HLA class II promoters (331).

Since CIITA appeared to be the critical molecule affected by the E_2-ER signaling (Figure 4.5 and 4.6) and downregulation of CIITA in ERα^+ cells is at the transcription level, we examined the effect of various treatments on CIITA transcription using real time PCR. The optimal time for IFN-γ induced CIITA mRNA expression was determined using VC5. The cells were treated with IFN-γ and RNA extraction was done every 2 hr up to 10 hr. In agreement with the results of others (332), we found that CIITA expression was first observed at 2 hr, followed by a sharp increase at 4 to 6 hr and plateau from 8 to 10 hr (Figure 4.8A). Thus, IFN-γ treatment for 4 hr was optimal for CIITA upregulation in this breast cancer cell model.

Next, we examined if E_2 and/or ICI affected CIITA transcription in VC5 and MC2. The cells were pre-treated with ICI and/or E_2, and stimulated with and without IFNγ for 4 hr, followed by RNA extraction. Quantitative real time PCR was performed using a primer specific for CIITA. As shown in Figure 4.8B, induced CIITA transcripts were significantly less in MC2 compared to VC5. E_2 further decreased CIITA mRNA transcripts in MC2, while ICI reversed the E_2-mediated effect, but did not restore CIITA transcripts to VC5 levels, consistent with the results (Figure 4.5) for protein

Figure 4.7 IFN-γ inducible HLA class II expression is downregulated in MC2 at the mRNA level.

VC5 and MC2 cells were cultured in estrogen-depleted medium and stimulated with IFN-γ (100 Units/ml) for 12 hr. Total RNA was isolated and subjected to RT-PCR analysis using primers specific for CIITA and HLA class II genes. Figure represents one experiment.

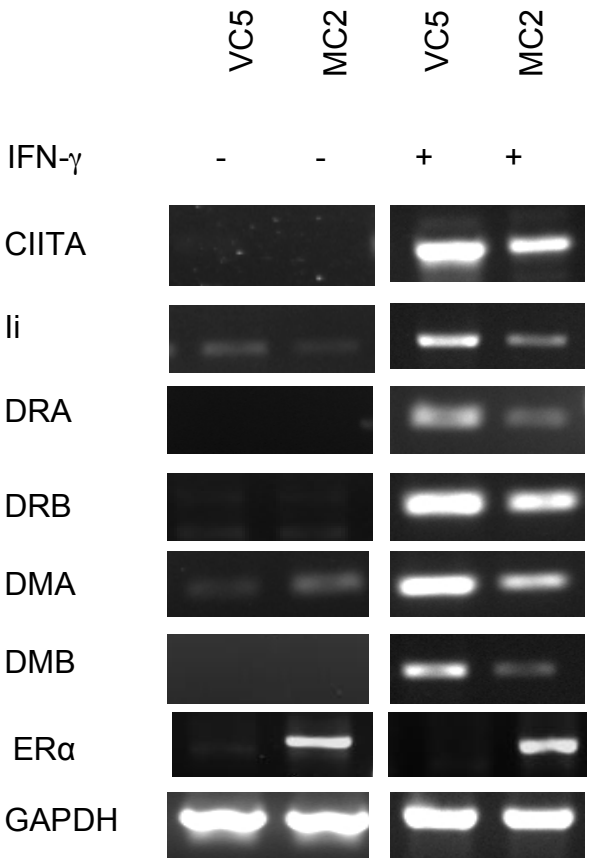
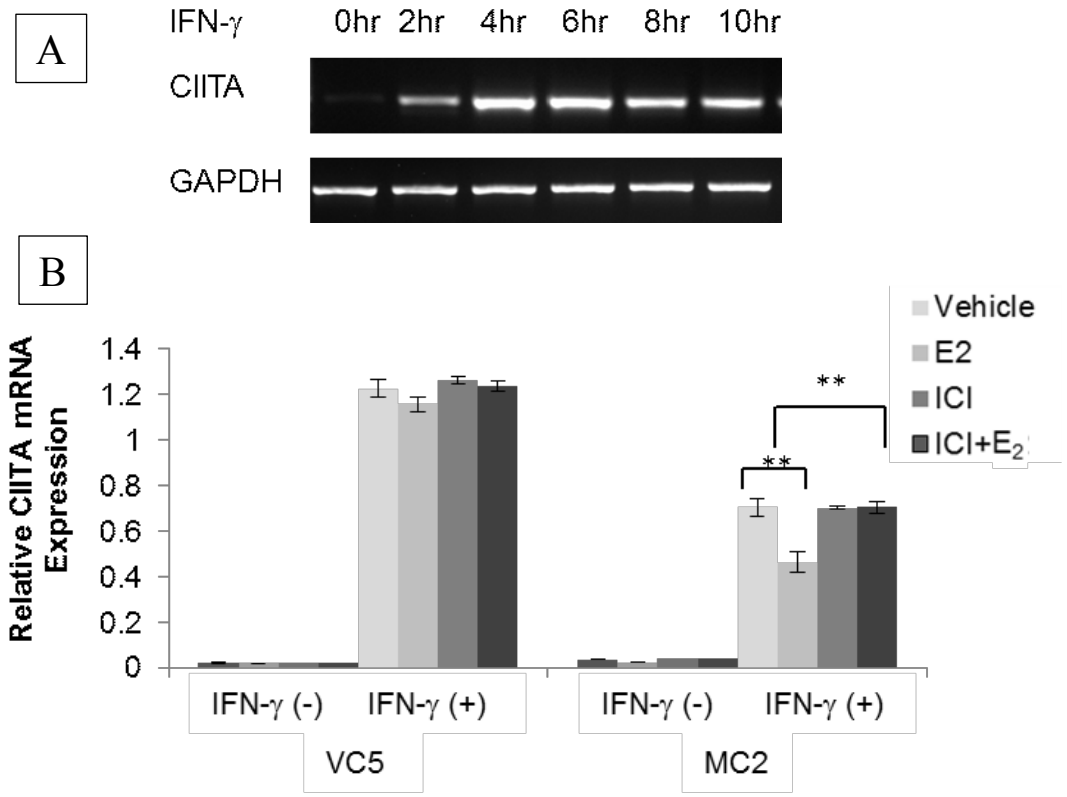

VC5
MC2
VC5
MC2
IFN-γ
-
-
+
+
CIITA
Ii
DRA
DRB
DMA
DMB
ERα
GAPDH

Figure 4.8 E_2-ER signaling downregulates CIITA mRNA expression in ERα^+ breast cancer cell lines.

(A) Kinetics of IFN-γ induced CIITA expression in VC5. Cells were treated with IFN-γ (100 Units/ml) at the indicated time points and assayed for CIITA mRNA expression. (B) VC5 and MC2 cells were cultured in estrogen-depleted media, treated with vehicle (ethanol), E_2 (10^{-9}M) or/and ICI (10^{-6}M) and stimulated or not with IFN-γ (100 Units/ml) for 4 hr. CIITA mRNA expression was measured by real time PCR using Taqman gene expression assay. GAPDH was used as an endogenous control and the data were expressed relative to a control B cell line (RAJI). Bar graphs represent the mean ± standards errors of the mean (SEM) of three replicate assays (**$p<0.01$).


A
IFN-γ
0hr 2hr 4hr 6hr 8hr 10hr
CIITA
GAPDH
B
Vehicle
E2
ICI
ICI+E2
Relative CIITA mRNA Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
IFN-γ (-)
IFN-γ (+)
IFN-γ (-)
IFN-γ (+)
VC5
MC2

expression. Very low CIITA transcripts were observed in the two cell lines in the non-IFN-γ induced cells, with no significant difference between them. These data suggest that ER and E_2 downregulate HLA class II expression by interfering with CIITA at the transcriptional level.

ICI had been reported to activate downstream signaling pathways independent of ER status through non-genomic ER signaling (315, 330, 333). To confirm that ICI reversed the downregulatory effect of E_2 on CIITA expression was through ER and not through the non-genomic effects of ICI, we used *ESR1*-siRNA to silence the ERα transgene. Validation of *ESR1* was done on MC2. The cells were transfected with the *ESR1*-siRNA and time-dependent response was assessed by preparing whole cell lysates and examining ERα protein expression at various time points using ERα antibody (HC-20) and immunoblotting. As depicted in Figure 4.9A, ERα protein was severely decreased in ESR1 siRNA-transfected MC2 by 48 hr and this persisted up to 144 hr. Thus, the optimal transfection time chosen for subsequent experiments was 48 hr to avoid cell toxicity due to longer exposure of the transfection reagents.

Next, we used *ESR1*-siRNA to silence the ERα transgene in MC2 treated or not with E_2, followed by IFN-γ stimulation for 24 hr. VC5, treated in the same manner, was used as a control. ERα was greatly diminished by transfection of *ESR1*-siRNA as compared to the scrambled siRNA in MC2 (Figure 4.9B). Although *ESR1*-siRNA silencing in MC2 did not upregulate CIITA protein level, it negated the effect of E_2-downregulation of CIITA that was apparent with the scrambled siRNA. By contrast, E_2

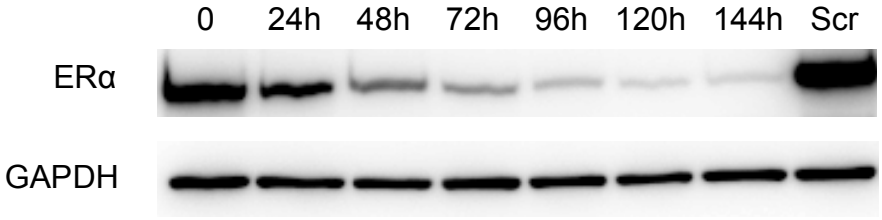
increased CIITA protein expression in the ERα$^-$ VC5, whether transfected with scrambled or *ESR1*-siRNA. Taken together, these results strongly support a role for E_2-activated ERα in regulating the CIITA pathway.

Subsequently, we corroborated these findings at the mRNA level. As shown in Figure 4.10A, *ESR1*-siRNA dramatically reduced ERα mRNA expression as compared to control siRNA without significant changes in GAPDH expression, as indicated by RT-PCR. Although CIITA transcripts were not increased (Figure 4.10B), *ESR1*-siRNA clearly abolished the inhibitory effect of E_2 on CIITA transcripts. These results, together with those showing that ICI-mediated degradation of ERα reverses the E_2-mediated inhibition of CIITA (Figure 4.5) and HLA class II (Figure 4.4), indicate that E_2-activated ER somehow interferes with CIITA expression at the transcription level.

Figure 4.9 Silencing the ERα by siRNA reversed the inhibitory effect of E_2 on CIITA protein expression.

(A) Temporal response to silencing *ESR1* by siRNA in MC2 and detection of ERα expression by immunoblotting. (B) ERα was silenced (*ESR1* siRNA) or not (scrambled siRNA) in MC2; VC5 served as a control. Cells were treated with vehicle (ethanol) or E_2 (10^{-9} M) and stimulated with IFN-γ (100 Units/ml) for 24 hr. Nuclear lysates were prepared and probed for CIITA (antiserum #21), ERα (HC-20), and p84. Figure represents one experiment.

A


0
24h
48h
72h
96h
120h
144h
Scr
ERα
GAPDH

B

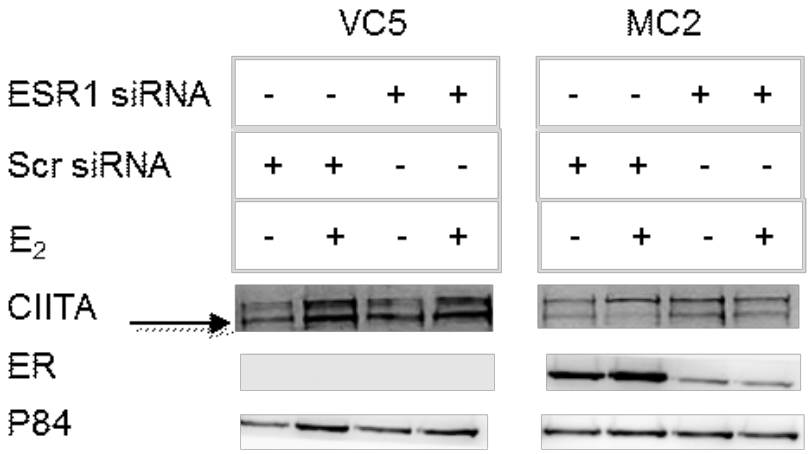
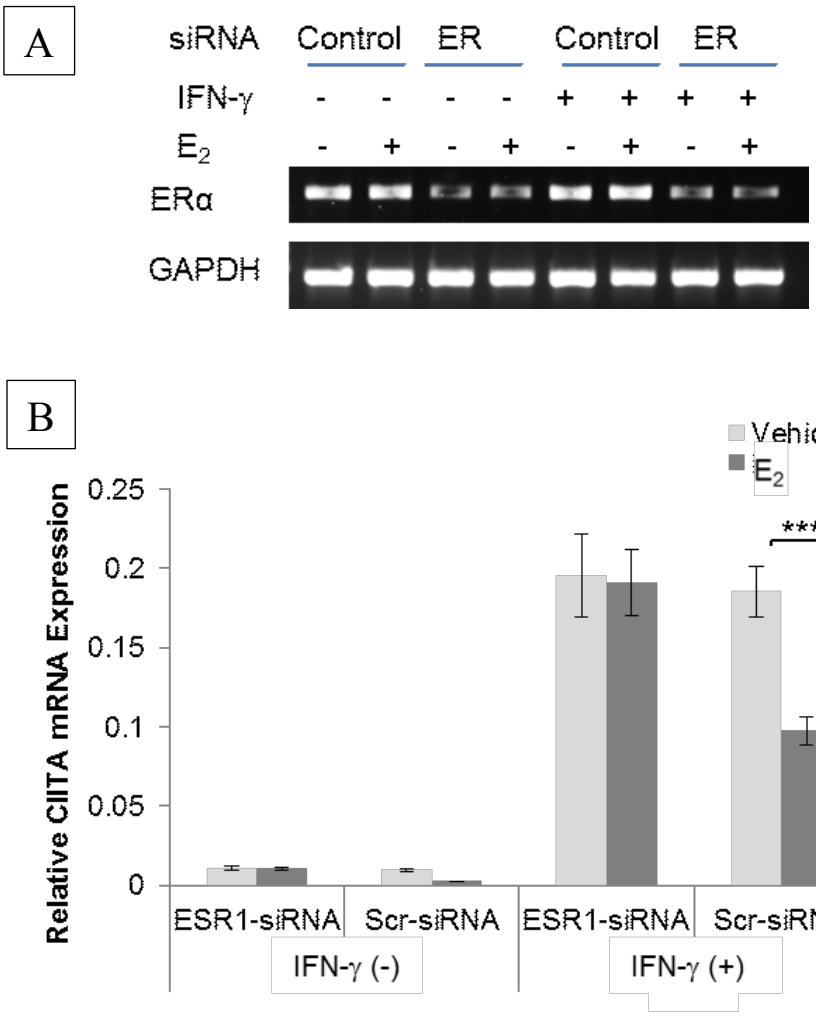

VC5
MC2
ESR1 siRNA
Scr siRNA
E2
CIITA
ER
P84

Figure 4.10 Silencing the *ESR1* by siRNA reversed the inhibitory effect of E_2 on CIITA mRNA expression.

ERα was silenced (*ESR1* siRNA) or not (scrambled siRNA) in MC2; VC5 served as a control. Cells were treated with vehicle (ethanol) or E_2 (10^{-9} M) and stimulated or not with IFN-γ (100 Units/ml) for 4 hr. (A) RT-PCR for ER mRNA showing the effect of siRNA on ER transcripts (B) CIITA mRNA was measured by real time PCR using Taqman gene expression assay. GAPDH was used as an endogenous control for and the data were expressed relative to a control B cell line (RAJI). Bar graphs represent the mean ± SEM of three replicate assays (*** $p<0.001$).


A
siRNA
Control
ER
Control
ER
IFN-γ
- - - - + + + +
E2
- + - + - + - +
ERα
GAPDH
B
Vehicle
E2
Relative CIITA mRNA Expression
0.25
0.2
0.15
0.1
0.05
0
***
ESR1-siRNA
Scr-siRNA
ESR1-siRNA
Scr-siRNA
IFN-γ (-)
IFN-γ (+)

4.7. Discussion

Regulation of HLA class II expression occurs primarily at the transcriptional level by CIITA (38, 334). Herein, we have shown that E_2 and TAM in the presence of ERα also result in downregulation of CIITA at the transcription level (Figure 4.7). The effect of E_2 on HLA class II and CIITA was reversed by ICI and ER-siRNA suggesting that E_2-ER signaling is interfering with HLA class II expression through its genomic signaling. Although 10^{-6} M TAM was effective in antagonising the E_2 effect in a number of studies including breast cancer cells (213, 328), it mimicked the E_2 effect in this study (Figure 4.1, 4.6). Johnson et al, (1989), also showed that TAM in estrogen depleted medium can increase proliferation and gene regulation and mimics the effect of E_2 in MCF-7 cells (335). Both TAM and E_2 increase ER binding to the estrogen response element (ERE) with similar affinities, which explains why TAM may produce effects similar to estrogen (263). On the other hand, ICI, which degrades the ER, as discussed in Section 4.2 and shown in Figures 4.4 and 4.5, successfully reversed the inhibitory effect of E_2 on HLA class II and CIITA expression.

The effective physiological level of TAM in serum and breast cancer tissue is 10^{-12} M and 10^{-11} M, respectively, which is reached after administration of 1 mg of TAM daily (336). Nevertheless, in our study we used 10^{-6} M, suggesting that *in vitro* blocking of the ER requires higher concentration. This could be due to reduced bioavailability of the drug during experiment time, however, the presence of cytochrome p450, which is abundant in breast adipose tissue (337), increases the drug bioavailability through conversion of the drug into more active metabolites (338). Additionally, the concentration of ICI was also higher than the physiological plasma level of ICI (10^{-10} M) when the drug

is administrated intramuscularly to patients with breast cancer (339), This is in agreement with other studies (213, 315), but not those suggesting a smaller dose (328, 340).

To the best of our knowledge, this is the first report showing that E_2-ER downregulates HLA class II expression through inhibiting CIITA expression. The inhibition of CIITA expression may be due to direct physical interaction of ER with CIITA or through activation of other signaling pathways. E_2 and TAM through their non-genomic effect results in MAPK upregulation and has been reported to interfere with class II expression by hypoacetylation of histone 3 (H3) and H4 (213, 315). Although, in Adamski et. al. (2004) had claimed that E_2 acts non-genomically by reducing class II expression; ER was recruited to the class II promoter in the presence of E_2 suggesting the involvement of the classical E_2-ER signaling effect (315). In agreement with our results, TAM has been shown to supress the maturation of DC, which expresses the ER and downregulates the expression of HLA class II molecules on the cell surface (341). On the other hand, the observation that TAM and E_2 downregulated CIITA expression was contradicted by other studies showing no effect on CIITA expression by PCR and western blot (213, 304, 315).

Although the effect of TAM and ICI on E_2-mediated downregulation of HLA class II was not the same in the current study, Adamski et al, (2005), found that neither ICI nor TAM reversed the inhibitory effect of E_2 on class II expression in immortalised brain endothelial (IBE) cells and thus, E_2, TAM and ICI act non-genomically in inhibiting class II expression (213). Moreover, this suggests that the action of SERM on HLA class II expression is cell specific and dose dependent, as E_2 used in the previous study was three fold higher. It has been reported that ICI prevents estrogen-induced MAPK

activation (342). However, another study reported that ICI activates MAPKs in breast cancer lines (256). MAPK is important in HLA class II regulation (Chapter 6), thus the action of ICI on HLA class II expression in E_2-treated cells may be due to its activation of MAPK. Yet, the results of theER-siRNA suggested that knocking down ER reversed the downregulatory effect of E_2, thus, suggesting implication of E_2-ER signaling in this downregulation. The failure of ICI and *ESR1*-siRNA to upregulate the IFN-γ induced HLA class II in MC2 to VC5 level could be due to residual ERα in MC2, since neither ICI nor *ESR1*-siRNA completely knocked down ERα. Moreover, Tzukerman et. al. (1990) had shown that very low levels of ERα can bind ERE even in the absence of ligand (263).

Ongoing and future studies would be useful to identify the mechanism(s) involved in downregulation of HLA class II expression in $ER\alpha^+$ breast, because downregulation of HLA class II expression occurs in $ER\alpha^+$ breast tumor with advanced age (106).

Chapter 5: Estradiol-estrogen receptor signaling downregulates class II trans activator expression by interfering with the interferon gamma signaling pathway

5.1. Rationale and objectives

Regulation of human leukocyte antigen (HLA) class II occurs mainly at the transcriptional level, through interaction of transcription factors with the class II transactivator (CIITA) (343). In the previous chapters, we have shown that estradiol (E_2) estrogen receptor (ER) signaling downregulates IFN-γ inducible HLA class II and CIITA expression. Downregulation of CIITA occurred at the transcriptional level, suggesting that activation of ER by its ligand inhibits CIITA transcription. IFN-γ induced CIITA transcription depends mainly on promoter IV (pIV) CIITA (343). Thus it is important to establish whether pIV CIITA is activated or repressed by ER activation. Repression of pIV CIITA may be due to interaction of ER with CIITA activation or due inhibitory effect of ER activation on IFN-γ signaling pathway. Based on our findings and previous reports from literature (304), it is plausible that activation of ER diminishes CIITA activation by interfering with the IFN-γ signaling pathway.

1. To test whether E_2-ER signaling interferes with CIITA expression at the promoter level in a breast cancer model.
2. To determine if ER directly inhibits pIV CIITA activity.
3. To test whether E_2-ER interferes with the interferon gamma (IFN-γ) signaling pathway.
4. To determine if E_2-ER interferes with the IFN-γ signaling pathway in breast cancer cells that endogenously express the ERα.

5.2. ERα and E_2 disrupts the activity of IFN-γ induced promoter IV CIITA activity

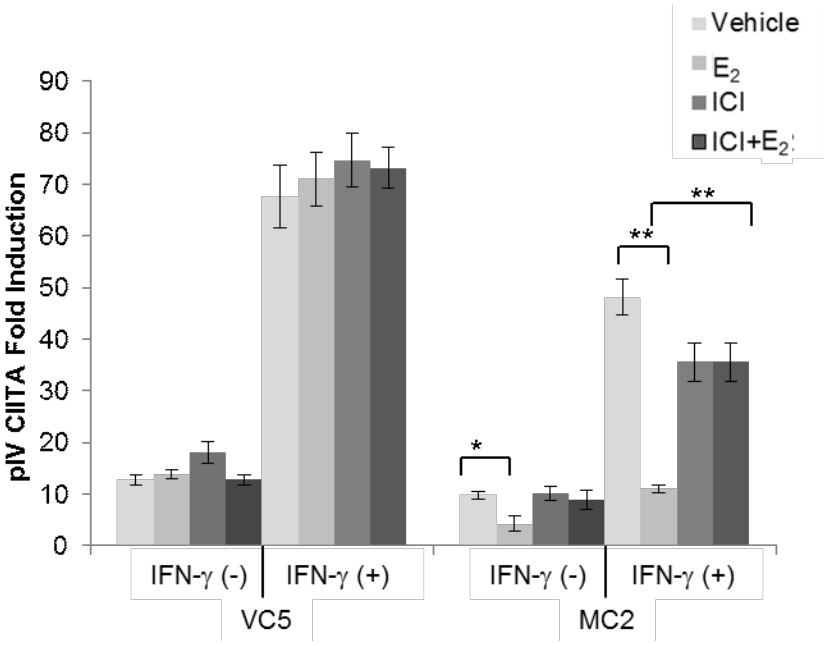
As previously described in Section 1.1.7.2, CIITA is under the control of three different promoters in professional antigen presenting cells (APC). IFN-γ primarily induces pIV, but pIII was shown to be constitutively expressed in some cancer cell lines including melanoma and breast cancer cells (203, 344). Therefore, to test which promoter is active and responsible for inducible CIITA expression in our breast cancer cell line model, we used pIII CIITA and pIV CIITA luciferase constructs.

VC5 and MC2 were transfected with a pIV CIITA luciferase construct and treated with E_2 and/or Fulvestrant (ICI 182,780; ICI), followed by stimulation with IFN-γ for 10 hr, which is optimal (89, 345) (Appendix 2). As expected, constitutive pIV CIITA activity was very low (Figure 5.1) while IFN-γ strongly induced pIV CIITA activation in both cells. However, inducible activity was greatly reduced in MC2, compared to VC5. E_2-treatment significantly reduced constitutive and IFN-γ induced pIV CIITA activity in MC2. ICI alone, or combined with E_2, did not restore the pIV CIITA activity in MC2 to the VC5 level, but reversed the inhibitory effect of E_2. Although there was a trend of decreased CIITA promoter activity with ICI-treatments compared to diluent control in MC2 cells, this was not significant (p0.06). Treatment with ICI and/or E_2 had no significant effect on luciferase activity in VC5. Downregulation of pIV CIITA activity by E_2-ER signaling was consistent with downstream HLA class II genes promoter activity (Appendix 3). These results parallel our previous findings described in Chapter 4, and suggest that E_2-ER activation is involved in CIITA regulation at the promoter level.

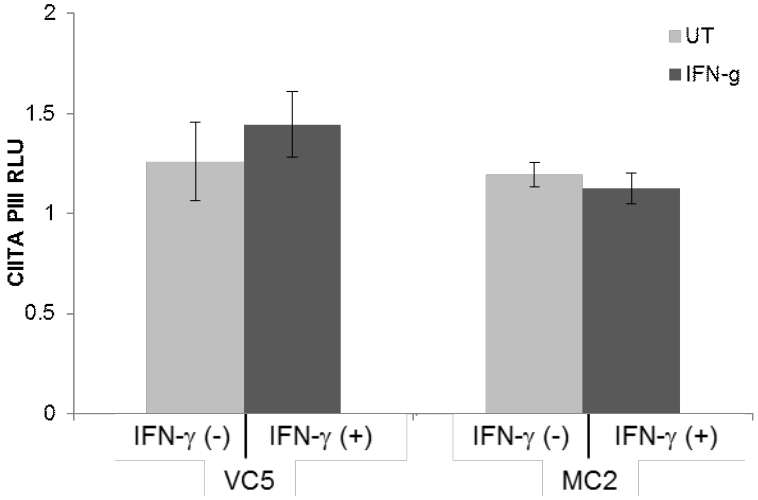
Figure 5.1 The E_2-ER signaling pathway interferes with promoter IV CIITA activity in MC2.

VC5 and MC2 cells were transfected with (A) pIV CIITA luciferase constructs or (B) pIII CIITA luciferase constructs. The next day, the cells were either treated or not with (E_2) or/and ICI stimulated or not with IFN-γ (100 Units/ml) for 10 hr. pIV CIITA activity is expressed as fold induction over the PGL2 basic empty plasmid after controlling for transfection efficiency using cells dual transfected with green fluorescence protein (GFP). Error bars represent the mean ± standard errors of the mean (SEM) of three independent experiments (**$p<0.0$, * $p<0.051$). pIII CIITA is expressed in the form of raw data (relative light unit (RLU)) and is a representative of one experiment.

A


Vehicle
E2
ICI
ICI+E2
pIV CIITA Fold Induction
90
80
70
60
50
40
30
20
10
0
*
**
**
IFN-γ (-)
IFN-γ (+)
IFN-γ (-)
IFN-γ (+)
VC5
MC2

B


UT
IFN-g
CIITA PIII RLU
2
1.5
1
0.5
0
IFN-γ (-)
IFN-γ (+)
IFN-γ (-)
IFN-γ (+)
VC5
MC2

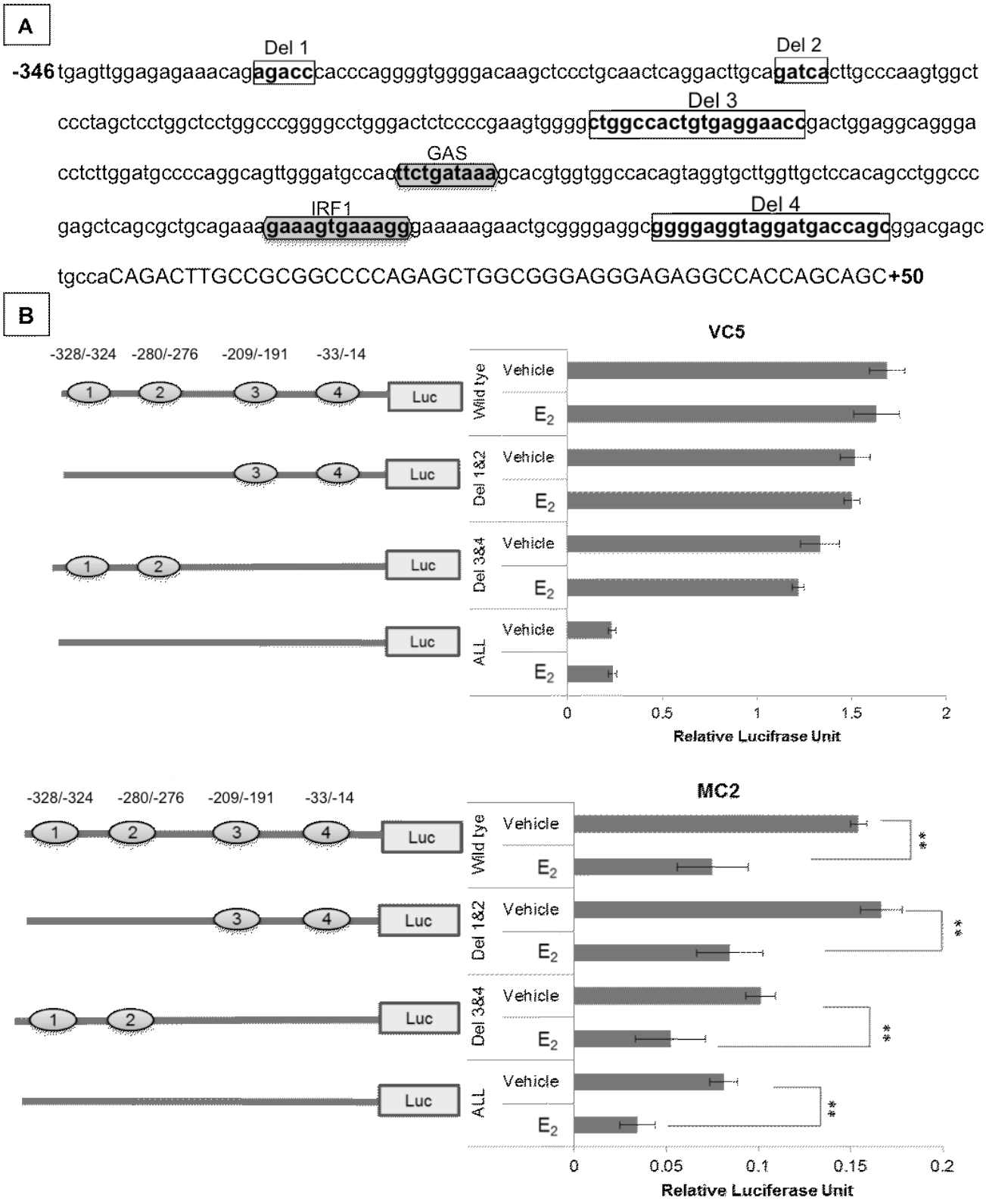
Since the IFN-γ induced pIII CIITA response depends on the cell context (345), we questioned if pIII was activated in response to IFN-γ in our BCCL model. VC5 and MC2 were transiently transfected with pIII CIITA as described for pIV CIITA. No significant difference was noted between the basal and the IFN-γ induced pIII CIITA activities in the two cell lines. Additionally, there was no significant difference in pIII CIITA activity between the VC5 and MC2 cell lines (Figure5.1B). These results suggest that ER does not interfere with CIITA at the pIII level.

5.3. Mutation of ERE in pIV CIITA does not enhance pIV CIITA activation in MC2.

As these data further support involvement of ERα signaling in repressing CIITA transactivation, we questioned whether ERα bound directly to pIV CIITA. Computer analysis software (Section 2.13.1) revealed four estrogen response element (ERE) in pIV CIITA (Figure 5.2A, bold letters), which could potentially bind ERα and repress pIV CIITA transactivation. To test this, three mutant constructs were created (Figure 5.2A, open boxes) and together with the wild type pIV CIITA plasmid were used to transfect VC5 and MC2 (Figure. 5.2B, left panel). Cells were treated with E_2 or vehicle control and stimulated with IFN-γ for 12 hr, followed by determination of luciferase activity. Comparing the mutants to wild type, it can be seen that the plasmid containing four successive deletion fragments significantly reduced promoter activity in both VC5 and MC2 (Figure 5.2 B, right panels). However, the plasmid containing deletions one and two and the plasmid containing deletions three and four did not affect pIV CIITA promoter activity.

Figure 5.2 Mutation of ERE in pIV CIITA does not enhance pIV CIITA activation in MC2.

(A) pIV CIITA sequence with the predicted ERE. Site directed mutagenesis was used to perform deletion of the predicted ERE. (B) VC5 and MC2 were transfected with pIV CIITA constructs with the selected deletions, followed by replacment with fresh medium containing vehicle (ethanol) or E_2 (10^{-9}M) and stimulated with IFN-γ (100 Units/ml) for 10 hr. Cells were lysed in passive lysis buffer and luciferase activity was measured and expressed in the form of raw data (relative light unit (RLU)). Bar graphs represent the mean ± SEM of three independent experiments (**$p<0.01$). Yumiko Komastsu created the deletion constructs in this study.


A
Del 1
Del 2
-346 tgagttggagagaaacagagacccacccaggggtggggacaagctccctgcaactcaggacttgcagatcacttgcccaagtggct
Del 3
ccctagctcctggctcctggcccggggcctgggactctccccgaagtggggctggccactgtgaggaaccgactggaggcaggga
GAS
cctcttggatgccccaggcagttgggatgccacttctgataaagcacgtggtggccacagtaggtgcttggttgctccacagcctggccc
IRF1
Del 4
gagctcagcgctgcagaaagaaagtgaaaggaaaaagaactgcggggaggcggggaggtaggatgaccagcggacgagc
tgccaCAGACTTGCCGCGGCCCCAGAGCTGGCGGGAGGGAGAGGCCACCAGCAGC+50
B
VC5
-328/-324
-280/-276
-209/-191
-33/-14
Luc
Wild tye
Del 1&2
Del 3&4
ALL
Vehicle
E2
0
0.5
1
1.5
2
Relative Lucifrase Unit
MC2
0
0.05
0.1
0.15
0.2
Relative Luciferase Unit

The deletion constructs in E_2-treated MC2 (Figure 5.2B, right panel) resulted in reduced activity, which was similar to that observed for transfection of the wild type pIV CIITA plasmid. This, combined with lack of restoration of pIV CIITA activity in MC2, makes it unlikely the mechanism by which ER-E_2 or ER mediates down regulation of pIV CIITA activity is ERα repression via binding to ERE in pIV CIITA. This does not preclude ER binding to other regulatory elements within CIITA. However, considering the negative impact of E_2-ER activation on pIV CIITA, which is activated by IFN-γ, we hypothesized that the E_2-ER activation may negatively impact another factor required for CIITA activation such as IRF1.

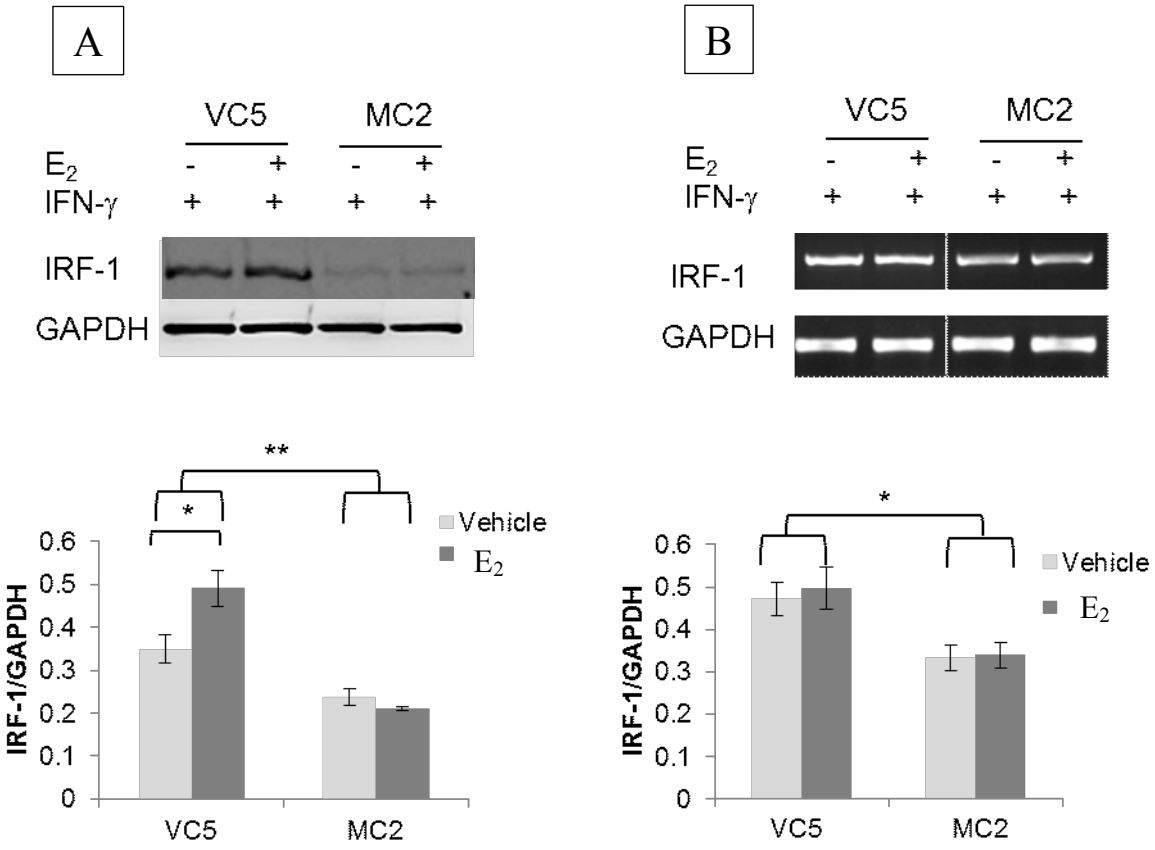
5.4. ERα interferes with IRF1 expression in ERα$^+$ breast cancer cells

IFN-γ-inducible expression of CIITA and subsequent HLA class II expression requires interferon regulatory factor I (IRF1) (35). IRF1 is induced early by IFN-γ through a signal transducer and transactivation 1 (STAT1) dependent mechanism and acts as a transcriptional activator for CIITA expression (37). To determine if E_2-ER signaling inhibits IRF1 expression in ERα$^+$ breast cancer cells, MC2 and VC5 were treated with E_2 or vehicle control and stimulated with IFN-γ. Whole cell lysates were prepared and analyzed by immunoblotting.

Overall expression of IRF1 was significantly reduced ($p<0.01$) in MC2 compared to VC5 (Figure 5.3A). Moreover, the relative expression of IRF1 for three independent experiments revealed that E_2-treatment increased constitutive IRF1 in VC5, and somewhat reduced its expression in MC2 (Figure 5.3A-lower panel, Appendix 4).

Figure 5.3 IRF1 is significantly reduced in MC2 compared to VC5.

VC5 and MC2 were treated with vehicle (ethanol), E_2 (10^{-9}M) stimulated or not with IFN-γ (100 Units/ml). A) Whole cell lysates after 96 hr of IFN-γ stimulation probed with IRF1 antibody and GAPDH as a loading control. B) RT-PCR using IRF1 specific primers after 4 hr of IFN-γ stimulation. IRF1 were normalized to GAPDH. Bar graphs represent the mean ± SEM of three independent experiments (**$p<0.01$, * $p<0.05$).


A
VC5
MC2
E2
IFN-γ
IRF-1
GAPDH
B
VC5
MC2
E2
IFN-γ
IRF-1
GAPDH
**
*
Vehicle
E2
IRF-1/GAPDH
0.6
0.5
0.4
0.3
0.2
0.1
0
VC5
MC2
*
Vehicle
E2
IRF-1/GAPDH
0.6
0.5
0.4
0.3
0.2
0.1
0
VC5
MC2

Further, analysis of IRF1 transcripts by RT-PCR (Figure 5.3B-upper panel) confirmed significant reduction of IRF1 in MC2 compared to VC5 ($p<0.05$) (Figure 5.3B-lower panel).

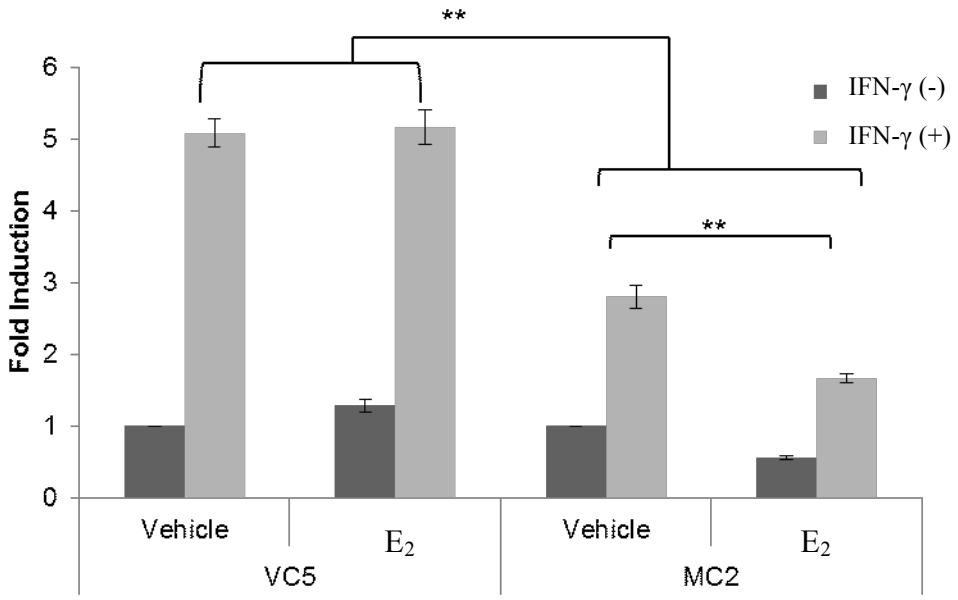
5.5. ERα interferes with IFN-γ signaling in MC2

Since IRF1 and CIITA expression are both dependent on IFN-γ signaling, we speculated that the downregulation observed in MC2 cells, was due to global downregulation of IFN-γ signaling. To test this hypothesis, VC5 and MC2 were transfected with the 8X IFN-γ activated sequence (GAS) luciferase plasmid, followed by E_2 treatment and stimulation, or not, with IFN-γ. We observed that GAS promoter activity in IFN-γ treated MC2 was about 50% of that for VC5. Moreover, E_2-treatment further diminished GAS activity in MC2, but not in VC5 (Figure. 5.4). As expected, GAS promoter activity was low in the absence of IFN-γ, however, it is notable that while E_2-treatment had a negligible effect on constitutive GAS activity in VC5, it significantly reduced activity in MC2.

These data suggest that E_2-ER signaling indirectly decreases CIITA transactivation, by repressing IFN-γ signaling. To determine which element in the IFN-γ pathway is involved, MC2 and VC5 were treated with either E_2 or vehicle control. The cells were stimulated with IFN-γ and IFN-γ receptor 1 (IFNGR1) expression was measured using CD119 antibody and analyzed by surface flow cytometry. As shown in Figure 5.5, both cells express IFNGR1 constitutively and the number of CD119 positive cells (Figure 5.5A) and staining intensity (Figure 5.5B) were approximately the same for

Figure 5.4 E_2-treatment reduces GAS promoter activity in MC2, but has no effect in VC5.

VC5 and MC2 were transfected with 8X GAS construct. On the subsequent day, the cells were treated with vehicle (ethanol), E_2 (10^{-9} M) and stimulated or not with IFN-γ (100 Units/ml) for 6 hr. Firefly luciferase activities in samples were normalized to Renilla luciferase activities in the same sample and expressed as fold induction over the un-stimulated mock. Error bars represent the mean ± SEM of three independent experiments (*$p<0.05$, ** $p<0.01$).

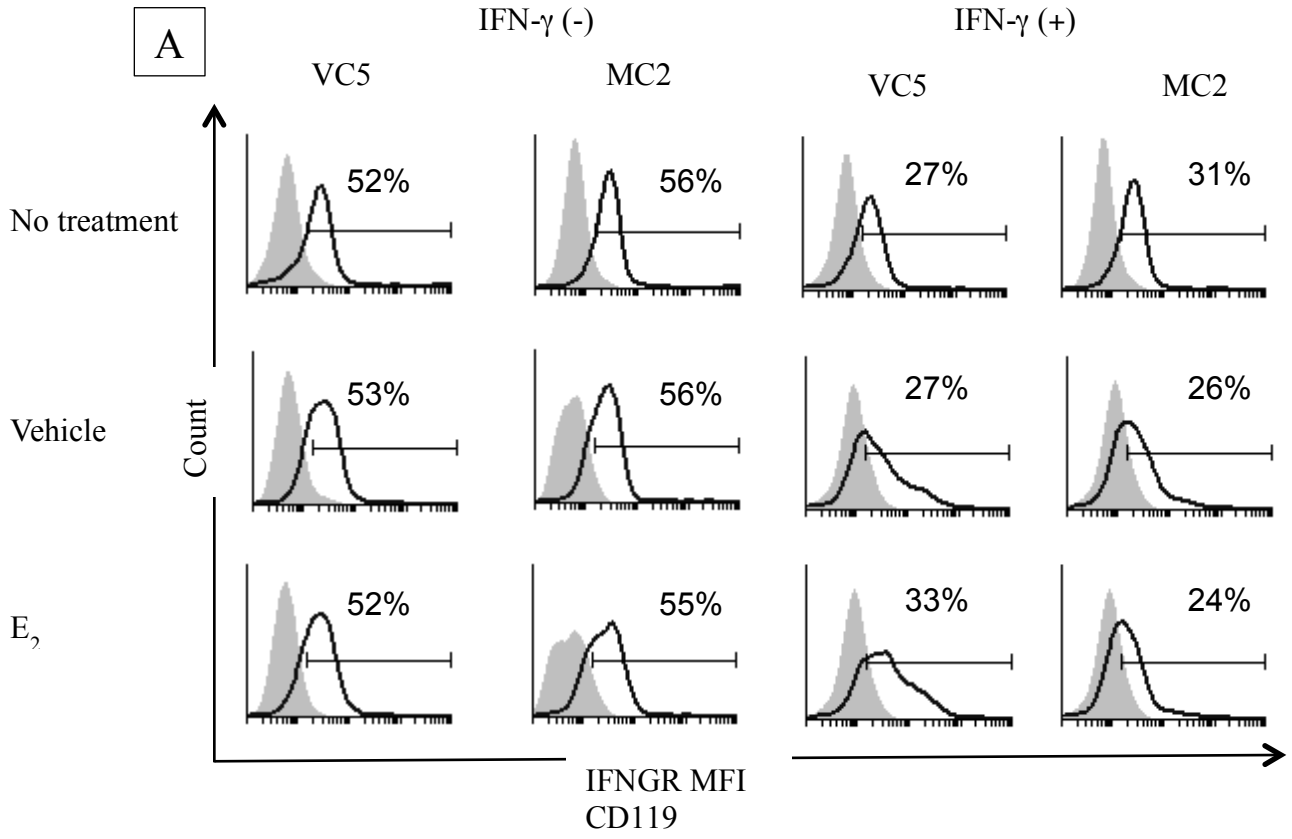

**
**
IFN-γ (-)
IFN-γ (+)
Fold Induction
0
1
2
3
4
5
6
Vehicle
E2
Vehicle
E2
VC5
MC2

both MC2 and VC5, regardless of E_2-treatment. IFN-γ stimulation resulted in decreased surface IFNGR1 as shown by the decreased number (Figure 5.5A) and staining intensity of CD119 positive cells (Figure 5.5B). This is likely due to increased internalisation of the receptor by IFN-γ treatment as has been described by others (346). Furthermore, no significant changes were detected in the E_2 treated cells, suggesting that E_2-ER signaling does not interfere with IFN-γ signaling by downregulation of IFNGR1 in this BCCL model.

IFNGR1 lacks intrinsic kinase/phosphatase activity and thus depends on other kinases like Janus kinase (JAK)1, JAK2 and STAT1 for signal transduction (346). To determine which IFN-γ pathway element is involved, MC2 and VC5 were stimulated, or not, with IFN-γ for 15 min and whole cell lysates were prepared and subjected to immunoblotting. JAK1 and JAK2 were constitutively phosphorylated and expressed in both VC5 and MC2 (Figure 5.6). Surprisingly, phosphorylation of both JAK1 and JAK2 was greater in MC2 than VC5 and correlated with JAK1 and JAK2 total expression. This indicates that ER interference with IFN-γ signaling is not because of lack of JAK1/2 phosphorylation. On the other hand, STAT1 phosphorylation at both sites, Y^{701} and S^{727}, was diminished in MC2 compared to VC5, and the same pattern was noticed in total STAT1. Interestingly, the activation of ERα in the absence of ligand as indicated by S^{118} phosphorylation suggests that, signaling pathways other than E_2 induce its activation (347). This activated ER may account for the downregulation of HLA class II expression seen in the absence of ligand activation (Chapter 3). Overall, these results suggest that ERα interferes with STAT1 activation and, thus, downregulates IFN-γ signaling in MC2.

Figure 5.5 No significant difference in expression of IRF1 in BCCL model.

VC5 and MC2 were cultured in estrogen-depleted media, treated with vehicle (ethanol) or E_2 (10^{-9} M) and stimulated or not with IFN-γ (100 Units/ml) for 96 hr. (A) IFNGR1 surface expression (detected by CD119) was analysed by flow cytometry. Shaded histogram = isotype control, black line = IFNGR1 expression with the indicated % of cells (B) Bar graphs represent the mean fluorescence intensity (MFI) for IFNGR1 expression. Figure indicates one experiment.


A
IFN-γ (-)
IFN-γ (+)
VC5
MC2
VC5
MC2
No treatment
Vehicle
E2
Count
52%
56%
27%
31%
53%
56%
27%
26%
52%
55%
33%
24%
IFNGR MFI
CD119

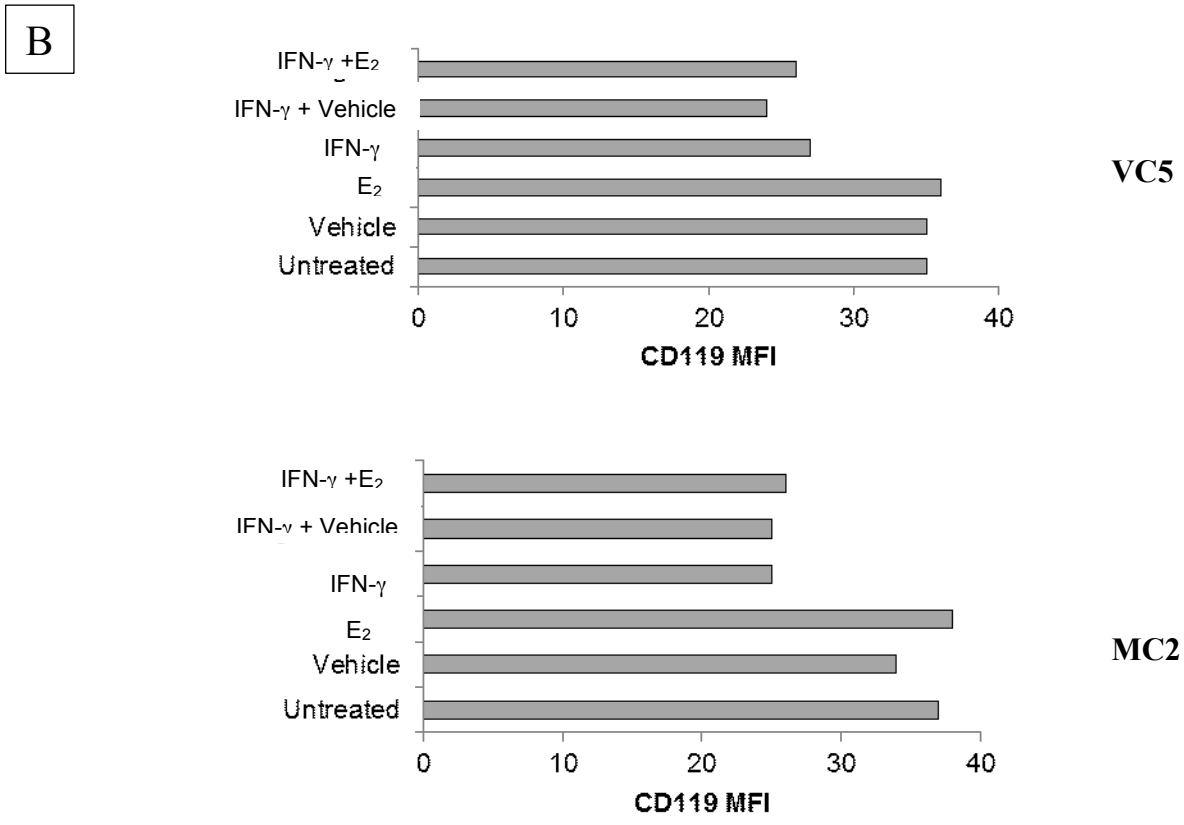
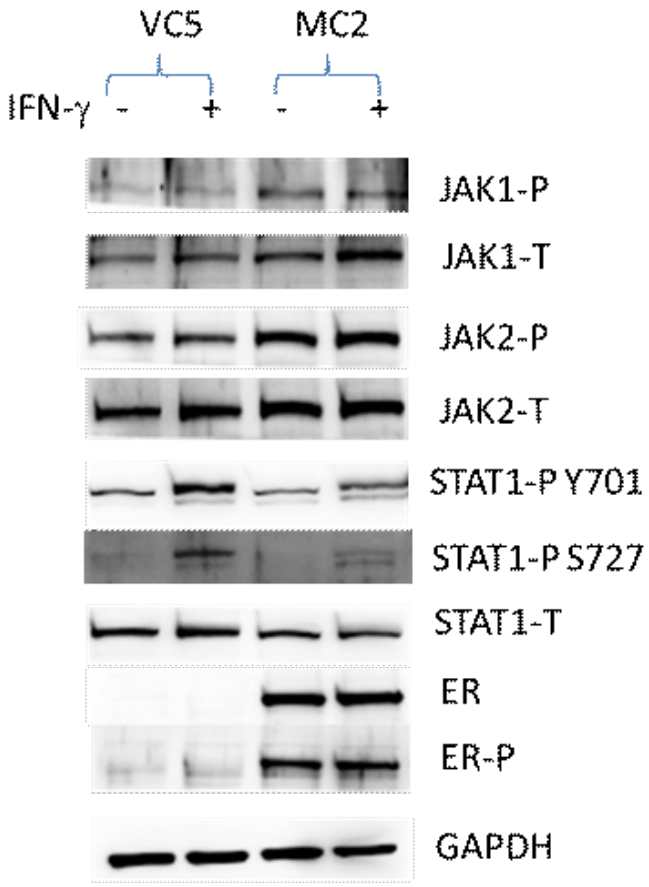

B
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
0
10
20
30
40
CD119 MFI
VC5
IFN-γ +E2
IFN-γ + Vehicle
IFN-γ
E2
Vehicle
Untreated
0
10
20
30
40
CD119 MFI
MC2

Figure 5.6 ERα interferes with STAT1 activation in MC2.

VC5 and MC2 cells were cultured in estrogen-depleted media and stimulated with or without IFN-γ (100 Units/ml) for 15 min and whole cell RIPA lysates were prepared and subjected for immunoblotting using the indicated antibodies. Figure indicates one experiment.


VC5
MC2
IFN-γ - + - +
JAK1-P
JAK1-T
JAK2-P
JAK2-T
STAT1-P Y701
STAT1-P S727
STAT1-T
ER
ER-P
GAPDH

We questioned whether E_2 further downregulates STAT1 phosphorylation in MC2 since E_2 downregulated inducible HLA class II expression (Chapter 3). To test this, MC2 and VC5 were treated with E_2 and/or ICI, followed by stimulation with IFN-γ. Immunoblotting of whole cell lysates showed overall STAT1 phosphorylation was diminished in MC2, no matter the treatment, compared to VC5 (Figure 5.7A). Surprisingly, E_2 treatment did not further downregulate STAT1 phosphorylation in MC2, and similar results were seen with ICI or the combination of ICI and E_2. These results may suggest that ER downregulates IFN-γ signaling by interfering with STAT1 phosphorylation, however, the additional downregulation of IFN-γ signaling by E_2 in $ERα^+$ BCCL is through an alternative mechanism.

We next asked whether STAT1 directly interacts with ERα in MC2 cells. STAT1 and ERα were co-immunoprecipitated from MC2 cells after stimulation with IFN-γ and immune complexes were probed for ER and STAT1, respectively, by ERα or STAT1 total antibody. Figure 5.7B showed that neither STAT1 nor ER co-immunoprecipitated, suggesting that there is no direct interaction between both proteins.

5.6. E_2 modulates IFN-γ signaling and IFN-γ induced proteins in endogenous $ERα^+$ BCCL

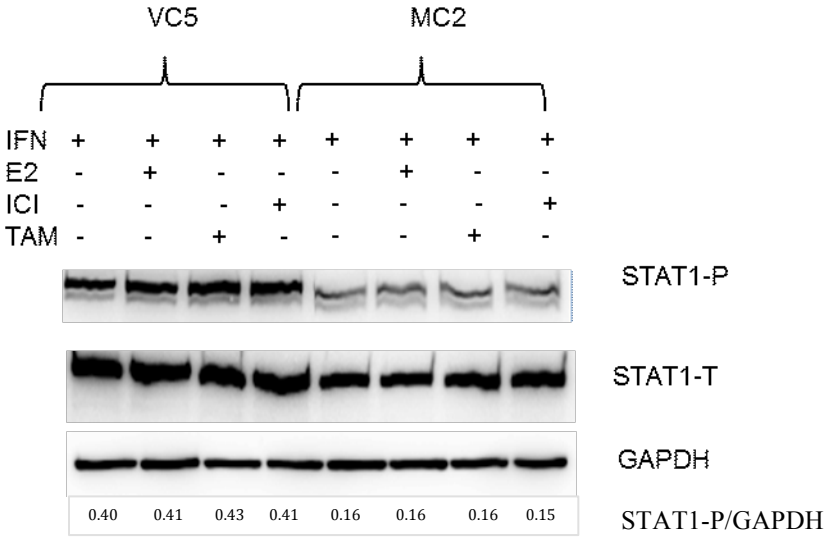
To ensure that diminished IFN-γ signaling in MC2 was not merely a peculiarity of the transfected cell model, we further analyzed GAS promoter activity in endogenous $ERα^+$ BCCL: MCF-7, BT-474 and T47D, and $ERα^-$ BCCL: MDA-MB-231 and SK-BR-3. Comparing E_2 to vehicle-treated cells, E_2 decreased constitutive and IFN-γ induced GAS

Figure 5.7 E_2-ER signaling doesn't impede with STAT1 phosphorylation.

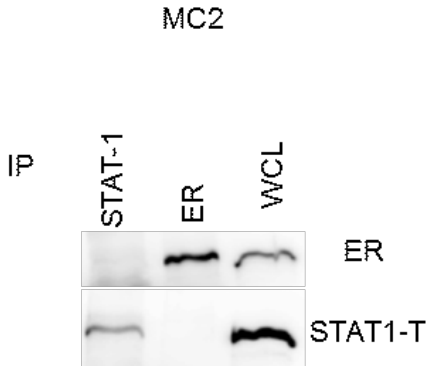
(A) VC5 and MC2 cells were cultured in estrogen-depleted media, treated with vehicle (ethanol), E_2 (10^{-9} M) and /or ICI (10^{-6} M), for 4 hr and stimulated with IFN-γ (100 Units/ml) for 15 min. Whole cell RIPA lysates were then prepared and subjected to immunoblotting. STAT1 phosphorylation was expressed relative to GAPDH using densitometry. Figure indicates one experiment.

(B) No direct interaction between STAT1 and ERα. MC2 was subjected to immunoprecipitation with anti-STAT1 and anti-ERα antibody followed by immunoblotting with antibodies as indicated. Whole cell lysate from MC2 was used as a positive control. Figure indicates one experiment.

A


VC5
MC2
IFN + + + + + + + +
E2 - + - - - + - -
ICI - - - + - - - +
TAM - - + - - - + -
STAT1-P
STAT1-T
GAPDH
0.40 0.41 0.43 0.41 0.16 0.16 0.16 0.15
STAT1-P/GAPDH

B


MC2
IP
STAT-1
ER
WCL
ER
STAT1-T

activity in all ERα$^+$ cell lines (Figure 5.8A-C), however, the difference between vehicle and E_2 was significant for IFN-γ inducible activity in MCF-7 and BT-474, (Figure 5.8A-B) but not for T47D (Figure 5.8C). To determine whether the diminished GAS activity resulted in reduction in IFN-γ inducible proteins other than HLA class II, we immunoblotted lysates described in Figure 3.1 and Figure 3.2 for STAT1 and selected downstream STAT1 activated components. These included IRF1, essential for pIV CIITA activity and subsequent HLA class II expression; IRF9, a member of the IRF family of transcription factors that is not implicated in CIITA expression (348); gamma-IFN-inducible lysosomal thiol reductase (GILT), an enzyme regulated by STAT1 but CIITA-independent protein, that is important for antigen processing(349).

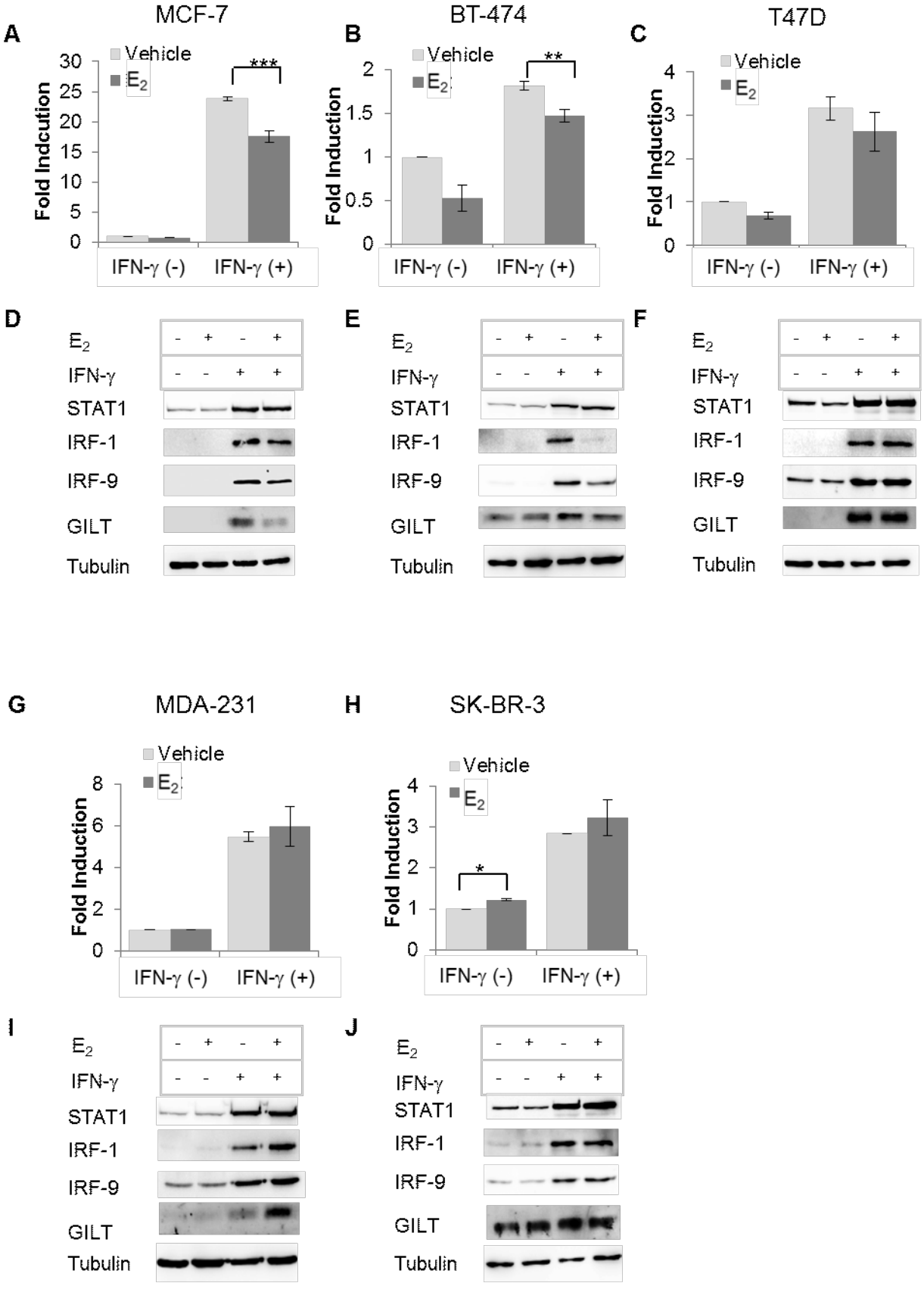
Despite the E_2-mediated reduced GAS activity, basal and IFN-γ inducible STAT1 levels were not substantially altered by E_2 in either cell line, (Figure 5.8D-F); however, STAT1 regulated proteins were differentially modulated in E_2-treated MCF-7 and BT-474 (Figure 5.8D-E). IRF1 and IRF9 were slightly decreased in MCF-7, while GILT was markedly reduced (Figure 5.8D). Notably, IRF1 was abolished in BT-474 (Figure 5.8E); IRF9 was reduced and GILT, which was present constitutively, was barely modulated by E_2. E_2-treated T47D cells (Figure 5.8F), which displayed decreased GAS promoter activity (Figure 5.8C), showed slightly reduced basal levels of STAT1 and IRF9, while IFN-γ inducible proteins were similar in E_2-treated and untreated cells.

In contrast to E_2-mediated inhibitory effect on GAS promoter activity in the ERα$^+$ lines, E_2 slightly enhanced GAS promoter activity in ERα$^-$ BCCL, MDA-MB-231, and SK-BR-3 (Figure. 5.8G-H). Furthermore, E_2-treated MDA-MB-231 augmented IRF1 and

GILT expression, whereas E_2-treated SK-BR-3 augmented STAT1 expression (Figure 5.8I-J). Taken together, the results support an opposing role for E_2 modulation of the IFN-γ and HLA II pathways in $ER\alpha^+$ and $ER\alpha^-$ BCCL.

Figure 5.8 E_2 differentially downregulates IFN-γ signaling and IFN-γ induced proteins in endogenous $ER\alpha^+$ BCCL.

(A) MCF-7, (B) BT-474, (C) T47D, (G) MDA-231, and (H) SK-BR-3 were cultured in estrogen-depleted media and transfected with 8X GAS construct. The next day, cells were treated with vehicle (ethanol), E_2 (10^{-9}M) and stimulated with IFN-γ (100 Units/ml) for 6 hr. Firefly luciferase activities in samples were normalized to Renilla luciferase activities in the same sample and expressed as fold induction over the un-stimulated control. (D) MCF-7, (E) BT-474, (F) T47D, (I) MDA-MB-231 and (J) SK-BR-3 were cultured in estrogen-depleted media, treated with vehicle (ethanol), or E_2 (10^{-9}M) and stimulated or not with IFN-γ (100 Units/ml) for 96 hr. Immunoblot analysis on cytoplasmic extracts was performed for expression of IFN-γ inducible proteins: STAT1 (06-501), IRF1 (BD-20), IRF9 (C-20), GILT (T-18), HLA-DRα (Tal 1B5). Each figure represents one of three independent experiments.


A
MCF-7
Vehicle
E2
***
Fold Indcution
30
25
20
15
10
5
0
IFN-γ (-)
IFN-γ (+)
B
BT-474
Vehicle
E2
**
Fold Induction
2
1.5
1
0.5
0
IFN-γ (-)
IFN-γ (+)
C
T47D
Vehicle
E2
Fold Induction
4
3
2
1
0
IFN-γ (-)
IFN-γ (+)
D
E2
IFN-γ
STAT1
IRF-1
IRF-9
GILT
Tubulin
E
E2
IFN-γ
STAT1
IRF-1
IRF-9
GILT
Tubulin
F
E2
IFN-γ
STAT1
IRF-1
IRF-9
GILT
Tubulin
G
MDA-231
Vehicle
E2
Fold Induction
8
6
4
2
0
IFN-γ (-)
IFN-γ (+)
H
SK-BR-3
Vehicle
E2
*
Fold Induction
4
3
2
1
0
IFN-γ (-)
IFN-γ (+)
I
E2
IFN-γ
STAT1
IRF-1
IRF-9
GILT
Tubulin
J
E2
IFN-γ
STAT1
IRF-1
IRF-9
GILT
Tubulin

5.7. Discussion

Expression of CIITA in antigen presenting cells is cell specific and under the control of 3 different promoters as discussed in Section 1.1.7.2. Similarly, in cancer cells the activation of these promoters is also cell specific and dependent on other activated signaling pathways (203, 344). Herein, we have shown that pIII CIITA luciferase activity was constitutively active and IFN-γ did not result in further activation. This was inconsistent with RT-PCR results using primers specific for pIII CIITA (Appendix 5). In the latter, IFN-γ induced CIITA isoform III expression in both VC5 and MC2, and the expression was less in MC2 compared to VC5. The mechanism of IFN-γ induction on pIII CIITA activity is somewhat controversial. It has been suggested that both pIII and pIV may act by enhancing each other due to the short genomic distance between the two promoters (77). However, Piskurich et. al. (1998) reported that the presence of a STAT1 binding site, which is located in the enhancer region of pIII that is 5 Kb from the transcription initiation site, is responsible for IFN-γ induced activation through JAK/STAT1 signaling pathway (89). This mechanism is likely to be cell specific and not a generalised mechanism (350-352). Inactivation of IFN-γ induced pIII CIITA is in agreement with others using breast cancer cells (353) and non-breast cancer cells (88). Others have shown that pIII is induced by IFN-γ in cancer cell lines such as U373-MG, a human glioblastoma astrocytoma, and 2fTGH, a human fibrosarcoma cell (89).

To the best of our knowledge, this is the first report, which shows that ER and E_2 reduce pIV CIITA activity. These results likely explain the reduced HLA class II surface

expression, signifying that downregulation of HLA class II expression is at the promoter level of CIITA.

One of the mechanisms responsible for downregulation of IFNγ-induced CIITA transcription is hypermethylation of pIV CIITA DNA (353, 354). In addition to hypermethylation, it has also been suggested that downregulation of IFN-γ induced CIITA in several cancer types is linked to increased histone deacetylase activity (71, 72). The ability of ER to influence gene activity depends on a number of co-activators, including SRC1, CBP, p300, PCAF, CARM1, co-repressors such as silencing mediator for retinoid or thyroid-hormone receptors (SMRT), nuclear receptor co-repressor (NCoR), metastasis associated gene 1 protein (MTA1), and histone modifying enzymes, which are frequently associated to form multi-protein complexes (355-357). Some of these co-activators are shared with IFN-γ signaling such as SRC1 (358), CBP/p300 (359) and CARM1 (360), which may explain why certain IFN-γ inducible genes, but not all of them are downregulated by E_2-ER signaling (Figure 5.8). E_2 and ER were shown to deregulate cellular epigenetic processes through alteration of DNA methylation and histone modifications (361). Moreover, E_2 in the absence of its cognate receptor results in hypoacetylation of histone 3 (H3) and H4, which results in gene repression (213). Since the only difference between MC2 and VC5 is the presence of ER, the diminished pIV activity and subsequently, the downregulation of HLA class II expression could be explained by E_2-ER epigenetically targeting pIV CIITA or other genes required for its activation like STAT1, IRF1 and USF1. This was strengthened by Ginter et. al. (2012), where they showed that hypoacetylation of STAT1 is required for IFN-γ to induce

STAT1 activation (362). ERα directly or indirectly may interact through the various acetylases/deacetylases and methylases/demethylases and affect essential post-translational histone modifications of CIITA. Thus future studies are needed to understand the mechanism(s) involved.

Seventy five percent of ER gene regulation occurs by binding of ER to ERE binding sites (363). We hypothesised that E_2-ER binds directly to the predicted ERE in pIV CIITA, and acts as a repressor for pIV CIITA. Our results did not support this hypothesis, but they do not exclude an alternative binding site such as AP1 or SP1. Furthermore, we only explored the 5' untranslated proximal CIITA region for ERE binding sites and it was recently shown using genome wide analysis that only 10% of ERE are identified in the 5' untranslated region. The majority of the ERE are present in the intronic or intergenic regions of all target genes (363). Thus, ER can regulate genes distally from their transcription initiation site, which could be further evaluated in the CIITA gene.

The suppresser effect of ER on IRF1 was not surprising since it was previously shown that IRF1 was constitutively expressed in the $ER\alpha^-$ BCCL: SK-BR-3, MDA-MB 468, and BT-20, but not in the $ER\alpha^+$ BCCL: MCF-7. Moreover, following IFN-γ stimulation, MCF-7 expressed 50% less nuclear staining for IRF1 and 50% more IRF2 when compared to the ER^- BCCL, suggesting that ER interferes with nuclear localisation of IRF1 (364). Activated IRF1 should be expressed in the nucleus while the inactivated form is in the cytoplasm (365). Indeed cytoplasmic IRF1 expression was shown to positively correlate with $ER\alpha^+$ breast tumors (366), suggesting that ER may regulate IRF1

expression, possibly by interfering with its synthesis or its cellular distribution. Parallel to this finding, ICI, which results in ER degradation, induces IRF1 expression in MCF-7 in a dose dependent manner. The latter results correlate with our finding (Appendix 6) in which ICI induced CIITA expression in MCF-7 in a dose dependent manner. $ER\alpha^+$ breast cancer cells are reported to suppress IRF1 as a way to develop anti-estrogen resistance (367), because upregulation of IRF1 by IFN-γ resulted in increased sensitivity of $ER\alpha^+$ BCCL sensitivity to ICI (368). The significant downregulation of IRF1 by E_2 detected in $ER\alpha^+$ cells was consistent with that reported by Bouker et al. (2004), who showed that E_2-treated MCF-7 had significantly decreased IRF1 expression (369).

The lack of transactivation of pIV CIITA is not due to global defective IFN-γ signaling. HLA class I, which is also induced by IFN-γ was not diminished by ER and E_2 (Figure 3.7). This suggests that ER-E_2 is targeting specific IFN-γ inducible genes. Although HLA class I is constitutively expressed in all nucleated cells, IFN-γ is still required for enhancement of expression by a number of mechanisms either directly or indirectly (370). IFN-γ via JAK/STAT signaling induces HLA class I expression by upregulating NFκβ and IRF1, which bind to enhancer A and ISRE binding sites, respectively, in the promoter of HLA class I genes to enhance transcription (371). Moreover, activation of HLA class I by IFN-γ is not only dependent on STAT1 and IRF1, but other factors have been shown to be involved and function independently of STAT1 and IRF1. Binding-IFN-γ activated factor (BIGAF) is a nuclear transcription factor which can bind to the enhancer in HLA class I genes and aid in the process of IFN-γ induced activation (372). Additionally, STAT1 may form a complex with STAT2 and IRF9 (P48)

and bind to ISRE in the HLA class I gene promoter (373). Recently, it was reported that nucleotide-binding oligomerization domain (NOD) like receptor family CARD domain containing 5 (NLRC5) acts in a manner similar to CIITA and binds to transcription factors bound to those regulatory elements (52). This suggests that HLA class I regulation is a multifaceted mechanism and depends on a number of regulatory factors. Unlike the CIITA, they are not totally dependent on STAT1 activation.

Although we did not find any difference with regards to IFNGR1 expression between MC2 and VC5, future studies are needed to localize IFNGR1 after IFN-γ stimulation. Nuclear localisation sequences (NLS) are present in the C-terminus of IFNGR1, which direct the receptor to the nucleus after IFN-γ stimulation for direct interaction with STAT1 (346). It is possible that this may result in cross talk with ER in the nucleus.

Upregulation of JAK1 and JAK2 in MC2 was somewhat surprising since it was previously shown that both JAKs correlate negatively with ERα status in breast tumors and cell lines (374, 375). Moreover, it was shown that E_2-ER signaling upregulates suppressors of cytokine signaling (SOCS)-2, which result in inhibition of JAK phosphorylation (376). These data suggest that E_2-ER can negatively regulate IFN-γ signaling by altering JAK2 phosphorylation through SOCS-2 upregulation. Cells that endogenously express ER may behave differently from cells that have been stably transfected to express the ER (287). Moreover, a human neuroblastoma cell line that had been stably transfected with ERα upregulated both JAK1 and JAK2 as confirmed by microarray analysis (377). Recently, it was shown E_2 activation of ER in MCF-7 resulted

upregulation of JAK2 expression as detected by immunoblotting (378). JAK activation was not reduced in MC2 compared to VC5 cells, thus, excluding any defect in upstream signalling such as IFNGR2, which is the limiting factor of IFNγ signalling. Since phosphorylation of STAT1 at Y701 and S727 was diminished in MC2, as compared to VC5 and correlated with reduced GAS activity, it is likely that reduced CIITA promoter activity in MC2 was due to decreased activation of STAT1, which is critical for induction of pIV CIITA. Although our results clearly showed that E_2 and ICI had no effect on STAT1 phosphorylation, this does not exclude the possibility that E_2 may interfere with STAT1 hypoacetylation, dimerization, nuclear localisation, and binding to a specific promoter (362). E_2 was reported to interfere with nuclear localisation of STAT1 and result in post-translational modification in the form of STAT1 truncation (379). Identifying STAT1 function by studying phosphorylation alone is not sufficient because, the level of phosphorylation may not be affected, but the recruitment to pIV CIITA can be reduced (380).

Estrogen can mediate its action by a non-genomic signaling pathway through interaction with other transcription factors. Therefore, we investigated the effect of direct interaction of ER with STAT1 after IFN-γ stimulation. There was no evidence of direct interaction of STAT1 and ER in the whole cell lysates but this did not exclude the possibility of interaction in the nuclear matrix or at the DNA binding site. Thus, future studies are needed to investigate the effect of E_2 on STAT1 and should look for STAT1 expression in different cell compartments.

The MC2 cell model of ERα$^+$ breast cancer cells showed that E_2-ER regulates inducible HLA class II expression by interfering with the IFN-γ signaling pathway. This finding was similar to the results seen in endogenously ERα$^+$ BCCL in which we showed that E_2 decreased GAS activity in MCF-7 and BT-474. These findings paralleled HLA-DR surface expression. Moreover, immunoblotting confirmed that other IFN-γ inducible proteins are reduced by E_2 treatment in the same ERα$^+$ breast cancer cells, but not in ERα$^-$ breast cancer cells. These results suggest the presence of common mechanisms for downregulation of HLA-DR expression in ER$^+$ breast cancer cells, which is probably by interfering with element(s) of IFN-γ signaling pathway. E_2 has been shown to interfere with IFN-γ genes and proteins in a number of occasions. For example in keratinocytes of psoriatic skin lesions, E_2 inhibited the production of interferon-induced protein of 10 kDa (IP-10), and indirectly inhibited STAT1 activation by stimulating adenylate cyclase, which resulted in upregulating 3', 5'-adenosine cyclic monophosphate and inhibition of STAT1 phosphorylation (381). Consistent with our data, E_2 was shown to result in downregulation of IRF1 (369) and HLA-DRα (304) in MCF-7 cells. The effect of estrogen on IFN-γ induced proteins was not only restricted to human and breast cancer cells, but male mice treated with estrogen resulted in decreased STAT1 expression (379). Furthermore, E_2-treatment of immortomouse brain endothelial (IBE) cell line resulted in downregulation of HLA class II expression (315). Likewise, E_2 treatment abolished IFN-γ inducible class II antigen expression in rat vascular allograft and improved graft survival (283).

In conclusion, E_2-ER signaling supresses specific IFN-γ inducible genes, especially genes involved in HLA class II antigen processing and presentation. This may explain why some breast tumors do not upregulate HLA class II molecules, in spite of high level of IFN-γ in tumor microenvironment.

Chapter 6: Abrogation of mitogen activation protein kinase activity in breast cancer cells results in loss of human leukocyte antigen class II expression on the cell surface.

6.1. Rationale and objectives

Patients with estrogen receptor alpha negative ($ER\alpha^-$) tumors have a worse prognosis as they are resistant to antiestrogens and commonly present with overexpression of growth factor receptors. Activation of certain pathways like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen activated protein kinase (MAPK) signaling is associated with abrogation of ERα expression, which suggests significant cross talk between ERα and these signaling pathways (382). Moreover, it has been suggested that breast cancer cells employ a number of different mechanisms to stop expressing ERα as the tumor progresses. These mechanisms include, promoter hypermethylation, estrogen withdrawal, hypoxia, or hyperactivation of MAPK (383).

In melanoma cells, MAPK- extracellular regulated kinase (ERK) and MAPK- c-jun N-terminal kinase (JNK) are required for the regulation of class II transactivator (CIITA), through binding to the activation protein 1 (AP1) responsive element in the enhancer of promoter III (pIII) CIITA (203). Moreover, MAPK activation is important in regulation of human leukocyte antigen (HLA) class II in professional antigen presenting cells (APC) such as dendritic cells (DC) and macrophages (176, 384) and non-professional APC such as fibroblast cells (214).

Based on a previous report, that MAPK activation drives HLA class II expression in melanoma cells and the finding that activation of MAPK results in downregulated ERα (382), my hypothesis is that MAPK activation leads to upregulation of HLA class II in the

ERα$^-$ breast cancer cells. To address this, I used MDA-MB-231 breast cancer cell line (BCCL), which is both ERα$^-$ and exhibits hyperactivation of the MAPK pathway (226).

1. To ascertain the role of MAPK in human leukocyte antigen (HLA) class II expression in ERα- breast cancer cells.
2. To identify the role of different classes of MAPK inhibitors on HLA class II regulation in breast cancer cells.

6.2. Preliminary experiments to assess the effect of U0126 on MDA-MB-231 viability

The recommended and effective concentration of U0126 for inhibiting MEK activity is 10 μM according to a number of different cell lines examined, including BCCL (225, 384, 385). Kinetics studies of U0126 were done on MDA-MB-231 cells. The cells were treated with either U0126 (10 μM) or DMSO (vehicle control). Cell confluence was examined every 24 hr up to 96 hr using an inverted phase microscope, and the estimated growth was recorded. On day 5, the cells were harvested and counted using a haemocytometer. As shown in Figure 6.1A, the percentage of cells growing in DMSO increased steadily, whereas U0126 suppressed cell proliferation. After 96 hr, U0126-treatment decreased cell counts compared to DMSO-treated cells (Figure 6.1B).

To confirm that 10 μM U0126 is effective in inhibiting MEK activation without affecting cell viability, MDA MB 231 cells were treated for 72 hr with serial dilutions of U0126. Crystal violet staining and immunoblotting were done to determine cell viability and ERK activation, respectively.

U0126 treatment resulted in a dose-dependent inhibition of proliferation as shown by gradual decrease of proliferation with increasing U0126 concentration (Figure 6.1C) with an IC-50 (half-maximal inhibitory concentration) of 10 μM. Furthermore, the activation of ERK as detected by immunoblotting for pERK was decreased in a dose dependent manner with activation barely detected at 10 μM U0126 (Figure 6.1D).

Overall, these preliminary experiments confirmed the previous literature and established that 10 μM was sufficient to inhibit MEK activation in MDA MB 231. In addition, the 72-hr time point was used in all our subsequent experiments for detecting the effect of U0126 on HLA-DR protein expression.

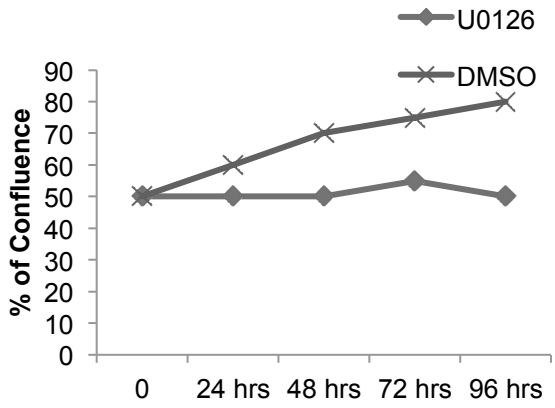
6.3. U0126 inhibited constitutive and IFN-γ induced surface HLA-DR expression

The aim of this experiment was to determine if ERK is involved in the regulation of HLA-DR in BCCL by blocking MEK activation with U0126. MDA-MB-231 was treated with U0126 or DMSO and stimulated or not with IFN-γ for 48 and 72 hr. HLA-DR expression was measured at the indicated time points using L243 antibody and flow cytometry. As shown in Figure 6.2A-B, approximately 20% of the cells constitutively expressed HLA-DR. IFN-γ strongly upregulated HLA-DR expression at 48 and 72 hr with 96% positive cells. U0126 down regulated constitutive and IFN-γ induced HLA-DR expression. Although the number of HLA-DR positive cells was reduced only by 16% in U0126 and IFN-γ treated cells, the staining intensity was significantly decreased ($p<0.01$) (Figure 6.2C). Similarly, U0126 had the same effect on surface HLA-DR expression on VC5 and MC2 cells (Appendix 7). These results suggest that ERK is important for

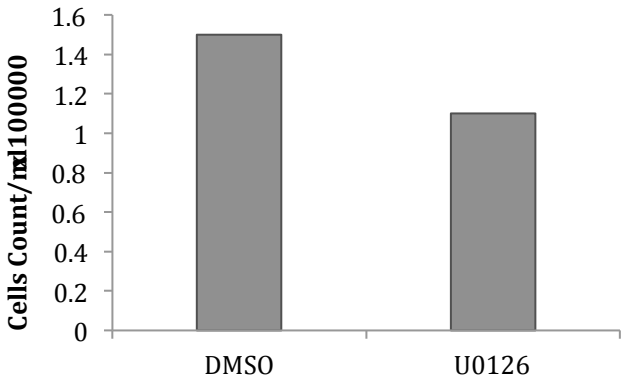
Figure 6.1 Effect of the MEK inhibitor, U0126, on proliferation of MDA-MB231 and ERK 1/2 activation.

MDA-MB-231 was treated with various concentrations of U0126 for the indicated time points (A) line graph showing the percentage of confluent cells as estimated by inverted phase microscope at the indicated time points and U0126 concentrations. (B) Bar graphs showing the cell count after 96 hr of treatment with the indicated U0126 concentrations. (C) Bar graphs showing the cell viability, as measured by crystal violet staining at the indicated concentrations of U0126. (D) ERK activity was detected by immunobloting using p-ERK 1/2 (Thr 202/Tyr 204) antibody from whole cell lysates. Figures indicate one experiment.

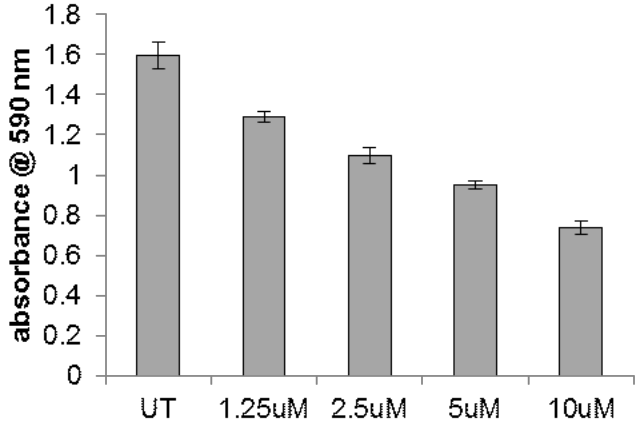
A


U0126
DMSO
% of Confluence
90
80
70
60
50
40
30
20
10
0
0
24 hrs
48 hrs
72 hrs
96 hrs

B


Cells Count/ml100000
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
U0126

C


absorbance @ 590 nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
UT
1.25uM
2.5uM
5uM
10uM

D

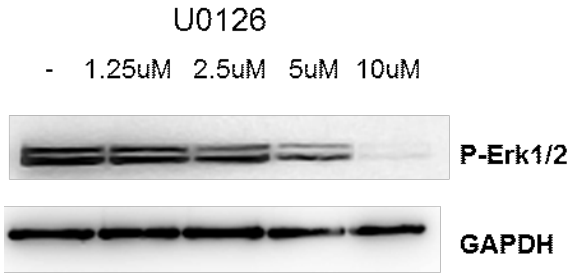
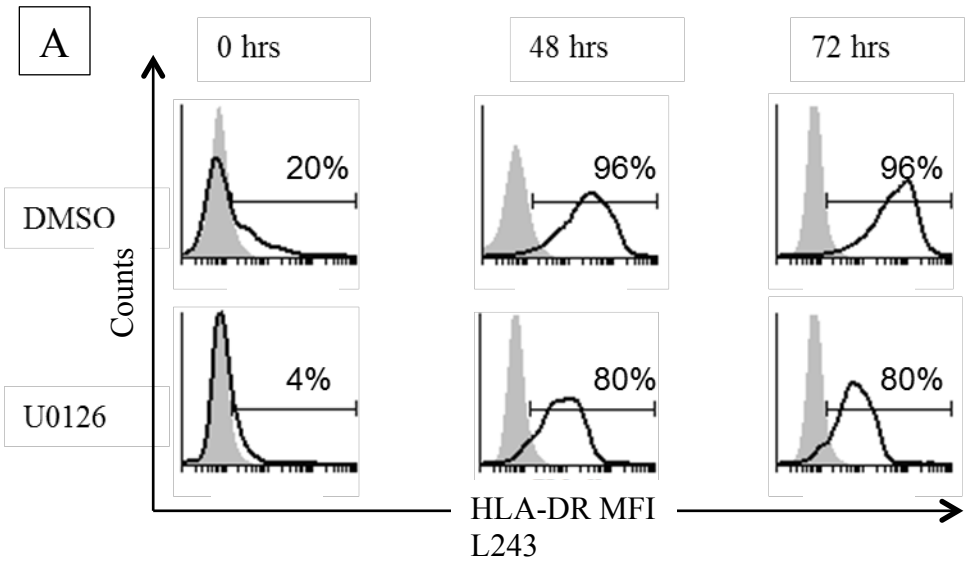
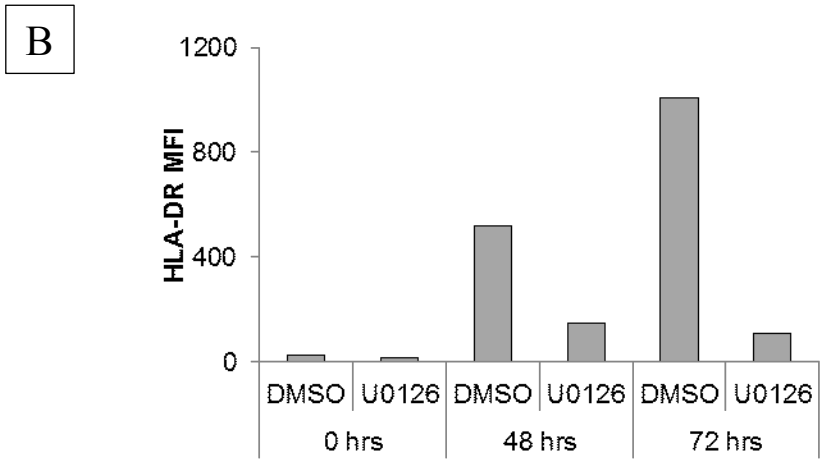
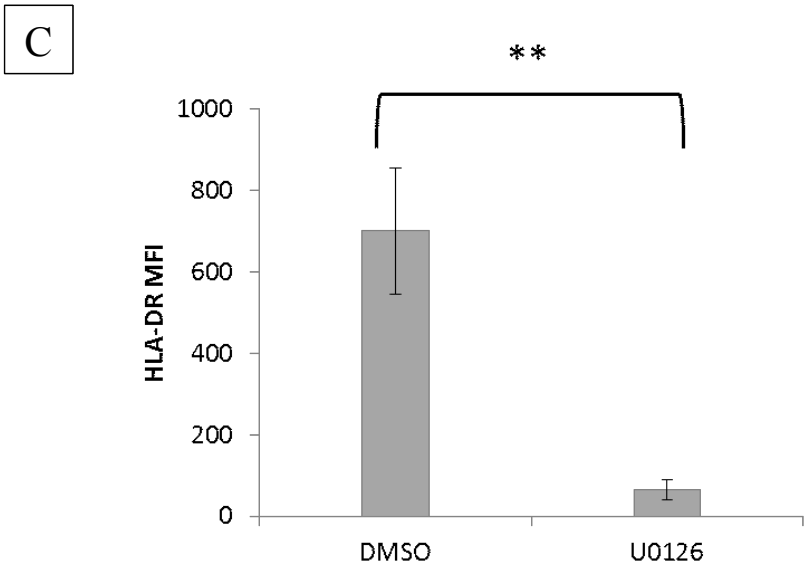

U0126
-
1.25uM
2.5uM
5uM
10uM
P-Erk1/2
GAPDH

Figure 6.2 Effect of MEK inhibitor, U0126, on HLA-DR expression in MDA-MB-231. MDA-MB-231 cells were treated with 10 μM U0126 or vehicle (DMS0) and stimulated or not IFN-γ (100 Units/ml) for various time points as indicated. (A) HLA-DR surface expression (detected by L243) was analysed by flow cytometry. Shaded histogram = isotype control, black line = HLA-DR expression with the indicated % of positive cells (B) Bar graphs represent the mean fluorescence intensity (MFI) for HLA-DR expression (C) Bar graphs represent the MFI ± standard errors of the mean (SEM) for HLA-DR expression of three independent experiments after 72 hr of IFN-γ and U0126 treatment (**$p<0.01$).


A
0 hrs
48 hrs
72 hrs
DMSO
U0126
Counts
20%
96%
96%
4%
80%
80%
HLA-DR MFI
L243


B
1200
800
400
0
HLA-DR MFI
DMSO
U0126
DMSO
U0126
DMSO
U0126
0 hrs
48 hrs
72 hrs


C
**
1000
800
600
400
200
0
HLA-DR MFI
DMSO
U0126

HLA-DR expression.

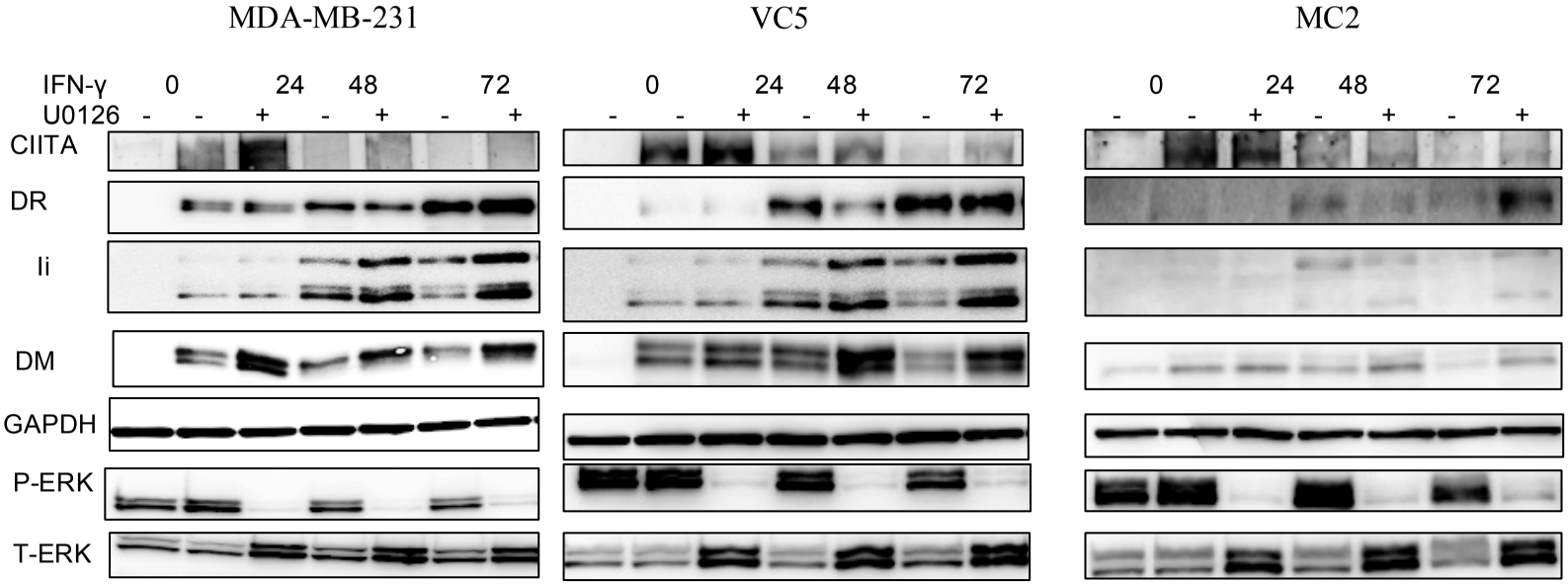
We next tested the effect of ERK inhibition on HLA class II expression in our $ER\alpha^-$ and $ER\alpha^+$ BCCL model. MDA-MB-231, VC5 and MC2 were treated with either DMSO or U0126 and stimulated with IFN-γ for 24, 48, and 72 hr. Whole cell RIPA lysates were prepared and examined by SDS-PAGE gel under reduced conditions and immunoblotted for CIITA, HLA class II, and ERK activation.

The results depicted in Figure 6.3 showed no constitutive expression of CIITA or HLA-class II proteins in VC5 and MC2 cell lines. CIITA basal expression was noticed only in MDA-MB-231 cells, which is consistent with Figure 3.5. IFN-γ stimulation upregulated CIITA and HLA class II proteins. Unexpectedly, the combined treatment of U0126 and IFN-γ resulted in augmented upregulation of CIITA and HLA class II expression at all time points in all the three cell lines examined.

Upregulation of HLA-DR expression by U0126 at 72 hr (Figure 6.3) was inconsistent with HLA-DR surface expression detected by flow cytometry (Figure 6.2). This suggests that ERK inhibition prevents transport of HLA-DR to the cell surface. HLA-DR could be trapped in the endocytic vesicles, or Golgi apparatus and prevented from being expressed. HLA-DM and Ii were coordinately upregulated by the combination of U0126 and IFN-γ, suggesting that this upregulation is secondary to increased CIITA expression. The effect of ERK was the same for all the three cell lines, suggesting that ERK-dependent modulation of HLA class II expression occurs regardless of ERα. We focused on MDA-MB-231 to address possible mechanisms.

Figure 6.3 U0126-treatment differentially modulates HLA class II expression in the BCCL model.

MDA-MB-231, VC5, MC2 cells were treated with vehicle (DMSO), 10 μM U0126 followed by stimulation with IFN-γ (100 Units/ml) at the indicated time points. HLA class II and CIITA proteins from whole cell extracts were analyzed by SDS-PAGE under reduced condition, followed by immunoblotting using CIITA (antiserum #21) anti-DRα (TAL 1B5), anti-DM (TAL18.1) and anti-Ii (LN2). P-ERK and T-ERK were used to test the efficiency of U0126. Figure indicates one experiment.

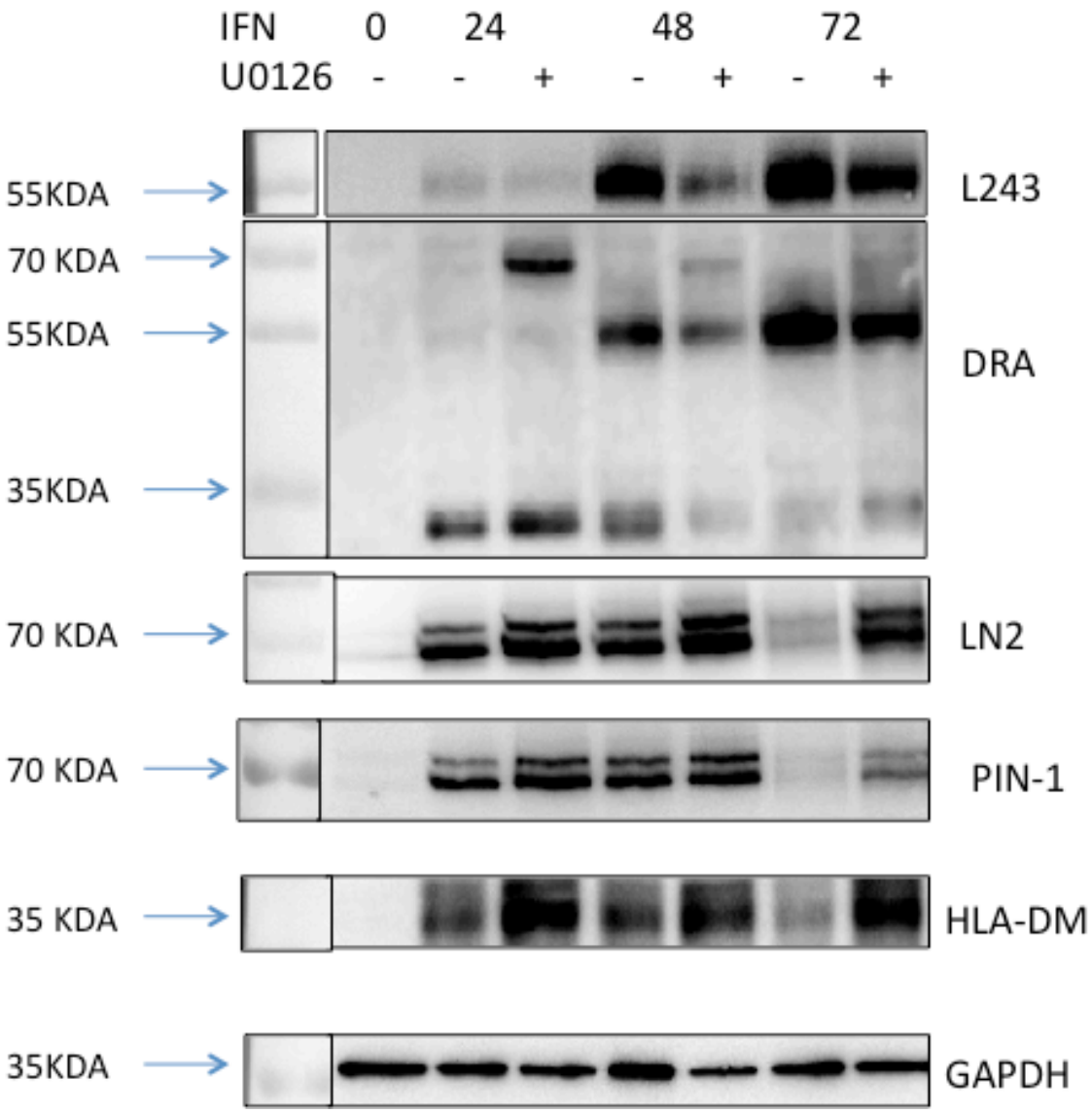

MDA-MB-231
VC5
MC2
IFN-γ 0 24 48 72
U0126 - - + - + - +
CIITA
DR
Ii
DM
GAPDH
P-ERK
T-ERK

The discrepancy between flow cytometry and immunoblotting for HLA-DR expression may be due to different antibody specificities. The L243 antibody used for flow cytometry detects a conformational epitope on HLA-DR molecules (386); however, the Tal 1B5 (HLA-DRα antibody) used for immunoblotting detects HLA-DRα monomer (387). Based on the fact that MAPK signaling modulates protein stability and cell surface expression of some proteins (388), we hypothesized that U0126-treatment decreases the stability of HLA-DR molecules, which may explain the increase of HLA-DRα at the expense of a decrease in HLA-DR dimers. To test this idea, we used the same whole cell lysates and examined them by SDS using non-reducing conditions. Notably, HLA-DRα monomer (29 kDa) was strongly upregulated after 24 hr of IFN-γ stimulation (Figure 6.4), but the HLA-DR dimer (55 kDa) was weakly expressed, which was expected as the mature molecule takes a longer time to form (389). The HLA-DRα antibody detected a higher molecular weight band (~70 kDa) after 24 and 48 hr of combined U0126 and IFN-γ treatment. This high MW band may suggest an associated invariant chain monomer, because it correlates with Ii upregulation as detected by two different antibody clones (LN2 and Pin1).

HLA-DR stable dimers (55 kDa) as detected by L243 appeared to have decreased after 48 and 72 hr of combined IFN-γ and U0126 treatment. Although, this may appear to be inconsistent with Figure 6.2, the sum of the three bands (~29 kDa, ~55 kDa and ~70 kDa) (Figure 6.4) detected by TAL 1B5 accounts for this ostensible loss. This suggests that U0126 treatment may interfere with formation of mature HLA-DR molecules. The results of HLA-DM and Ii followed the same pattern as in the reduced

Figure 6.4 U0126-treatment differentially modulates HLA class II expression in MDA-MB-231 cells.

MDA MB 231 cells were treated with vehicle (DMSO) or 10 μM U0126 followed by stimulation with IFN-γ (100 Units/ml) at the indicated time points. HLA class II protein from whole cell extracts was analyzed by SDS-PAGE under non-reduced condition, followed by immunoblotting using pan HLA-DR antibody (L243), anti-DRα (TAL 1B5), anti-DM (TAL18.1) and anti-Ii (LN2). Figure indicates one experiment.


IFN 0 24 48 72
U0126 - - + - + - +
55KDA
L243
70 KDA
55KDA
DRA
35KDA
70 KDA
LN2
70 KDA
PIN-1
35 KDA
HLA-DM
35KDA
GAPDH

SDS-PAGE condition.

Overall, U0126-treatment downregulated HLA-DR surface expression and resulted in its intracellular accumulation, which suggests a possible role of ERK in HLA-DR trafficking. Moreover, upregulation of CIITA, HLA-DM, and Ii by U0126 treatment suggested that ERK rather than upregulate interferes with CIITA expression, possibly at the mRNA level since its regulation occurs mainly at the transcription level.

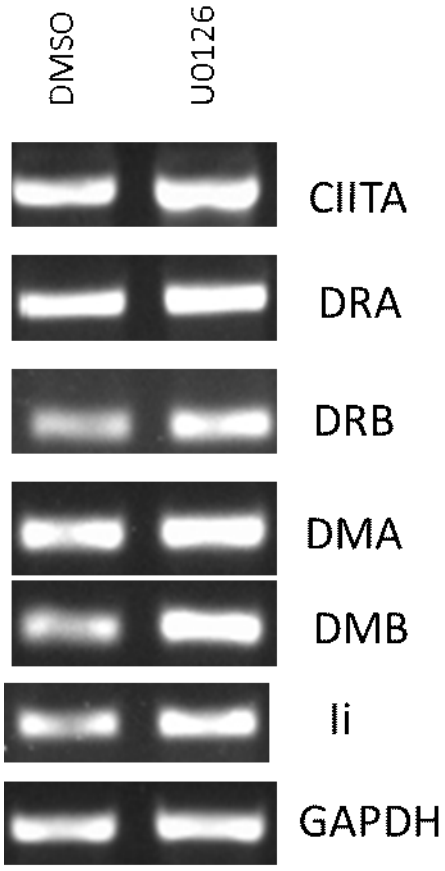
6.4. U0126 upregulated IFN-γ induced HLA class II mRNA expression

Since inhibition of ERK activity by U0126 increased CIITA and intracellular accumulation of HLA class II molecules, we questioned whether this occurred at the level of CIITA or HLA class II gene transcription. To address this, MDA-MB-231 was treated with vehicle control (DMSO) or U0126 and stimulated with IFN-γ for 12 hr, followed by RNA extraction. RT-PCR was performed using primers specific for CIITA, HLA class II, and co-chaperone genes. As shown in Figure 6.5, CIITA, and downstream genes controlled by CIITA (HLA DR [A,B], HLA DM [A,B] and Ii) were upregulated by U0126.

In conclusion, ERK pathway in MDA-MB-231 downregulates IFN-γ inducible CIITA transcription (and consequently, HLA class II transcription), but, interestingly, ERK activiton is required for expression of stable HLA-DR/peptide complexes and their cell surface expression.

Figure 6.5 CIITA and HLA class II expression is upregulated by U0126 in MDA-MB-231 at the mRNA level.

MDA-MB-231 was treated with either DMSO or 10 μM U0126 and stimulated with IFN-γ (100 Units/ml) for 12 hr. Total RNA was isolated and subjected to RT-PCR analysis using primers specific for CIITA and HLA class II genes. Figure indicates one experiment.


DMSO
U0126
CIITA
DRA
DRB
DMA
DMB
Ii
GAPDH

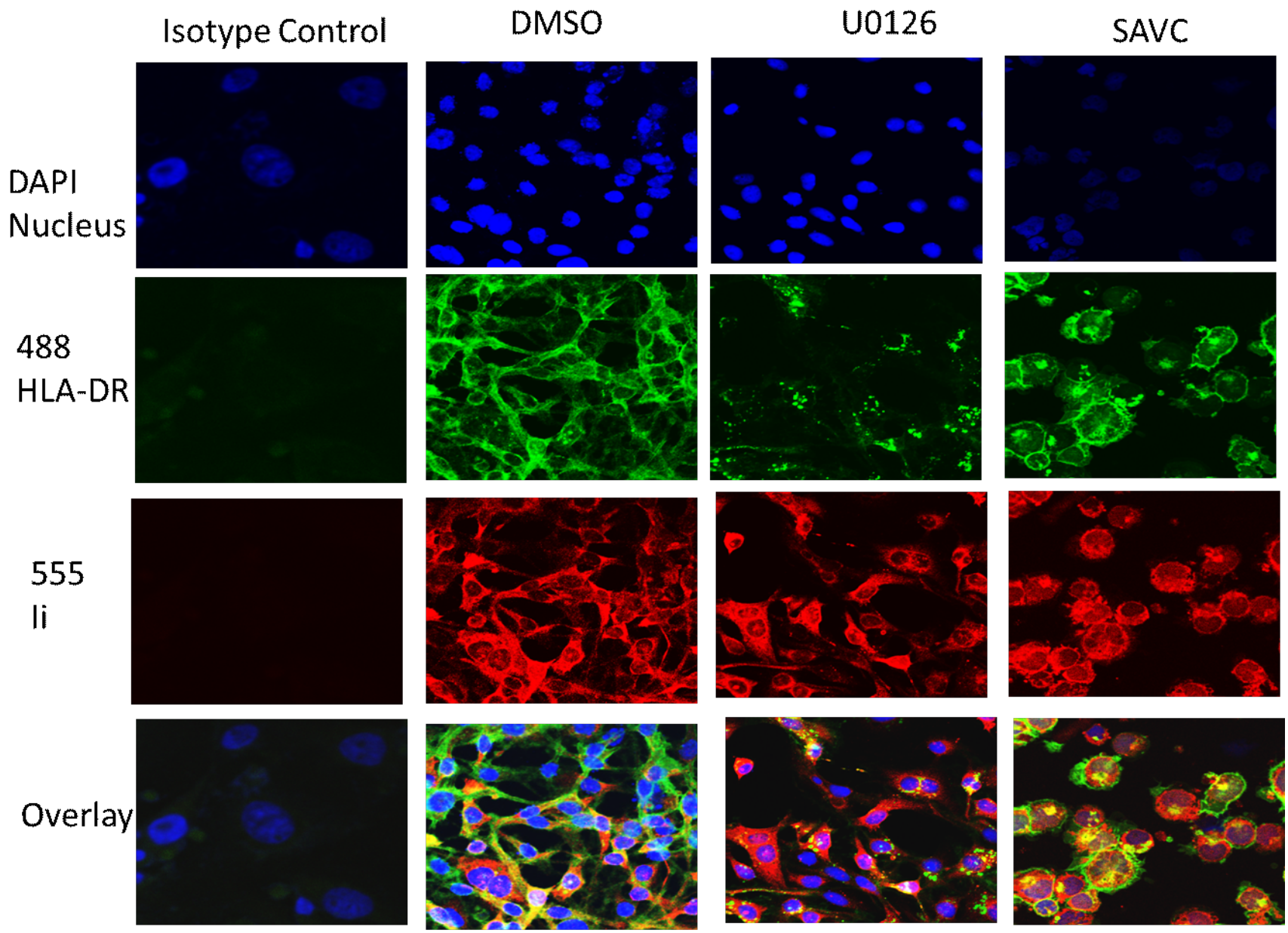
6.5. ERK is important for HLA-DR trafficking

ERK 1/2 was previously shown to regulate HLA class I trafficking through a clathrin-independent pathway (390). To test whether ERK plays a role in HLA-DR trafficking, we performed immunocytochemistry (ICC) and confocal microscopy to visualise and localize HLA-DR and Ii molecules before and after inhibiting ERK activation. MDA-MB-231 cells were grown in an 8-well chamber slide and treated with DMSO or U0126, followed by stimulation with IFN-γ for 72 hr. Cells were acetone-fixed and parallel immunofluorescence was performed using HLA-DR and Ii antibodies.

In DMSO-treated cells, HLA-DR expression was seen as strong green fluorescence and homogenous staining of the cytoplasm and membrane, while Ii was seen as red fluorescence and homogeneous cytoplasmic staining around the nucleus. DAPI was used for nuclear staining (Figure 6.6). The association of Ii and HLA-DR was detected in the overlayed image in the form of yellow localised areas. In contrast to DMSO, U0126-treated cells showed noticeable downregulation of HLA-DR membrane expression, but it was strongly expressed in endocytic vesicles. HLA-DR and Ii were strongly expressed in SAVC with strong membranous and intra-cytoplasmic staining respectively. The negative controls, isotype matched irrelevant antibodies, showed minimal background. In conclusion, these results suggest that either HLA-DRα/β dimers are trapped in the endosomes, having never made their way to the cell surface, or they rapidly recycle from the cell surface back to early endosomes. This will need to be addressed in future studies.

Figure 6.6 ERK is important for HLA-DR trafficking.

MDA-MB-231 was treated with either DMSO or U0126 and stimulated with IFN-γ (100 Units/ml) for 72 hr. Cytopreps were prepared and acetone fixed, HLA-DR was detected using L243 and visualised by Alexafluor 488 (green stain), Ii was detected by LN2 and visualised by Alexafluor 555 (red stain). Nuclei were stained blue with DAPI. Figure represents one of three experiments.


Isotype Control
DMSO
U0126
SAVC
DAPI
Nucleus
488
HLA-DR
555
Ii
Overlay

6.6. The role of other MAPK proteins on HLA class II expression

JNK and p38 are other members of the MAPK that were previously shown to play a significant role in HLA class II regulation in macrophages (176). We wished to examine the effect of these members on HLA class II expression in ERα$^-$ breast cancer cells. Preliminary experiments were done to ensure that optimal inhibitory concentrations for SP 600125 (JNK inhibitors) and SB 202190 (p38 inhibitors) did not significantly reduce cell viability. MDA-MB-231 cells were treated for 72 hr with serial dilutions (50 – 3.125 μM) of either SP 600125 or SB 202190; DMSO was used as mock control. The results depicted in Figure 6.7A-B showed that SP 600125 or SB 202190 resulted in a dose dependent inhibition of proliferation. The IC-50 ranged between 25- 50 μM for SB 202190 and was approximately 50 μM for SP 600125.

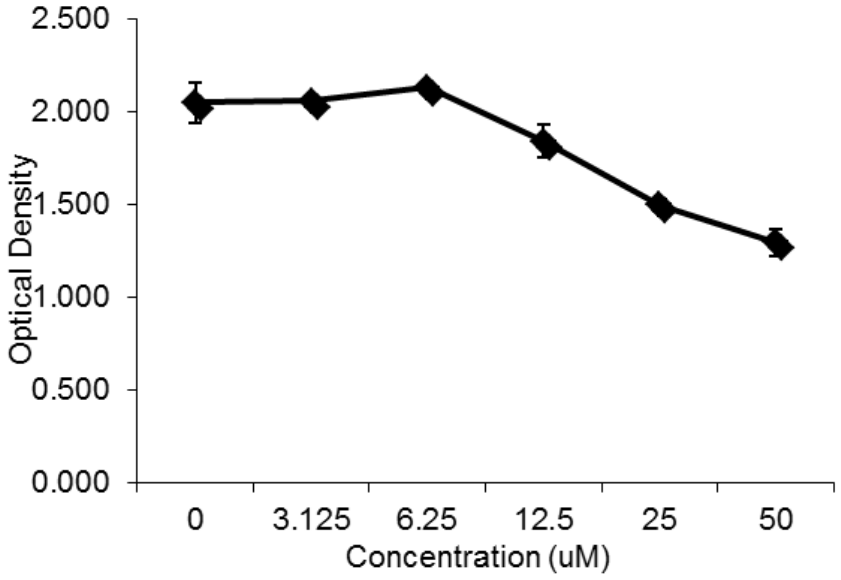
Next, MDA-MB-231 was treated with SP600125 and SB202190 or DMSO and stimulated with IFN-γ for 72 hr. Whole cell RIPA lysates were prepared and examined by immunoblotting. As shown in Figure 6.8 CIITA, HLA-DR, HLA-DM, and Ii were downregulated with both drugs. Although there was downregulation of all HLA class II proteins, SP600125 and SB202190 were only effective at 25μM as seen by a decrease of the phosphorylation of p38 and JNK respectively. These results suggested that both p38 and JNK have a positive effect on CIITA and HLA class II expression.

We next examined the effect of SP600125 and SB202190 on CIITA and HLA class II mRNA expression. MDA-MB-231 was treated as above and stimulated with IFN-γ for 12 hr. SP600125 and SB202190 did not modulate CIITA, HLA-DMA or Ii expression at the mRNA level, but resulted in decreased HLA-DRA and HLA-DRB

Figure 6.7 Cell viability assay for SP 600125 (JNK inhibitors) and SB 202190 (p38 inhibitors).

MDA-MB-231 was treated with indicated concentrations of (A) SP 600125 and (B) SB 202190 for 72 hr. Line graph show the absorbance reading for crystal violet staining. Figures indicate one experiment, which was conducted by Jillian Green.

A


2.500
2.000
1.500
1.000
0.500
0.000
Optical Density
0
3.125
6.25
12.5
25
50
Concentration (uM)

B

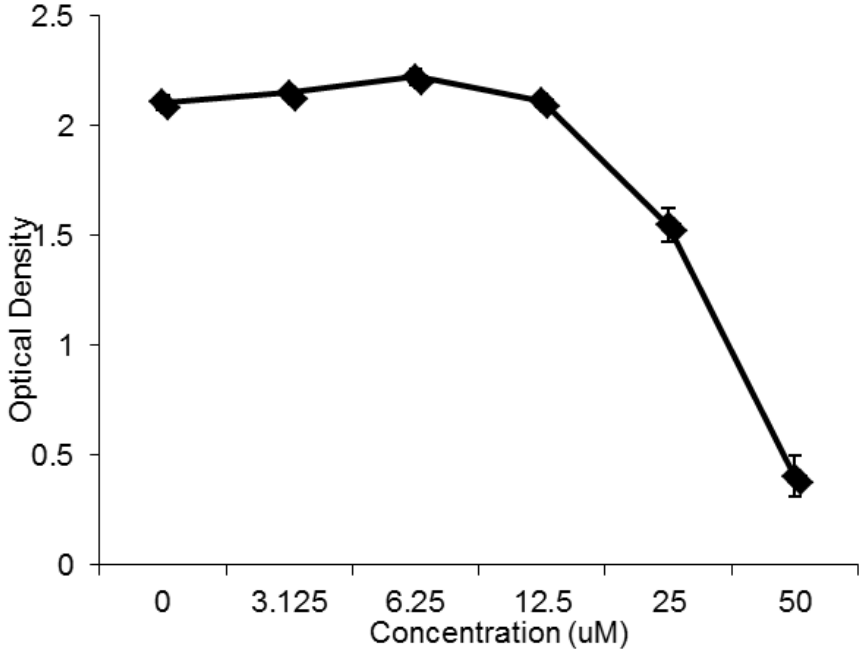
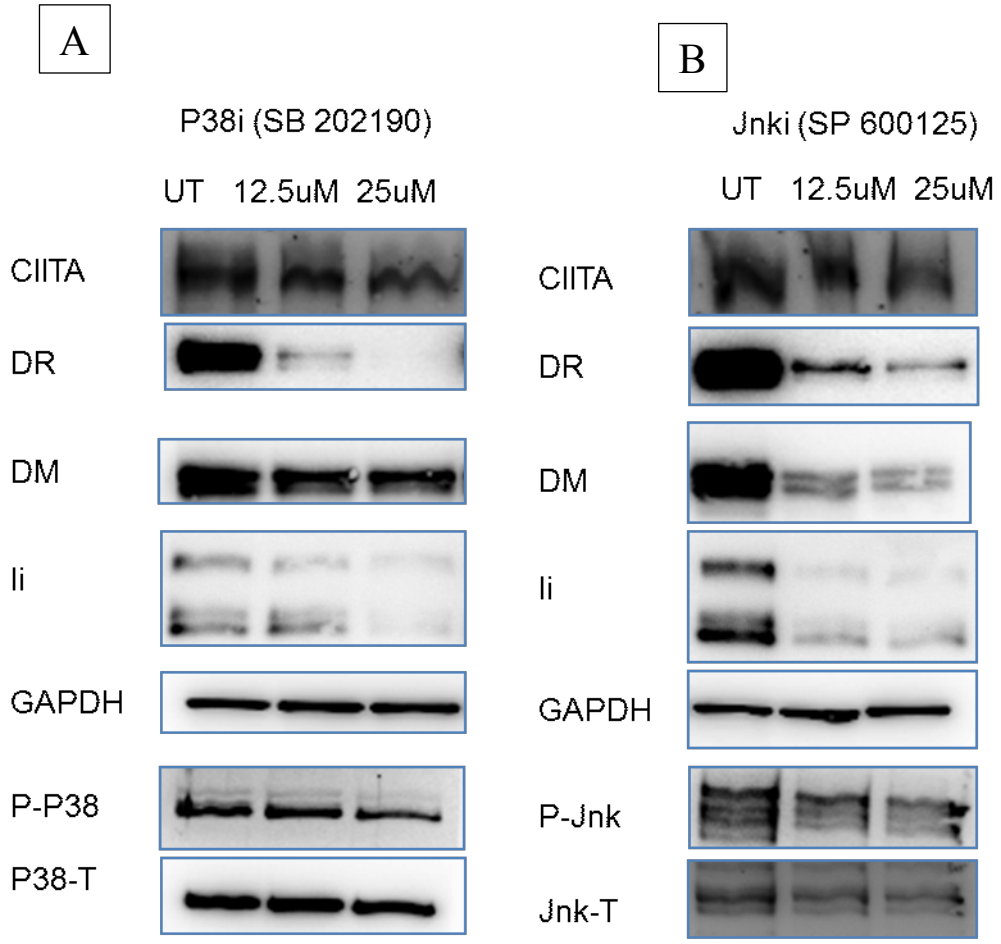

2.5
2
1.5
1
0.5
0
Optical Density
0
3.125
6.25
12.5
25
50
Concentration (uM)

Figure 6.8 SP 600125 and SB 202190 differentially modulate human leukocyte class II expression.

MDA MB 231 cells were treated with vehicle (DMSO), 12.5 and 50 μM (A) SB 202190 and (B) SP 600125 followed by stimulation with IFN-γ (100 Units/ml) for 72 hr. HLA class II expression was analyzed by immunoblotting from whole cell extracts using anti-DRα (TAL 1B5), anti-DM (TAL18.1) and anti-Ii (LN2), anti P-p38, and anti p-JNK. Figures indicate one experiment, which was conducted by Jillian Green.

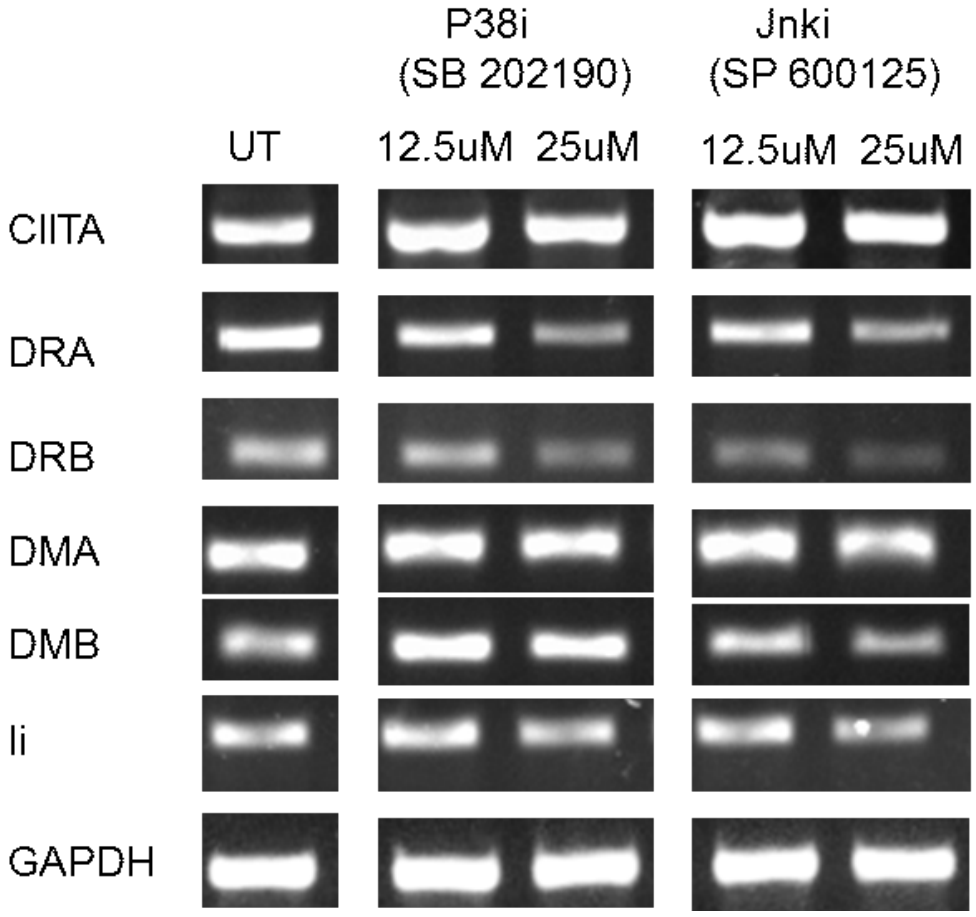

A
P38i (SB 202190)
UT 12.5uM 25uM
CIITA
DR
DM
Ii
GAPDH
P-P38
P38-T
B
Jnki (SP 600125)
UT 12.5uM 25uM
CIITA
DR
DM
Ii
GAPDH
P-Jnk
Jnk-T

mRNA expression (Figure 6.9), which was consistent with downregulation of HLA-DR protein expression (Figure 6.8). As opposed to pERK, which decreases CIITA and HLA class II expression and affects transport of HLA-DR molecules to the cell surface, p38 and JNK are associated with increased expression (Figure 7.2).

The mechanism of MAPK regulation of HLA class II has only been partially resolved and future experiments are need to study HLA class II promoter activity, since the AP1 binding site is present in most of HLA class II gene promoters.

Figure 6.9 p38 and JNK inhibitors selectively down regulate HLA class II genes.

MDA MB 231 cells were treated with vehicle (DMSO), 12.5 and 25 μM SP 600125 and SB 202190 followed by stimulation with IFN-γ (100 Units/ml) for 12hr. Total RNA was isolated and subjected to RT-PCR analysis using primers specific for CIITA and HLA class II genes. Figure indicates one experiment.


P38i
(SB 202190)
Jnki
(SP 600125)
UT
12.5uM
25uM
12.5uM
25uM
CIITA
DRA
DRB
DMA
DMB
Ii
GAPDH

6.7. Discussion

In the present study, we clearly demonstrated that ERK plays an important role in regulating HLA-DR surface expression in MDA-MB-231. Using U0126 to inhibit the ERK pathway, we showed downregulation of HLA-DR surface expression was not the result of ERK-inhibition of CIITA. In fact ERK inhibition resulted in increased CIITA and synthesis of downstream HLA class II proteins. The observed downregulation of surface HLA-DR expression by U0126 was not due to technical issues as HLA-DR stable dimers were also reduced as shown by immunoblotting under non-reducing conditions. Immunoblotting displays stable HLA-DR/peptide complexes, both intracellularly and at the plasma membrane. Possible explanations for the difference in cell surface and intracellular expression of HLA-DR are: 1) ERK is important in the stability of HLA-DR molecules, 2) ERK is important in the transport of HLA-DR molecule to cell surface. Overall, ERK appears to have dual opposing mechanisms in regulating HLA-DR expression; ERK negatively regulates CIITA expression and positively regulates HLA-DR surface expression.

The role of MAPK in regulating HLA class II has been studied in professional and non-professional APC with contradictory findings. These differences may be due to a number of reasons; lack of studying all HLA class II molecules (HLA-DR, Ii and HLA-DM), the use of various MAPK inhibitors, which result in different outcomes, the difference between human and mouse model system examined, and the use of different cell lines and tissues. For example: in mesenchymal stem cells (MSCs), activation of ERK resulted in upregulation of basal HLA-DR surface expression secondary to increased CIITA transcription, but had no effect on IFN-γ-induced CIITA expression

(391). In contrast, Yongxue Yao et. al. (2006) demonstrated that activation of ERK in murine macrophages resulted in hypoacetylation of histone 4 (H4) at the CIITA promoter and decreased CIITA expression (384). Martins et. al. (2007) showed activation of ERK and JNK in melanoma cell lines are required for regulation of constitutive CIITA expression. This occurs by binding of the AP1 responsive element in the enhancer of pIII CIITA (203). However, this mechanism was not sufficient in CIITA regulation and other factors like CXCL1, CXCL8 and NFκB p50 subunit were later shown to be required (319). In another study, ERK inactivation resulted in augmentation of IFN-γ induced CIITA expression in mouse bone marrow-derived DC (384). This suggests that ERK activation negatively regulates CIITA expression, probably through inhibition of pIV CIITA (77). These results propose that the difference in regulation of CIITA by ERK is species and cell specific and depends on which CIITA promoter is involved. Further studies are required to investigate the potential mechanisms involved in this regulation.

Although CIITA is regulated at the transcription level (392), post-translational modification events can still control its activation and nuclear transport (63, 64). Voong et al 2008, (214) demonstrated that ERK 1/2 could directly interact with CIITA and target phosphorylated S^{288} in the amino terminal end. This phosphorylation can enhance binding of the CIITA to the nuclear export factor, CARM1 which results in decreased nuclear concentration. As a result of decreased CIITA nuclear localisation, HLA-DR promoter activity is reduced. We noticed that inhibition of ERK 1/2 by U0126 in MDA-MB-231 resulted in upregulation of CIITA as determined by RT-PCR and immunoblotting using whole cell lysates (WCL). In WCL, the concentration of the nuclear protein is usually

diluted by the vast array of cytoplasmic proteins. Thus, further studies are needed to determine levels of nuclear and cytoplasmic CIITA, and properly address the mechanism by which MAPK regulates CIITA in BCCL

The significant U0126-mediated downregulation of surface HLA-DR expression, despite abundant intracellular proteins in MDA-MB-231 suggested that ERK 1/2 is important in the transport of HLA-DR to the cell surface. Indeed this was supported by immunofluorescence results showing abundant membrane and homogeneous HLA-DR staining in untreated cells as compared to little or no surface HLA-DR staining and large clumps of intracellular HLA-DR in the presence of U0126. The fact that Ii and HLA-DR colocalized in these endocytic-like vesicles, also suggests they are trapped in endocytic vesicles or alternatively, recycle to these vesicles. Future studies using endocytic tracker or colocalization with other endocytic markers are necessary to properly identify these vesicles. Although, we did not identify the mechanism by which HLA-DR transport is modulated by ERK, it may be similar to a mechanism described for HLA class I. Robertson et. al. (2006) showed that ERK signaling regulates endosomal trafficking through the clathrin-independent, ADP-ribosylation factor 6 (Arf6) GTPase-regulated endosomal pathways (390). U0126 resulted in inactivation of ERK and expansion of Arf6 compartment, which negatively correlated with HLA class I expression. ERK may regulate recycling of HLA class II under the same mechanism involved in class I, since clathrin independent endocytosis is responsible for recycling of HLA class II from the cell surface (393).

The global downregulation of the all HLA class II molecules seen by p38 and JNK inhibitors is due to downregulation of CIITA, the master regulator of HLA class II

molecules at the protein level with no effect on mRNA. The discrepancy between the protein and mRNA expression of CIITA in MDA-MB-231 may suggest a post-transcriptional regulation of mRNA stability. These findings are supported by Valledor et al 2008, where they showed that JNK inhibitor decreased CIITA mRNA stability in macrophages and subsequently resulted in loss of class II induction (176).

Overall, our original hypothesis that MAPK activation is important in upregulating HLA class II expression in ERα$^-$ could not be confirmed. Apparently, MAPK is important for HLA class II expression regardless the ERα status. The role of MAPK on HLA class II regulation is still an open area of research and more studies are needed to determine the potential mechanism. This may aid in the development of successful anti-tumor therapy by targeting component of MAPK signaling and thus modulating antigen processing and presentation in breast tumor cells.

Chapter 7 Summary and future directions

7.1.Overview

The immune response in humans is regulated by the interaction of $CD4^+$ T-cells with human leukocyte antigen (HLA)-DR molecules that display peptides from antigens that have undergone proteolysis in the endocytic pathway. Peptide loading of HLA-DR molecules also requires the co-chaperones, invariant chain (Ii) and HLA-DM. Previous studies in our laboratory showed that HLA-DR and Ii were expressed in 40-50% of breast carcinomas, whereas HLA-DM was expressed in only 10% (106). Moreover, HLA-DR and Ii, but not HLA-DM, correlated with estrogen receptor (ER) α^- tumors and reduced age at diagnosis. On the other hand, coordinate expression of HLA class II molecules and co-chaperones were associated with increased levels of intratumoral T-cells and interferon gamma (IFN-γ). As these findings suggested an important role for cytokines and hormones in HLA class II regulation in breast cancer, a major goal of this project was to directly assess the individual and combined effects of estradiol and ERα on constitutive and IFN-γ inducible HLA class II expression in breast cancer cell lines. A secondary goal was to evaluate the role of mitogen-activated protein kinase (MAPK) on HLA class II expression. The rationale for this was based on the knowledge that the MAPK pathway is frequently over activated in breast cancer, especially in $ER\alpha^-$ tumors (226, 227) and reports showing that MAPK regulates HLA class II expression in professional antigen presenting cells (APC) (176, 384) and melanoma (203).

7.2. Summary of major findings

The research work described in this thesis examined the mechanisms involved in regulation of HLA class II expression in $ER\alpha^+$ and $ER\alpha^-$ breast cancer cell lines (BCCL). Regulation of HLA class II in professional APC has been examined in detail and is reviewed in (37). Nevertheless, there remain large gaps in our knowledge of the mechanism(s) involved in regulation of HLA class II expression in cancer cells.

To test the hypothesis that estrogen differentially regulates HLA class II expression in $ER\alpha^+$ and $ER\alpha^-$ and breast cancer cells, a panel of an established $ER\alpha^+$ and $ER\alpha^-$ breast cancer cell lines was used. E_2 downregulated IFN-γ induced HLA-DR expression in two $ER\alpha^+$ breast cancer cells (MCF-7 and BT474), but not in T47D, suggesting that the effect of E_2-ER signaling on IFN-γ induced HLA-DR expression expression may be cell specific, perhaps depending on what other pathways may be activated. Interestingly, we found a negative correlation between amounts of ERα and expression of HLA-DR in T47D and MCF-7 suggesting that any potential impact of E_2-activation of ERα on HLA-DR expression, could depend on the levels of ERα or as discussed in Chapter 3, the expression of other receptors such as ERβ. Although BT474 is $ER\alpha^+$ it over expresses HER2 receptors, which could be an alternative mechanism responsible for HLA class II regulation. In support of that, blocking HER2 receptor by Lapatinib resulted in upregulation of CIITA and HLA class II expression in BT474 (Tracey Dyer, Honour thesis). Moreover, $ER\alpha^+HER2^+$ breast tumors were completely HLA-DR negative (unpublished data), whereas a significant proportion of other breast cancer subtypes expressed HLA-DR (106). In addition, several studies reported that

overexpression of HER2 downregulates HLA class I expression and IFN regulated genes such as, PA28, LMP, and TAP, which are involved in the HLA class I antigen-processing pathway (394-396). In conclusion, these data suggest crosstalk between the ERα, growth factor receptor pathways and components of IFN-γ signaling.

As opposed to the $ER\alpha^+$ BCCL, estradiol (E_2) upregulated HLA-DR expression in $ER\alpha^-$ BCCL (MDA-MB-231 and SK-BR-3), implying the involvement of non-genomic estrogen signaling in the IFN-γ inducible HLA class II pathway. These results indicate that E_2 differentially modulates HLA-DR expression in $ER\alpha^-$ and $ER\alpha^+$ BCCL, which supported our previous finding in breast carcinoma (106).

To further study the mechanism(s) involved in downregulation of HLA-DR expression in $ER\alpha^+$ breast cancer cells, we used a cell line model system of $ER\alpha^+$ line, MC2 (MDA-MB-231 transfected with the wild type ERα gene) and $ER\alpha^-$ line, VC5 (MDA-MB-231 transfected with the empty vector). As described in Chapter 3, transfection of the $ER\alpha^+$ gene into an $ER\alpha^-$ BCCL resulted in significant reduction of IFN-γ inducible HLA-DR expression. Supporting our initial findings, the addition of exogenous E_2 further downregulated IFN-γ induced HLA-DR expression in $ER\alpha^+$ MC2 but not in $ER\alpha^-$ VC5. Indeed, the inhibitory effect of E_2 and ER was not only restricted to IFN-γ induced HLA-DR, but included the two co-chaperones (HLA-DM and Ii), which are normally coordinately expressed with HLA-DR. We thought it unlikely that the inhibitory effect of E_2-ER on IFN-γ induced HLA class II expression was due to global interference with IFN-γ signaling because constitutive and IFN-γ induced HLA class I surface expression were not downregulated by E_2 in MC2.

Since our data supported a mechanism by which E_2-activation of ER interfered with HLA class II expression, we predicted that blocking the ER signaling pathway with Tamoxifen (TAM) would overcome the inhibitory effect of E_2. Unexpectedly, TAM mimicked the downregulatory effect of E_2 on HLA-DR expression. However, further analysis of immunoblotting results revealed that both TAM and E_2 treatments increased nuclear translocation of ER from the cytoplasm with no functional ER degradation. The upregulation of nuclear ERα negatively correlated with CIITA expression. Thus, TAM may not be a suitable ERα blocker for this study; this is also supported by the fact that TAM non-genomically inhibits MAPK (397, 398), which plays a role in HLA class II regulation (as discussed in Chapter 6).

Because TAM blocks E_2-ER activation but does not degrade ER, we investigated whether the pure anti-estrogen compound Fulvestrant (ICI), which degrades ERα, would restore HLA-DR in MC2. Although ICI-treatment clearly knocked down ERα in MC2, it did not restore HLA class II protein to the levels of VC5; however, ICI in the absence and presence of E_2 reversed the downregulatory effect of E_2 on HLA class II and co-chaperones in MC2, which was detected by immunocytochemistry, flow cytometry and immunoblotting. Moreover, the degradation of ERα by ICI inversely correlated with CIITA protein and mRNA expression. Overall, these findings suggest that E_2-activated ERα clearly mediates decreased HLA class II and can be restored by ICI and silencing of ER by siRNA. On the other hand, ER downregulates HLA class II expression but ICI failed to be restore this downregulatory effect. The latter may suggest a physical interaction with CIITA, because ICI inhibited ER in a dose dependent manner and correlated with CIITA expression in MCF-7 cells. Alternatively, neither ICI nor ER-

siRNA completely knocked down ERα and thus, the amount of remaining ERα may have been sufficient to bind to the ER binding sites in target genes that are implicated in CIITA transactivation (Chapter 5).

To the best of our knowledge, this study is the first to show that E_2 downregulated CIITA expression in $ER\alpha^+$ breast cancer cells. pIV CIITA activity was inhibited by E_2 in MC2. ICI alone, or combined with E_2 did not restore the pIV CIITA activity in MC2 to the VC5 level. These results are in parallel with our HLA class II protein expression and further suggest that E_2-ER activation is implicated in CIITA regulation at the promoter level. Inhibition of pIV CIITA activity in MC2 was not due to direct binding of ER to the predicted ERE binding site in PIV CIITA as detected by the deletion studies. Since pIV CIITA is primarily regulated by IFN-γ we examined the IFN-γ signaling pathway. IFN-γ activated sequence (GAS) luciferase activity was inhibited by E_2 in $ER\alpha^+$ breast cancer cells suggesting attenuation of IFN-γ signaling pathway by E_2-ER signaling; however, the inhibition is not due to global attenuation of IFN-γ signaling, as other important functions mediated by this cytokine, such as signal transducer and transactivation 1 (STAT1) activation (Chapter 5) and HLA class I expression, remained intact in the presence of E_2 (Chapter 3). E_2 decreased GAS luciferase activity without inhibiting STAT1 phosphorylation as detected by immunoblotting. Although STAT1 phosphorylation was not affected by E_2-ER activation, this doesn't exclude the possibility that E_2-ER activation may interfere with STAT1 binding to the GAS promoter (Figure 7.1).

One of our goals was to discover if MAPK, which is frequently over activated in $ER\alpha^-$ breast cancer cells, might be responsible for increased HLA class II, as detected in

the human ERα$^-$ breast tumors (218) and in the ERα$^-$ cell lines MDA-MB-231 and VC5 (Chapter 3). Indeed the work described in chapter 6, showed that MAPK plays an important role in regulating IFN-γ inducible HLA class II expression in the BCCL; however, this was independent of ER status. By using MAPK inhibitors that targeted different components of the MAPK pathway, I showed a differential effect on HLA class II expression. For example ERK is responsible for HLA-DR surface expression, while both p38 and JNK are important for HLA class II expression at the transcription level. These initial data aided in better understanding the role of different MAPK in HLA class II regulation and future work is needed to identify the possible mechanisms involved (Figure 7.2).

Thus, while my thesis strongly supports a role for E_2-ER signaling in repressing HLA class II expression in ERα$^+$ breast cancer cells through interference with the IFN-γ pathway, the mechanism is likely to be more complex and may involve other pathways such as MAPK or EGFR, which are overexpressed in MC2, or HER2 signaling pathway, which is overexpressed in BT474.

Figure 7.1 Activation of ER by E_2 interferes with IFN-γ signalling pathway

Estradiol (E_2) enters the cell by simple diffusion, binds to ER, which results in activation of ER. Activated ER dimerizes, dissociates from HSP and enters the nucleus. The activated ER decreases activation of STAT1 regulated genes and diminishes CIITA transcription.

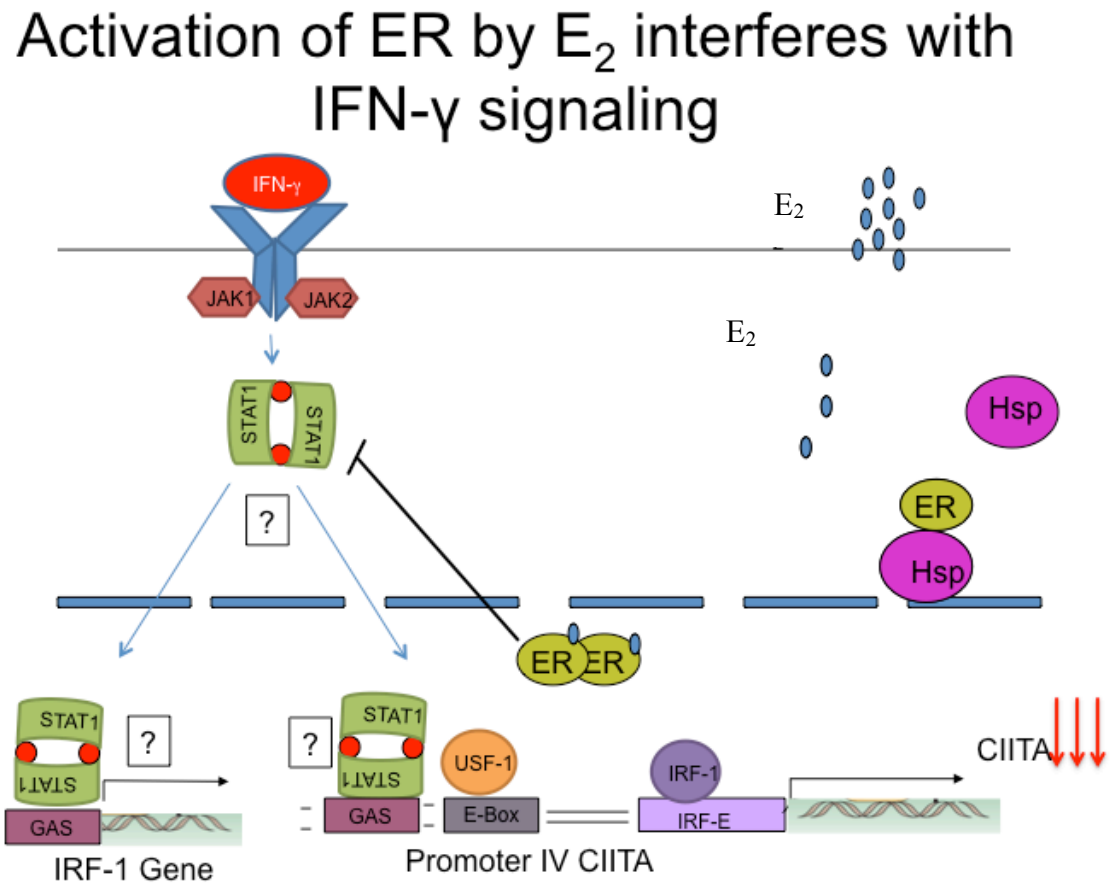
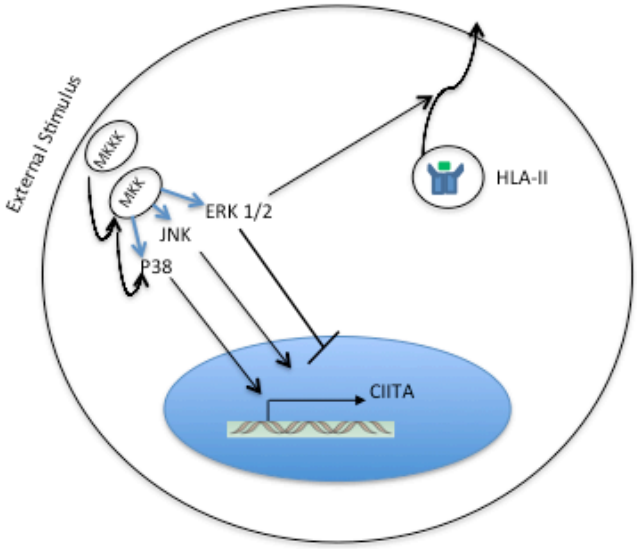

Activation of ER by E_2 interferes with IFN-γ signaling
IFN-γ
JAK1
JAK2
E_2
E_2
STAT1
STAT1
?
Hsp
ER
Hsp
ER
ER
STAT1
STAT1
GAS
?
IRF-1 Gene
?
STAT1
STAT1
GAS
USF-1
E-Box
IRF-1
IRF-E
CIITA
Promoter IV CIITA

Figure 7.2 Differential regulation of HLA class II by MAPK in breast cancer cells.

ERK activation has dual role in regulation of HLA class II. First, ERK is important for the transport of HLA-DR to the cell surface. Second, ERK inhibits CIITA and HLA class II expression. P38 and JNK are important in inducing CIITA and HLA class II protein expression.

MAPK differentially activate HLA class II expression



7.3. Potential implications of findings

In the last few years, we have seen substantial progress in the field of tumor immunotherapy; however, there are still many gaps and challenges. While there have been numerous successes at the bench, in clinical trials and even to the bedside, it is still difficult to predict which responses are sustained. Moreover, some of responses are short lived and cause autoimmunity in some cases (399). For example treatment of melanoma with adoptive immunotherapy that involves the isolation of lymphocytes from patients and their activation and expansion in vitro, followed by infusion into patients resulted in melanocyte destruction, vitiligo, ocular toxicity and autoimmune thyroiditis, as reviewed by Amos et. al. (2011) (400). Conversely, immunotherapies such as those targeting B-cells (Anti CD19) for treating lymphomas can lead to immunodeficiency (401).

If these hurdles are cleared, then many cancer patients could benefit from a strategy to boost their immune system. Proper understanding of the mechanism of tumor immunity is key for development of proper vaccine therapy. The mechanisms responsible for modulation of HLA class II in breast cancer cells is still uncertain. Herein, we provided experimental evidence that E_2-activation of ERα adversely affected STAT1 activated genes as shown by reduced GAS and CIITA promoter activity in MC2, but not in VC5. Importantly, similar results were observed in $ER\alpha^+$ BCCL, MCF-7 and BT-474, in which GAS activity, STAT1 regulated genes and HLA-DR were all downregulated by E_2. In contrast E_2 augemented GAS activity and STAT1 regulated genes in the $ER\alpha^-$ BCCL, MDA-MB-231 and SK-BR-3.

One pathway by which tumors escape the immune system involves deregulation of cell surface HLA class II expression, which is crucial for $CD4^+$ T cell activation (402).

Some tumors fails to upregulate of HLA class II molecules even in the presence of IFN-γ, which has been attributed to defect in CIITA synthesis, the master regulator of HLA class II genes (403). Such mechanisms include impaired CIITA synthesis by pIV CIITA hypermythlation and decreased transcription, as was shown by De Lerma Barbaro et., al., 2008 as a mechanism for reduced CIITA expression in promyelocytic cancer (404). Recently, epigenetic targeting of CIITA in breast cancer cells through recruitment of histone methyltransferase resulted in inhibition of CIITA expression (74). Deficient class II expression in the mouse adencocarcinoma cell line was due to decreased CIITA protein stability (405).

Dysregulation of CIITA expression is not only restricted to tumors as a way to escape immune recognition, but pathogens evade the immune system by similar mechanisms. For example, the IFN-γ signaling pathway is altered by Cytomegalovirus, which results in CIITA repression and, thus, downregulation of HLA class II molecules (406). Moreover, murine macrophages infected by Mycobacterium bovis BCG, have decreased STAT1α phosphorylation, thus decreasing CIITA expression (407). Likewise, Chlamydia infection results in degradation of the upstream stimulatory factor 1 (USF1), an important transcription factor for CIITA synthesis (408). All these previous studies provide strong evidence that cancer cells and different pathogens have targeted CIITA as a way to evade the immune system, suggesting its important role in regulating the immune response.

The significance of CIITA expression and subsequently, HLA class II in cancer remains controversial. In some studies, overexpression of HLA class II in different types of tumors, including breast, has been associated with poor outcome and tumor

progression (409, 410). It has been speculated that HLA class II positive tumors escape from immune surveillance through activating regulatory T-cells results in suppression of the immune system (411). On the other hand, hypermethylation of CIITA promoter and subsequently, the loss of IFN-γ induced HLA class II expression has been regarded as a way of generating immune privilege to cancer cells and preventing their recognition by cells of the immune system (404). A possible hypothesis for the different anti-tumor immune response may be related to which isoforms of CIITA is activated. For example pIII CIITA is responsible for constitutive HLA class II expression, while pIV CIITA is responsible for IFN-γ induced HLA class II expression. Yet, IFN-γ is an indicator of better immune responses. (106). This suggests that IFN-γ plays a significant role in upregulating these molecules in breast cancer cells. Since pIV CIITA is dependent on IFN-γ, which is produced by Th1 and cytotoxic T cells that may be present in the tumor microenvironment and functions to kill tumor cells, one would expect that the tumor will try to shut down the mechanism of IFN-γ induced activation by hypermethylation of the CIITA promoter. On the other hand, pIII CIITA, which is constitutively activated and correlates with a bad prognosis (203), could be due to the activation of other signaling pathways. An example is MAPK in cancer cells, which discordantly express HLA class II molecules in the absence of co-stimulatory molecules and thus, stimulate T regulatory cells (203, 412). Thus, future studies could attempt to correlate differential class II expression and antigen processing with the expression of pIII or pIV CIITA transcripts.

There is strong experimental evidence to support a positive role for class II expression in anti-tumor immunity. For example, studies show that transfecting a murine

mammary adenocarcinoma cell line with either the IFN-γ (413) or the CIITA gene (117) resulted in tumor rejection in a $CD4^+$ T cell dependent manner by upregulating class II antigens. In contrast, other studies suggest that overexpression of CIITA may be harmful in tumor therapy due to increased expression of Ii, which favours presentation of exogenous antigens and possibly suggests that class II-transfected tumors are more immunogenic due to their ability to present endogenous peptide (414, 415). In agreement, upregulation of Ii expression in tumor cells was associated with poor prognosis (119, 416). HLA-DR^+ Ii^+ and HLA-DM^- breast tumors were associated with worse prognosis regardless of the ER status (106). The upregulation of Ii may impede an effective HLA class II restricted anti-tumor immune response. Probably, Ii blocks the HLA class II peptide binding-groove, thus, resulting in inefficient presentation of the endogenous tumor specific antigen (TSA) or tumor-associated antigen (TAA) by HLA class II molecules (414, 417, 418). Moreover, in the absence of DM, stable exogenous peptides (e.g. peptides from tumor antigens that intersected the endocytic pathway) are unable to bind. The negative association of HLA-DR and Ii expression with ER in breast carcinoma (106), together with our finding that MC2 expressed significantly less HLA class II than VC5, suggests that ER negatively modulates HLA class II expression in cancer (Figure 7.3).

Normal proteasomal degradation of Ii by cathepsin S in the endosomal compartment results in degradation of the Ii chain such that only the CLIP peptide remains bound to the peptide-binding groove of HLA class II molecules (18). HLA-DM, which acts as a 'peptide editor', exchanges CLIP and low affinity binding peptides from the peptide-binding groove, with stable and high affinity peptides (11). Although the role

of HLA-DM in antigen presentation in the APC is significant, its role in tumor cells is the subject of argument. In one study, sarcoma cells transfected with class II without coexpression of Ii and DM was highly immunogenic, while tumors cells, which coordinately expresses the class II, and two co-chaperones were not effective immunogens (414). In contrast, our lab demonstrated that coordinate expression of HLA-DR and the co-chaperone in breast carcinoma is an independent predictor of survival and is associated with Th1 cytokine profile (106). This was also supported by another study on ovarian cancer cells that showed high levels of HLA-DMB were associated with improved survival (419). HLA-DM is important in preventing surface CLIP expression, thus avoiding Th2 type responses (420). In support of that, van Luijn et. al. (2011) showed that leukemic blasts of AML patients, which lacked surface CLIP expression, stimulated activation of $CD4^+$ Th1 response (421).

In this study, it was clearly shown that IFN-γ induced CIITA and HLA class II were differentially expressed between $ER\alpha^-$ and $ER\alpha^+$. This followed on a study from our lab showing an increased frequency of HLA class II positive tumor cells in $ER\alpha^-$ breast carcinoma from younger women as compared to $ER\alpha^+$ tumors from older women (106). Since breast carcinomas contain high levels of estrogen, irrespective of menopausal status (100), these observations implied that E_2 negatively regulates HLA class II in $ER\alpha^+$ but not in $ER\alpha^-$ tumor cells. Although *in vitro* studies showed that breast cancer cells upregulated HLA class II and behaved as an APC and engaged $CD4^+$ T cells (114), it is still not known whether the same mechanism occurs *in vivo*. However, since several studies have shown that HLA class II positive tumors are associated with favourable outcomes (103, 106, 410), it suggests that breast tumors can engage $CD4^+$ T cells

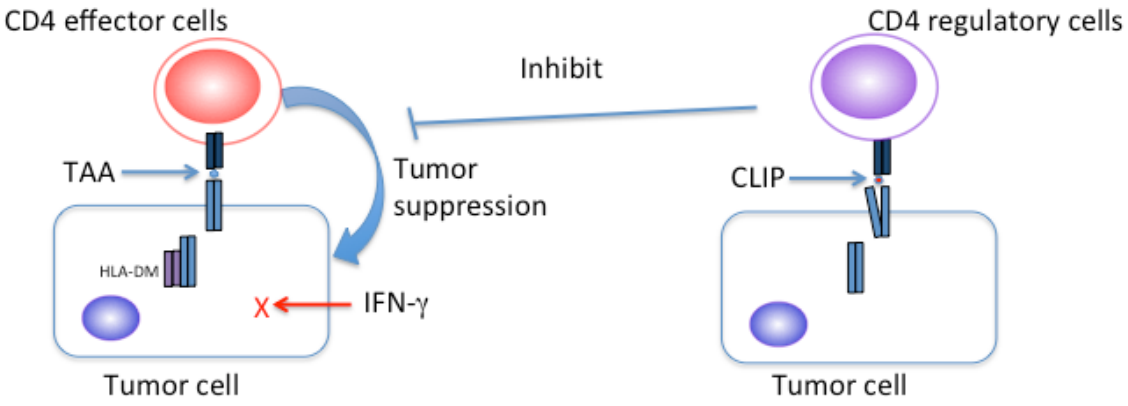
efficaciously. On the other hand, there are other mechanisms by which tumor cells escape immune surveillance. For example, tumor cells that express HLA class II in the absence of the co-stimulatory molecules may anergize tumor specific T cells (111, 422).

Notably, in our study E_2-ERα activation interfered with IFN-γ signaling, explaining the absence of HLA class II molecules in $ER\alpha^+$ breast tumors even in the presence of high IFN-γ (106). Investigations such as this provide improved understanding that $ER\alpha^-$ and $ER\alpha^+$ breast cancer cells differentially regulate HLA class II expression and hence, have different antigen processing and presentation capacities. Thus, targeting antigen-processing deviation for the development of successful tumor immunotherapy may be different for $ER\alpha^-$ and $ER\alpha^+$ breast tumors.

Figure 7.3 Significance of HLA class II expression in breast cancer cells

Coordinate expression of HLA class II (HLA-DR, DM and Ii) are important in proper presentation of tumor associated antigen (TAA) to $CD4^+$ Th1 cells. Activation of Th1 cells supresses tumour growth directly by Fas-Fas Ligand inhibition or indirectly by secretion of IFN-γ. Cancer cells can evade immune elimination by modulation of IFN-γ signalling pathway.

Expression of HLA-DR on the cell surface in the absence of HLA-DM, results in the formation of unstable HLA-DRα/β dimers. Morover, nonspecific peptides or CLIP may be presented in the context of HLA-DR and activate $CD4^+$ T regulatory cells. $CD4^+$ T regulatory cells may inhibit tumour suppression by inhibiting activation of $CD4^+$ Th1 cells.


CD4 effector cells
CD4 regulatory cells
Inhibit
TAA
Tumor
suppression
CLIP
HLA-DM
X
IFN-γ
Tumor cell
Tumor cell

7.4. Study limitations

7.4.1. Cell lines

A panel of ERα$^-$ and ERα$^+$ breast cancer cells were used in this study as in vitro models for breast cancer. These established cell lines are representative of the initial breast tumors as described in Table 2.1. The prognosis and treatment are different for each subtype (423). Luminal A and luminal B subtypes are ERα$^+$, thus, they can be targeted by hormonal therapy (424). Similarly, the HER2 breast cancer can be treated with trasuszumab therapy (425). Basal subtype is characterized by the absence of ER, PR and HER2 expression and they are known as triple negative, which represents the most aggressive, and challenging group for treatment (426). Although the BCCL are reportedly representative of the various breast cancer subtypes and have the advantage of immortality, easy handling and culturing, they have the disadvantage that prolonged culture may modify the cells and alter their genetic properties. For example, MCF-7 cells obtained from different laboratories appear morphologically normal, but they differ in their rate of growth and response to hormones. This is due to different levels of ER being expressed by the cells (427). In addition, some argue that MDA-MB-231, MCF-7, T47D and SK-BR-3, which are not derived from the primary tumor, but rather from pleural effusion, may behave differently because they are more aggressive and more metastatic (294).

7.4.2. Transfection of ESR1 into an ERα$^-$ cell line may not be representative of endogenously expressed ERα

Transfection is a potent tool used to study gene expression, protein function and regulation by introducing genetic materials into the cells. In most of my studies, I used a BCCL model of $ERα^+$ line, MC2 (MDA-MB-231 stably transfected with the wild type ERα gene) and $ERα^-$ line, VC5 (MDA-MB-231 stably transfected with the empty vector), to study the mechanism of regulation of HLA class II by ERα. In addition, transient transfection of ERα was also carried in a number of experiments. Stable transfections of ER are integrated into the host genome and expressed after the cells replicate. In contrast, transient transfections of ER are only expressed for a limited period of time and lost by cell division (428). Overexpression of ER is toxic to the cells, possibly through squelching effects that result in alteration of gene function (429). Moreover, growth was inhibited when MDA-MB-231 was transfected with an exogenous ERα cDNA plasmid (286). Thus, experimental results obtained by ectopic expression of ERα in cells that lack ERα receptor expression might not be entirely applicable to cells that endogenously express the receptors. Stable transfection of ERα into MDA-MB-231 resulted in morphological changes in the cells to become more luminal than basal (430); however, this resulted in decreased cell proliferation as noticed by our lab (unpublished) and other (431), which was claimed due to suppression of COX-2 and VEGF-C.

7.4.3. Reporter gene assays

As an indirect way to measure gene promoter activity, identify estrogen response element (ERE) regions and do mutational analysis, luciferase reporter gene assays were performed. Luciferase activity has a high sensitivity, is an easy technique, and not normally expressed in mammalian tissues (432). However, some of the drawbacks

include lack of chromatin structure, regulatory coding regions and distal regulatory elements in the promoters; moreover, addition of exogenous components to the cells may results in cell toxicity(433). Thus, in order to overcome these issues, we validated the reporter assay by testing the downstream effect on genes and proteins by immunoblotting and PCR.

7.5.Future directions

The work described in this thesis provides insight into the complexity of, and cross talk between different signaling pathways and HLA class II regulation in $ER\alpha^+$ and $ER\alpha^-$ breast cancer cells. Despite a large number of experiments and important findings, there are still several questions to be addressed in future studies.

1) We demonstrated that E_2-ER signaling adversely affected STAT1 activated genes as shown by reduced GAS activity in $ER\alpha^+$. However, due to the complexity of the pathways involved and time limitations, the exact mechanisms were not totally resolved. To better understand and identify the pathways dysregulated by ERα in the presence of IFN-γ, global gene expression analysis by microarray would be the standard technique to apply. Microarray analysis identifies gene expression profiling and measures the differential downstream gene expression between $ER\alpha^+$ and $ER\alpha^-$ breast cancer cell lines. MC2 and VC5 cells should be the perfect models for microarray analysis, since they only differ by ERα expression. Next, a panel of established $ER\alpha^+$ and $ER\alpha^-$ cell lines can be examined by microarray analysis and the genes with statistically increased or decreased expression between the two groups can be identified. Gene ontology analysis can be

performed to determine the biological process associated with the significant dysregulated genes as well as in identifying the possible pathways involved.

2) Although our results clearly showed that E_2 and ICI had no effect on STAT1 phosphorylation, they do not exclude the possibility that E_2 may interfere with STAT1 dimerization, nuclear localisation, and binding to a specific promoter. Fluorescence microscopy can be done using antibodies specific for phosphorylated STAT1 to identify subcellular localization. Furthermore, this technique would allow us to determine if ERα interferes with the normal nuclear localisation of STAT1 as compared to ERα$^-$ cell lines. In order to assess if ERα interferes with STAT1 binding to GAS sequence located in the regulatory element of STAT1 activated genes, chromatin immuneprecipitation would be the gold standard technique to apply.

3) We determined that the suppressive effect of E_2-ER on CIITA promoter activity is unlikely to be through direct binding of the predicted ERE in pIV CIITA. This does not exclude an alternative-binding site such as AP1 or SP1. We only explored the 5' untranslated proximal CIITA region for ERE binding sites; however, it was recently shown that only 10% of ERE are identified in this region and the majority of the ERE are present in the intronic or intergenic regions (363). Thus, ER can regulate genes distally from their transcription initiation site, which could be further evaluated in the CIITA gene using binding site prediction programs and evaluated them directly by site directed mutagenesis and chromatin immunoprecipitation assays.

4) Studying the effect of other hormones such as prolactin in the regulation of HLA class II expression in breast cancer is of great importance. Prolactin contributes to the initiation or progression of breast cancer and is important in HLA class II expression in benign breast tumours and MCF-7 cells (91, 93, 434). Several studies have suggested cross talk of prolactin and ERα signaling pathways in breast cancer cells (435-437). Moreover, prolactin has been implicated directly in several pathways such as; JAK2/STAT5 and JAK1/STAT3 and indirectly through cross talk with other signaling such as; Src family kinases (PI3K)/Akt and Raf/MEK/ERK in cancer cell lines as reviewed in (438). Some of these pathways have been involved in regulation of HLA class II expression as previously mentioned (Chapter 6). This may suggest that prolactin interacts with one of these pathways to regulate HLA class II expression in breast cancer cells.

5) IRF-2 in contrast to IRF1 has oncolytic activity, thus the ratio between IRF1 and IRF-2 is very important in cells to maintain proper cell cycle and any imbalance in this ratio may lead to cancer (439). Both IRF1 and IRF-2 compete for the same binding site in the promoter of the genes, but in CIITA, both transcription factors bind simultaneously to the same sequence (440). Due to technical problems with antibody detection, we were not able to detect specific bands for IRF-2 in immunoblotting. In fact, others have discussed similar technical issues (364). Future studies will need to address optimization of the antibody as it was shown previously that IRF-2 was the causative factor for downregulation of IRF1 in $ER\alpha^{+}$ cells (441).

6) The mechanism behind regulation of HLA-DR transport by ERK is not fully understood. However one study established that ERK signaling regulates endosomal trafficking through the clathrin-independent, ADP-ribosylation factor 6 (Arf6) GTPase-regulated endosomal pathways. U0126 resulted in inactivation of ERK and expansion of the Arf6 compartment, which negatively correlated with HLA class I expression (390). ERK may regulate recycling of HLA class II under the same mechanism involved in class I, since clathrin independent endocytosis is responsible for recycling of HLA class II from the cell surface (393). Thus further identification of the mechanism by which ERK regulates HLA-DR should form the basis of future studies.

Chapter 8 References

1. Horton R, Wilming L, Rand V, Lovering RC, Bruford EA, Khodiyar VK, et al. Gene map of the extended human MHC. Nat Rev Genet. 2004 Dec;5(12):889-99.

2. Bodmer WF. The HLA system: Structure and function. J Clin Pathol. 1987 Sep;40(9):948-58.

3. Complete sequence and gene map of a human major histocompatibility complex. the MHC sequencing consortium. Nature. 1999 Oct 28;401(6756):921-3.

4. Riteau B, Menier C, Khalil-Daher I, Martinozzi S, Pla M, Dausset J, et al. HLA-G1 co-expression boosts the HLA class I-mediated NK lysis inhibition. Int Immunol. 2001 Feb;13(2):193-201.

5. Lepin EJ, Bastin JM, Allan DS, Roncador G, Braud VM, Mason DY, et al. Functional characterization of HLA-F and binding of HLA-F tetramers to ILT2 and ILT4 receptors. Eur J Immunol. 2000 Dec;30(12):3552-61.

6. Jinushi M, Takehara T, Kanto T, Tatsumi T, Groh V, Spies T, et al. Critical role of MHC class I-related chain A and B expression on IFN-alpha-stimulated dendritic cells in NK cell activation: Impairment in chronic hepatitis C virus infection. J Immunol. 2003 Feb 1;170(3):1249-56.

7. Geppert TD, Lipsky PE. Antigen presentation by interferon-gamma-treated endothelial cells and fibroblasts: Differential ability to function as antigen-presenting cells despite comparable ia expression. J Immunol. 1985 Dec;135(6):3750-62.

8. Boyer CM, Dawson DV, Neal SE, Winchell LF, Leslie DS, Ring D, et al. Differential induction by interferons of major histocompatibility complex-encoded and non-major histocompatibility complex-encoded antigens in human breast and ovarian carcinoma cell lines. Cancer Res. 1989 Jun 1;49(11):2928-34.

9. Basham TY, Nickoloff BJ, Merigan TC, Morhenn VB. Recombinant gamma interferon differentially regulates class II antigen expression and biosynthesis on cultured normal human keratinocytes. J Interferon Res. 1985 Winter;5(1):23-32.

10. Kropshofer H, Vogt AB, Thery C, Armandola EA, Li BC, Moldenhauer G, et al. A role for HLA-DO as a co-chaperone of HLA-DM in peptide loading of MHC class II molecules. EMBO J. 1998 Jun 1;17(11):2971-81.

11. Denzin LK, Cresswell P. HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. Cell. 1995 Jul 14;82(1):155-65.

12. Pamer E, Cresswell P. Mechanisms of MHC class I--restricted antigen processing. Annu Rev Immunol. 1998;16:323-58.

13. Robinson J, Halliwell JA, McWilliam H, Lopez R, Parham P, Marsh SG. The IMGT/HLA database. Nucleic Acids Res. 2013 Jan 1;41(D1):D1222-7.

14. Klein J, Sato A. The HLA system. first of two parts. N Engl J Med. 2000 Sep 7;343(10):702-9.

15. Pieters J. MHC class II restricted antigen presentation. Curr Opin Immunol. 1997 Feb;9(1):89-96.

16. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. Nature. 1990 Jun 14;345(6276):615-8.

17. Peters PJ, Neefjes JJ, Oorschot V, Ploegh HL, Geuze HJ. Segregation of MHC class II molecules from MHC class I molecules in the golgi complex for transport to lysosomal compartments. Nature. 1991 Feb 21;349(6311):669-76.

18. Ghosh P, Amaya M, Mellins E, Wiley DC. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. Nature. 1995 Nov 30;378(6556):457-62.

19. Kropshofer H, Vogt AB, Moldenhauer G, Hammer J, Blum JS, Hammerling GJ. Editing of the HLA-DR-peptide repertoire by HLA-DM. EMBO J. 1996 Nov 15;15(22):6144-54.

20. Arndt SO, Vogt AB, Markovic-Plese S, Martin R, Moldenhauer G, Wolpl A, et al. Functional HLA-DM on the surface of B cells and immature dendritic cells. EMBO J. 2000 Mar 15;19(6):1241-51.

21. Kropshofer H, Arndt SO, Moldenhauer G, Hammerling GJ, Vogt AB. HLA-DM acts as a molecular chaperone and rescues empty HLA-DR molecules at lysosomal pH. Immunity. 1997 Mar;6(3):293-302.

22. Riberdy JM, Newcomb JR, Surman MJ, Barbosa JA, Cresswell P. HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides. Nature. 1992 Dec 3;360(6403):474-7.

23. Young JA, Trowsdale J. The HLA-DNA (DZA) gene is correctly expressed as a 1.1 kb mature mRNA transcript. Immunogenetics. 1990;31(5-6):386-8.

24. Tonnelle C, DeMars R, Long EO. DO beta: A new beta chain gene in HLA-D with a distinct regulation of expression. EMBO J. 1985 Nov;4(11):2839-47.

25. Denzin LK, Sant'Angelo DB, Hammond C, Surman MJ, Cresswell P. Negative regulation by HLA-DO of MHC class II-restricted antigen processing. Science. 1997 Oct 3;278(5335):106-9.

26. Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. Immunol Today. 2000 Sep;21(9):455-64.

27. Guce AI, Mortimer SE, Yoon T, Painter CA, Jiang W, Mellins ED, et al. HLA-DO acts as a substrate mimic to inhibit HLA-DM by a competitive mechanism. Nat Struct Mol Biol. 2013 Jan;20(1):90-8.

28. Boss JM, Jensen PE. Transcriptional regulation of the MHC class II antigen presentation pathway. Curr Opin Immunol. 2003 Feb;15(1):105-11.

29. Zika E, Ting JP. Epigenetic control of MHC-II: Interplay between CIITA and histone-modifying enzymes. Curr Opin Immunol. 2005 Feb;17(1):58-64.

30. Steimle V, Durand B, Barras E, Zufferey M, Hadam MR, Mach B, et al. A novel DNA-binding regulatory factor is mutated in primary MHC class II deficiency (bare lymphocyte syndrome). Genes Dev. 1995 May 1;9(9):1021-32.

31. Masternak K, Barras E, Zufferey M, Conrad B, Corthals G, Aebersold R, et al. A gene encoding a novel RFX-associated transactivator is mutated in the majority of MHC class II deficiency patients. Nat Genet. 1998 Nov;20(3):273-7.

32. Durand B, Sperisen P, Emery P, Barras E, Zufferey M, Mach B, et al. RFXAP, a novel subunit of the RFX DNA binding complex is mutated in MHC class II deficiency. EMBO J. 1997 Mar 3;16(5):1045-55.

33. Nagarajan UM, Louis-Plence P, DeSandro A, Nilsen R, Bushey A, Boss JM. RFX-B is the gene responsible for the most common cause of the bare lymphocyte syndrome, an MHC class II immunodeficiency. Immunity. 1999 Feb;10(2):153-62.

34. Moreno CS, Beresford GW, Louis-Plence P, Morris AC, Boss JM. CREB regulates MHC class II expression in a CIITA-dependent manner. Immunity. 1999 Feb;10(2):143-51.

35. Mantovani R. The molecular biology of the CCAAT-binding factor NF-Y. Gene. 1999 Oct 18;239(1):15-27.

36. Jabrane-Ferrat N, Fontes JD, Boss JM, Peterlin BM. Complex architecture of major histocompatibility complex class II promoters: Reiterated motifs and conserved protein-protein interactions. Mol Cell Biol. 1996 Sep;16(9):4683-90.

37. Reith W, LeibundGut-Landmann S, Waldburger JM. Regulation of MHC class II gene expression by the class II transactivator. Nat Rev Immunol. 2005 Oct;5(10):793-806.

38. Ting JP, Trowsdale J. Genetic control of MHC class II expression. Cell. 2002 Apr;109 Suppl:S21-33.

39. Zhu XS, Linhoff MW, Li G, Chin KC, Maity SN, Ting JP. Transcriptional scaffold: CIITA interacts with NF-Y, RFX, and CREB to cause stereospecific regulation of the class II major histocompatibility complex promoter. Mol Cell Biol. 2000 Aug;20(16):6051-61.

40. Masternak K, Muhlethaler-Mottet A, Villard J, Zufferey M, Steimle V, Reith W. CIITA is a transcriptional coactivator that is recruited to MHC class II promoters by multiple synergistic interactions with an enhanceosome complex. Genes Dev. 2000 May 1;14(9):1156-66.

41. Fontes JD, Jiang B, Peterlin BM. The class II trans-activator CIITA interacts with the TBP-associated factor TAFII32. Nucleic Acids Res. 1997 Jun 15;25(12):2522-8.

42. Mahanta SK, Scholl T, Yang FC, Strominger JL. Transactivation by CIITA, the type II bare lymphocyte syndrome-associated factor, requires participation of multiple regions of the TATA box binding protein. Proc Natl Acad Sci U S A. 1997 Jun 10;94(12):6324-9.

43. Kanazawa S, Okamoto T, Peterlin BM. Tat competes with CIITA for the binding to P-TEFb and blocks the expression of MHC class II genes in HIV infection. Immunity. 2000 Jan;12(1):61-70.

44. Kretsovali A, Agalioti T, Spilianakis C, Tzortzakaki E, Merika M, Papamatheakis J. Involvement of CREB binding protein in expression of major histocompatibility complex class II genes via interaction with the class II transactivator. Mol Cell Biol. 1998 Nov;18(11):6777-83.

45. Fontes JD, Kanazawa S, Jean D, Peterlin BM. Interactions between the class II transactivator and CREB binding protein increase transcription of major histocompatibility complex class II genes. Mol Cell Biol. 1999 Jan;19(1):941-7.

46. Riley JL, Westerheide SD, Price JA, Brown JA, Boss JM. Activation of class II MHC genes requires both the X box region and the class II transactivator (CIITA). Immunity. 1995 May;2(5):533-43.

47. Reith W, Mach B. The bare lymphocyte syndrome and the regulation of MHC expression. Annu Rev Immunol. 2001;19:331-73.

48. Chang CH, Flavell RA. Class II transactivator regulates the expression of multiple genes involved in antigen presentation. J Exp Med. 1995 Feb 1;181(2):765-7.

49. Martin BK, Chin KC, Olsen JC, Skinner CA, Dey A, Ozato K, et al. Induction of MHC class I expression by the MHC class II transactivator CIITA. Immunity. 1997 May;6(5):591-600.

50. Gobin SJ, Peijnenburg A, Keijsers V, van den Elsen PJ. Site alpha is crucial for two routes of IFN gamma-induced MHC class I transactivation: The ISRE-mediated route and a novel pathway involving CIITA. Immunity. 1997 May;6(5):601-11.

51. Gobin SJ, Peijnenburg A, van Eggermond M, van Zutphen M, van den Berg R, van den Elsen PJ. The RFX complex is crucial for the constitutive and CIITA-mediated transactivation of MHC class I and beta2-microglobulin genes. Immunity. 1998 Oct;9(4):531-41.

52. Neerincx A, Rodriguez GM, Steimle V, Kufer TA. NLRC5 controls basal MHC class I gene expression in an MHC enhanceosome-dependent manner. J Immunol. 2012 May 15;188(10):4940-50.

53. Ting JP, Davis BK. CATERPILLER: A novel gene family important in immunity, cell death, and diseases. Annu Rev Immunol. 2005;23:387-414.

54. Inohara, Chamaillard, McDonald C, Nunez G. NOD-LRR proteins: Role in host-microbial interactions and inflammatory disease. Annu Rev Biochem. 2005;74:355-83.

55. Kretsovali A, Spilianakis C, Dimakopoulos A, Makatounakis T, Papamatheakis J. Self-association of class II transactivator correlates with its intracellular localization and transactivation. J Biol Chem. 2001 Aug 24;276(34):32191-7.

56. Linhoff MW, Harton JA, Cressman DE, Martin BK, Ting JP. Two distinct domains within CIITA mediate self-association: Involvement of the GTP-binding and leucine-rich repeat domains. Mol Cell Biol. 2001 May;21(9):3001-11.

57. Sisk TJ, Roys S, Chang CH. Self-association of CIITA and its transactivation potential. Mol Cell Biol. 2001 Aug;21(15):4919-28.

58. Raval A, Howcroft TK, Weissman JD, Kirshner S, Zhu XS, Yokoyama K, et al. Transcriptional coactivator, CIITA, is an acetyltransferase that bypasses a promoter requirement for TAF(II)250. Mol Cell. 2001 Jan;7(1):105-15.

59. Zhu XS, Linhoff MW, Li G, Chin KC, Maity SN, Ting JP. Transcriptional scaffold: CIITA interacts with NF-Y, RFX, and CREB to cause stereospecific regulation of the class II major histocompatibility complex promoter. Mol Cell Biol. 2000 Aug;20(16):6051-61.

60. Kretsovali A, Agalioti T, Spilianakis C, Tzortzakaki E, Merika M, Papamatheakis J. Involvement of CREB binding protein in expression of major histocompatibility complex class II genes via interaction with the class II transactivator. Mol Cell Biol. 1998 Nov;18(11):6777-83.

61. Fontes JD, Kanazawa S, Jean D, Peterlin BM. Interactions between the class II transactivator and CREB binding protein increase transcription of major histocompatibility complex class II genes. Mol Cell Biol. 1999 Jan;19(1):941-7.

62. Hake SB, Masternak K, Kammerbauer C, Janzen C, Reith W, Steimle V. CIITA leucine-rich repeats control nuclear localization, in vivo recruitment to the major histocompatibility complex (MHC) class II enhanceosome, and MHC class II gene transactivation. Mol Cell Biol. 2000 Oct;20(20):7716-25.

63. Cressman DE, O'Connor WJ, Greer SF, Zhu XS, Ting JP. Mechanisms of nuclear import and export that control the subcellular localization of class II transactivator. J Immunol. 2001 Oct 1;167(7):3626-34.

64. Cressman DE, Chin KC, Taxman DJ, Ting JP. A defect in the nuclear translocation of CIITA causes a form of type II bare lymphocyte syndrome. Immunity. 1999 Feb;10(2):163-71.

65. Harton JA, Cressman DE, Chin KC, Der CJ, Ting JP. GTP binding by class II transactivator: Role in nuclear import. Science. 1999 Aug 27;285(5432):1402-5.

66. Wright KL, Ting JP. Epigenetic regulation of MHC-II and CIITA genes. Trends Immunol. 2006 Sep;27(9):405-12.

67. Zika E, Ting JP. Epigenetic control of MHC-II: Interplay between CIITA and histone-modifying enzymes. Curr Opin Immunol. 2005 Feb;17(1):58-64.

68. Zika E, Fauquier L, Vandel L, Ting JP. Interplay among coactivator-associated arginine methyltransferase 1, CBP, and CIITA in IFN-gamma-inducible MHC-II gene expression. Proc Natl Acad Sci U S A. 2005 Nov 8;102(45):16321-6.

69. Ni Z, Abou El Hassan M, Xu Z, Yu T, Bremner R. The chromatin-remodeling enzyme BRG1 coordinates CIITA induction through many interdependent distal enhancers. Nat Immunol. 2008 Jul;9(7):785-93.

70. Greer SF, Zika E, Conti B, Zhu XS, Ting JP. Enhancement of CIITA transcriptional function by ubiquitin. Nat Immunol. 2003 Nov;4(11):1074-82.

71. Kanaseki T, Ikeda H, Takamura Y, Toyota M, Hirohashi Y, Tokino T, et al. Histone deacetylation, but not hypermethylation, modifies class II transactivator and MHC class II gene expression in squamous cell carcinomas. J Immunol. 2003 May 15;170(10):4980-5.

72. Magner WJ, Kazim AL, Stewart C, Romano MA, Catalano G, Grande C, et al. Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. J Immunol. 2000 Dec 15;165(12):7017-24.

73. Zika E, Greer SF, Zhu XS, Ting JP. Histone deacetylase 1/mSin3A disrupts gamma interferon-induced CIITA function and major histocompatibility complex class II enhanceosome formation. Mol Cell Biol. 2003 May;23(9):3091-102.

74. Londhe P, Zhu B, Abraham J, Keller C, Davie J. CIITA is silenced by epigenetic mechanisms that prevent the recruitment of transactivating factors in rhabdomyosarcoma cells. Int J Cancer. 2012 Aug 15;131(4):E437-48.

75. De Lerma Barbaro A, Procopio FA, Mortara L, Tosi G, Accolla RS. The MHC class II transactivator (CIITA) mRNA stability is critical for the HLA class II gene expression in myelomonocytic cells. Eur J Immunol. 2005 Feb;35(2):603-11.

76. Schnappauf F, Hake SB, Camacho Carvajal MM, Bontron S, Lisowska-Grospierre B, Steimle V. N-terminal destruction signals lead to rapid degradation of the major histocompatibility complex class II transactivator CIITA. Eur J Immunol. 2003 Aug;33(8):2337-47.

77. Muhlethaler-Mottet A, Otten LA, Steimle V, Mach B. Expression of MHC class II molecules in different cellular and functional compartments is controlled by differential usage of multiple promoters of the transactivator CIITA. EMBO J. 1997 May 15;16(10):2851-60.

78. Barbieri G, Deffrennes V, Prod'homme T, Vedrenne J, Baton F, Cortes C, et al. Isoforms of the class II transactivator protein. Int Immunol. 2002 Aug;14(8):839-48.

79. Dong Y, Rohn WM, Benveniste EN. IFN-gamma regulation of the type IV class II transactivator promoter in astrocytes. J Immunol. 1999 Apr 15;162(8):4731-9.

80. Muhlethaler-Mottet A, Di Berardino W, Otten LA, Mach B. Activation of the MHC class II transactivator CIITA by interferon-gamma requires cooperative interaction between Stat1 and USF-1. Immunity. 1998 Feb;8(2):157-66.

81. Landmann S, Muhlethaler-Mottet A, Bernasconi L, Suter T, Waldburger JM, Masternak K, et al. Maturation of dendritic cells is accompanied by rapid transcriptional silencing of class II transactivator (CIITA) expression. J Exp Med. 2001 Aug 20;194(4):379-91.

82. Lennon AM, Ottone C, Rigaud G, Deaven LL, Longmire J, Fellous M, et al. Isolation of a B-cell-specific promoter for the human class II transactivator. Immunogenetics. 1997;45(4):266-73.

83. Ghosh N, Piskurich JF, Wright G, Hassani K, Ting JP, Wright KL. A novel element and a TEF-2-like element activate the major histocompatibility complex class II transactivator in B-lymphocytes. J Biol Chem. 1999 Nov 5;274(45):32342-50.

84. van der Stoep N, Quinten E, van den Elsen PJ. Transcriptional regulation of the MHC class II trans-activator (CIITA) promoter III: Identification of a novel regulatory region in the 5'-untranslated region and an important role for cAMP-responsive element binding protein 1 and activating transcription factor-1 in CIITA-promoter III transcriptional activation in B lymphocytes. J Immunol. 2002 Nov 1;169(9):5061-71.

85. van der Stoep N, Quinten E, Marcondes Rezende M, van den Elsen PJ. E47, IRF-4, and PU.1 synergize to induce B-cell-specific activation of the class II transactivator promoter III (CIITA-PIII). Blood. 2004 Nov 1;104(9):2849-57.

86. Piskurich JF, Lin KI, Lin Y, Wang Y, Ting JP, Calame K. BLIMP-I mediates extinction of major histocompatibility class II transactivator expression in plasma cells. Nat Immunol. 2000 Dec;1(6):526-32.

87. Ghosh N, Gyory I, Wright G, Wood J, Wright KL. Positive regulatory domain I binding factor 1 silences class II transactivator expression in multiple myeloma cells. J Biol Chem. 2001 May 4;276(18):15264-8.

88. Nikcevich KM, Piskurich JF, Hellendall RP, Wang Y, Ting JP. Differential selectivity of CIITA promoter activation by IFN-gamma and IRF-1 in astrocytes and macrophages: CIITA promoter activation is not affected by TNF-alpha. J Neuroimmunol. 1999 Oct 29;99(2):195-204.

89. Piskurich JF, Wang Y, Linhoff MW, White LC, Ting JP. Identification of distinct regions of 5' flanking DNA that mediate constitutive, IFN-gamma, STAT1, and TGF-beta-regulated expression of the class II transactivator gene. J Immunol. 1998 Jan 1;160(1):233-40.

90. Piskurich JF, Linhoff MW, Wang Y, Ting JP. Two distinct gamma interferon-inducible promoters of the major histocompatibility complex class II transactivator gene are differentially

regulated by STAT1, interferon regulatory factor 1, and transforming growth factor beta. Mol Cell Biol. 1999 Jan;19(1):431-40.

91. Bartek J, Petrek M, Vojtesek B, Bartkova J, Kovarik J, Rejthar A. HLA-DR antigens on differentiating human mammary gland epithelium and breast tumours. Br J Cancer. 1987 Dec;56(6):727-33.

92. Newman RA, Ormerod MG, Greaves MF. The presence of HLA-DR antigens on lactating human breast epithelium and milk fat globule membranes. Clin Exp Immunol. 1980 Sep;41(3):478-86.

93. Bernard DJ, Maurizis JC, Chassagne J, Chollet P, Plagne R. Effect of prolactin on class II HLA antigen expression by MCF7 cell line. Anticancer Res. 1986 Jan-Feb;6(1):79-83.

94. Campoli M, Chang CC, Oldford SA, Edgecombe AD, Drover S, Ferrone S. HLA antigen changes in malignant tumors of mammary epithelial origin: Molecular mechanisms and clinical implications. Breast Dis. 2004;20:105-25.

95. Jabrane-Ferrat N, Faille A, Loiseau P, Poirier O, Charron D, Calvo F. Effect of gamma interferon on HLA class-I and -II transcription and protein expression in human breast adenocarcinoma cell lines. Int J Cancer. 1990 Jun 15;45(6):1169-76.

96. Tabibzadeh SS, Sivarajah A, Carpenter D, Ohlsson-Wilhelm BM, Satyaswaroop PG. Modulation of HLA-DR expression in epithelial cells by interleukin 1 and estradiol-17 beta. J Clin Endocrinol Metab. 1990 Sep;71(3):740-7.

97. Sedlak J, Speiser P, Zeillinger R, Krugluger W, Wiltschke C, Kubista E, et al. Cytokine (IFN-alpha, IFN-gamma, IL-1-alpha, TNF-alpha)-induced modulation of HLA cell surface expression in human breast cancer cell lines. Neoplasma. 1992;39(5):269-72.

98. Obiri NI, Siegel JP, Varricchio F, Puri RK. Expression of high-affinity IL-4 receptors on human melanoma, ovarian and breast carcinoma cells. Clin Exp Immunol. 1994 Jan;95(1):148-55.

99. Lee AH, Happerfield LC, Millis RR, Bobrow LG. Inflammatory infiltrate in invasive lobular and ductal carcinoma of the breast. Br J Cancer. 1996 Sep;74(5):796-801.

100. Simpson ER. Sources of estrogen and their importance. J Steroid Biochem Mol Biol. 2003 Sep;86(3-5):225-30.

101. Moller P, Mattfeldt T, Gross C, Schlosshauer P, Koch A, Koretz K, et al. Expression of HLA-A, -B, -C, -DR, -DP, -DQ, and of HLA-D-associated invariant chain (ii) in non-neoplastic mammary epithelium, fibroadenoma, adenoma, and carcinoma of the breast. Am J Pathol. 1989 Jul;135(1):73-83.

102. Whitwell HL, Hughes HP, Moore M, Ahmed A. Expression of major histocompatibility antigens and leucocyte infiltration in benign and malignant human breast disease. Br J Cancer. 1984 Feb;49(2):161-72.

103. Zuk JA, Walker RA. HLA class II sublocus expression in benign and malignant breast epithelium. J Pathol. 1988 Aug;155(4):301-9.

104. Koretz K, Moldenhauer G, Majdic O, Moller P. Correlation of HLA-D/ii antigen expression in breast carcinoma with local lymphohistiocytic infiltration reveals considerable dysregulation in a subset of tumors. Int J Cancer. 1989 Nov 15;44(5):816-22.

105. Oldford SA, J DR, Peter HW, Drover S. HLA-DRB alleles are differentially expressed by tumor cells in breast carcinoma. International journal of cancer Journal international du cancer. 2004 11/10;112(3):399-406.

106. Oldford SA, J DR, Codner D, Gadag V, Peter HW, Drover S. Tumor cell expression of HLA-DM associates with a Th1 profile and predicts improved survival in breast carcinoma patients. Int Immunol. 2006 11;18(11):1591-602.

107. Singal DP, Qiu X. Polymorphism in both X and Y box motifs controls level of expression of HLA-DRB1 genes. Immunogenetics. 1996;43(1-2):50-6.

108. Sheen-Chen SM, Chou FF, Eng HL, Chen WJ. An evaluation of the prognostic significance of HLA-DR expression in axillary-node-negative breast cancer. Surgery. 1994 Sep;116(3):510-5.

109. Becker JC, Brabletz T, Czerny C, Termeer C, Brocker EB. Tumor escape mechanisms from immunosurveillance: Induction of unresponsiveness in a specific MHC-restricted CD4+ human T cell clone by the autologous MHC class II+ melanoma. Int Immunol. 1993 Dec;5(12):1501-8.

110. Driessens G, Kline J, Gajewski TF. Costimulatory and coinhibitory receptors in anti-tumor immunity. Immunol Rev. 2009 May;229(1):126-44.

111. Croft M, Dubey C. Accessory molecule and costimulation requirements for CD4 T cell response. Crit Rev Immunol. 1997;17(1):89-118.

112. Dadmarz R, Sgagias MK, Rosenberg SA, Schwartzentruber DJ. CD4+ T lymphocytes infiltrating human breast cancer recognise autologous tumor in an MHC-class-II restricted fashion. Cancer Immunol Immunother. 1995 Jan;40(1):1-9.

113. Sotiriadou R, Perez SA, Gritzapis AD, Sotiropoulou PA, Echner H, Heinzel S, et al. Peptide HER2(776-788) represents a naturally processed broad MHC class II-restricted T cell epitope. Br J Cancer. 2001 Nov 16;85(10):1527-34.

114. Perez SA, Sotiropoulou PA, Sotiriadou NN, Mamalaki A, Gritzapis AD, Echner H, et al. HER-2/neu-derived peptide 884-899 is expressed by human breast, colorectal and pancreatic adenocarcinomas and is recognized by in-vitro-induced specific CD4(+) T cell clones. Cancer Immunol Immunother. 2002 Jan;50(11):615-24.

115. Armstrong TD, Clements VK, Ostrand-Rosenberg S. Class II-transfected tumor cells directly present endogenous antigen to CD4+ T cells in vitro and are APCs for tumor-encoded antigens in vivo. J Immunother. 1998 May;21(3):218-24.

116. Armstrong TD, Clements VK, Ostrand-Rosenberg S. MHC class II-transfected tumor cells directly present antigen to tumor-specific CD4+ T lymphocytes. J Immunol. 1998 Jan 15;160(2):661-6.

117. Meazza R, Comes A, Orengo AM, Ferrini S, Accolla RS. Tumor rejection by gene transfer of the MHC class II transactivator in murine mammary adenocarcinoma cells. Eur J Immunol. 2003 May;33(5):1183-92.

118. van Luijn MM, Chamuleau ME, Thompson JA, Ostrand-Rosenberg S, Westers TM, Souwer Y, et al. Class II-associated invariant chain peptide down-modulation enhances the immunogenicity of myeloid leukemic blasts resulting in increased CD4+ T-cell responses. Haematologica. 2010 Mar;95(3):485-93.

119. Jiang Z, Xu M, Savas L, LeClair P, Banner BF. Invariant chain expression in colon neoplasms. Virchows Arch. 1999 Jul;435(1):32-6.

120. Kropshofer H, Hammerling GJ, Vogt AB. The impact of the non-classical MHC proteins HLA-DM and HLA-DO on loading of MHC class II molecules. Immunol Rev. 1999 Dec;172:267-78.

121. ISAACS A, LINDENMANN J. Virus interference. I. the interferon. Proc R Soc Lond B Biol Sci. 1957 Sep 12;147(927):258-67.

122. George PM, Badiger R, Alazawi W, Foster GR, Mitchell JA. Pharmacology and therapeutic potential of interferons. Pharmacol Ther. 2012 Jul;135(1):44-53.

123. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: An overview of signals, mechanisms and functions. J Leukoc Biol. 2004 Feb;75(2):163-89.

124. Thoreau E, Petridou B, Kelly PA, Djiane J, Mornon JP. Structural symmetry of the extracellular domain of the cytokine/growth hormone/prolactin receptor family and interferon receptors revealed by hydrophobic cluster analysis. FEBS Lett. 1991 Apr 22;282(1):26-31.

125. Bach EA, Aguet M, Schreiber RD. The IFN gamma receptor: A paradigm for cytokine receptor signaling. Annu Rev Immunol. 1997;15:563-91.

126. Bernabei P, Coccia EM, Rigamonti L, Bosticardo M, Forni G, Pestka S, et al. Interferon-gamma receptor 2 expression as the deciding factor in human T, B, and myeloid cell proliferation or death. J Leukoc Biol. 2001 Dec;70(6):950-60.

127. Farrar MA, Fernandez-Luna J, Schreiber RD. Identification of two regions within the cytoplasmic domain of the human interferon-gamma receptor required for function. J Biol Chem. 1991 Oct 15;266(29):19626-35.

128. Farrar MA, Campbell JD, Schreiber RD. Identification of a functionally important sequence in the C terminus of the interferon-gamma receptor. Proc Natl Acad Sci U S A. 1992 Dec 15;89(24):11706-10.

129. Kotenko SV, Izotova LS, Pollack BP, Mariano TM, Donnelly RJ, Muthukumaran G, et al. Interaction between the components of the interferon gamma receptor complex. J Biol Chem. 1995 Sep 8;270(36):20915-21.

130. Pestka S, Kotenko SV, Muthukumaran G, Izotova LS, Cook JR, Garotta G. The interferon gamma (IFN-gamma) receptor: A paradigm for the multichain cytokine receptor. Cytokine Growth Factor Rev. 1997 Sep;8(3):189-206.

131. Krause CD, Mei E, Xie J, Jia Y, Bopp MA, Hochstrasser RM, et al. Seeing the light: Preassembly and ligand-induced changes of the interferon gamma receptor complex in cells. Mol Cell Proteomics. 2002 Oct;1(10):805-15.

132. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene. 2002 Feb 20;285(1-2):1-24.

133. Yeh TC, Dondi E, Uze G, Pellegrini S. A dual role for the kinase-like domain of the tyrosine kinase Tyk2 in interferon-alpha signaling. Proc Natl Acad Sci U S A. 2000 Aug 1;97(16):8991-6.

134. Schindler C, Strehlow I. Cytokines and STAT signaling. Adv Pharmacol. 2000;47:113-74.

135. Vinkemeier U, Cohen SL, Moarefi I, Chait BT, Kuriyan J, Darnell JE,Jr. DNA binding of in vitro activated Stat1 alpha, Stat1 beta and truncated Stat1: Interaction between NH2-terminal domains stabilizes binding of two dimers to tandem DNA sites. EMBO J. 1996 Oct 15;15(20):5616-26.

136. Horvath CM. STAT proteins and transcriptional responses to extracellular signals. Trends Biochem Sci. 2000 Oct;25(10):496-502.

137. Strehlow I, Schindler C. Amino-terminal signal transducer and activator of transcription (STAT) domains regulate nuclear translocation and STAT deactivation. J Biol Chem. 1998 Oct 23;273(43):28049-56.

138. Horvath CM, Stark GR, Kerr IM, Darnell JE,Jr. Interactions between STAT and non-STAT proteins in the interferon-stimulated gene factor 3 transcription complex. Mol Cell Biol. 1996 Dec;16(12):6957-64.

139. Begitt A, Meyer T, van Rossum M, Vinkemeier U. Nucleocytoplasmic translocation of Stat1 is regulated by a leucine-rich export signal in the coiled-coil domain. Proc Natl Acad Sci U S A. 2000 Sep 12;97(19):10418-23.

140. Chen X, Vinkemeier U, Zhao Y, Jeruzalmi D, Darnell JE,Jr, Kuriyan J. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. Cell. 1998 May 29;93(5):827-39.

141. Yang E, Wen Z, Haspel RL, Zhang JJ, Darnell JE,Jr. The linker domain of Stat1 is required for gamma interferon-driven transcription. Mol Cell Biol. 1999 Jul;19(7):5106-12.

142. Schindler U, Wu P, Rothe M, Brasseur M, McKnight SL. Components of a stat recognition code: Evidence for two layers of molecular selectivity. Immunity. 1995 Jun;2(6):689-97.

143. Levy DE, Kessler DS, Pine R, Darnell JE,Jr. Cytoplasmic activation of ISGF3, the positive regulator of interferon-alpha-stimulated transcription, reconstituted in vitro. Genes Dev. 1989 Sep;3(9):1362-71.

144. Taniguchi T, Ogasawara K, Takaoka A, Tanaka N. IRF family of transcription factors as regulators of host defense. Annu Rev Immunol. 2001;19:623-55.

145. Harada H, Takahashi E, Itoh S, Harada K, Hori TA, Taniguchi T. Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: Implications for a gene network in the interferon system. Mol Cell Biol. 1994 Feb;14(2):1500-9.

146. Watanabe N, Sakakibara J, Hovanessian AG, Taniguchi T, Fujita T. Activation of IFN-beta element by IRF-1 requires a posttranslational event in addition to IRF-1 synthesis. Nucleic Acids Res. 1991 Aug 25;19(16):4421-8.

147. Ealick SE, Cook WJ, Vijay-Kumar S, Carson M, Nagabhushan TL, Trotta PP, et al. Three-dimensional structure of recombinant human interferon-gamma. Science. 1991 May 3;252(5006):698-702.

148. Briscoe J, Rogers NC, Witthuhn BA, Watling D, Harpur AG, Wilks AF, et al. Kinase-negative mutants of JAK1 can sustain interferon-gamma-inducible gene expression but not an antiviral state. EMBO J. 1996 Feb 15;15(4):799-809.

149. Decker T, Kovarik P, Meinke A. GAS elements: A few nucleotides with a major impact on cytokine-induced gene expression. J Interferon Cytokine Res. 1997 Mar;17(3):121-34.

150. Wen Z, Zhong Z, Darnell JE,Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell. 1995 Jul 28;82(2):241-50.

151. Sekimoto T, Imamoto N, Nakajima K, Hirano T, Yoneda Y. Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1. EMBO J. 1997 Dec 1;16(23):7067-77.

152. Lillemeier BF, Koster M, Kerr IM. STAT1 from the cell membrane to the DNA. EMBO J. 2001 May 15;20(10):2508-17.

153. Vinkemeier U, Cohen SL, Moarefi I, Chait BT, Kuriyan J, Darnell JE,Jr. DNA binding of in vitro activated Stat1 alpha, Stat1 beta and truncated Stat1: Interaction between NH2-terminal domains stabilizes binding of two dimers to tandem DNA sites. EMBO J. 1996 Oct 15;15(20):5616-26.

154. Greenlund AC, Farrar MA, Viviano BL, Schreiber RD. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). EMBO J. 1994 Apr 1;13(7):1591-600.

155. van Boxel-Dezaire AH, Stark GR. Cell type-specific signaling in response to interferon-gamma. Curr Top Microbiol Immunol. 2007;316:119-54.

156. Zhou F. Molecular mechanisms of IFN-gamma to up-regulate MHC class I antigen processing and presentation. Int Rev Immunol. 2009;28(3-4):239-60.

157. Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol. 1993;11:571-611.

158. Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, et al. A family of cytokine-inducible inhibitors of signalling. Nature. 1997 Jun 26;387(6636):917-21.

159. Zhang JG, Farley A, Nicholson SE, Willson TA, Zugaro LM, Simpson RJ, et al. The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. Proc Natl Acad Sci U S A. 1999 Mar 2;96(5):2071-6.

160. Song MM, Shuai K. The suppressor of cytokine signaling (SOCS) 1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferative activities. J Biol Chem. 1998 Dec 25;273(52):35056-62.

161. You M, Yu DH, Feng GS. Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated jak/STAT pathway. Mol Cell Biol. 1999 Mar;19(3):2416-24.

162. You M, Yu DH, Feng GS. Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated jak/STAT pathway. Mol Cell Biol. 1999 Mar;19(3):2416-24.

163. Fornerod M, Ohno M, Yoshida M, Mattaj IW. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell. 1997 Sep 19;90(6):1051-60.

164. Gil MP, Bohn E, O'Guin AK, Ramana CV, Levine B, Stark GR, et al. Biologic consequences of Stat1-independent IFN signaling. Proc Natl Acad Sci U S A. 2001 Jun 5;98(12):6680-5.

165. Ramana CV, Gil MP, Schreiber RD, Stark GR. Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. Trends Immunol. 2002 Feb;23(2):96-101.

166. Matsumoto M, Tanaka N, Harada H, Kimura T, Yokochi T, Kitagawa M, et al. Activation of the transcription factor ISGF3 by interferon-gamma. Biol Chem. 1999 Jun;380(6):699-703.

167. Qing Y, Stark GR. Alternative activation of STAT1 and STAT3 in response to interferon-gamma. J Biol Chem. 2004 Oct 1;279(40):41679-85.

168. Meinke A, Barahmand-Pour F, Wohrl S, Stoiber D, Decker T. Activation of different Stat5 isoforms contributes to cell-type-restricted signaling in response to interferons. Mol Cell Biol. 1996 Dec;16(12):6937-44.

169. Gough DJ, Levy DE, Johnstone RW, Clarke CJ. IFNgamma signaling-does it mean JAK-STAT? Cytokine Growth Factor Rev. 2008 Oct-Dec;19(5-6):383-94.

170. Hu J, Roy SK, Shapiro PS, Rodig SR, Reddy SP, Platanias LC, et al. ERK1 and ERK2 activate CCAAAT/enhancer-binding protein-beta-dependent gene transcription in response to interferon-gamma. J Biol Chem. 2001 Jan 5;276(1):287-97.

171. Gough DJ, Sabapathy K, Ko EY, Arthur HA, Schreiber RD, Trapani JA, et al. A novel c-jun-dependent signal transduction pathway necessary for the transcriptional activation of interferon gamma response genes. J Biol Chem. 2007 Jan 12;282(2):938-46.

172. Xia K, Mukhopadhyay NK, Inhorn RC, Barber DL, Rose PE, Lee RS, et al. The cytokine-activated tyrosine kinase JAK2 activates raf-1 in a p21ras-dependent manner. Proc Natl Acad Sci U S A. 1996 Oct 15;93(21):11681-6.

173. Roy SK, Hu J, Meng Q, Xia Y, Shapiro PS, Reddy SP, et al. MEKK1 plays a critical role in activating the transcription factor C/EBP-beta-dependent gene expression in response to IFN-gamma. Proc Natl Acad Sci U S A. 2002 Jun 11;99(12):7945-50.

174. Sun D, Ding A. MyD88-mediated stabilization of interferon-gamma-induced cytokine and chemokine mRNA. Nat Immunol. 2006 Apr;7(4):375-81.

175. Halfter UM, Derbyshire ZE, Vaillancourt RR. Interferon-gamma-dependent tyrosine phosphorylation of MEKK4 via Pyk2 is regulated by annexin II and SHP2 in keratinocytes. Biochem J. 2005 May 15;388(Pt 1):17-28.

176. Valledor AF, Sanchez-Tillo E, Arpa L, Park JM, Caelles C, Lloberas J, et al. Selective roles of MAPKs during the macrophage response to IFN-gamma. J Immunol. 2008 Apr 1;180(7):4523-9.

177. Schaeffer HJ, Weber MJ. Mitogen-activated protein kinases: Specific messages from ubiquitous messengers. Mol Cell Biol. 1999 Apr;19(4):2435-44.

178. Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol. 1997 Apr;9(2):180-6.

179. Flaherty PT, Chopra I, Jain P, Yi S, Allen E, Cavanaugh J. Identification of benzimidazole-based inhibitors of the mitogen activated kinase-5 signaling pathway. Bioorg Med Chem Lett. 2010 May 1;20(9):2892-6.

180. Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. Adv Cancer Res. 1998;74:49-139.

181. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev. 2001 Apr;81(2):807-69.

182. Whitmarsh AJ, Davis RJ. Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. Trends Biochem Sci. 1998 Dec;23(12):481-5.

183. Canagarajah BJ, Khokhlatchev A, Cobb MH, Goldsmith EJ. Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. Cell. 1997 Sep 5;90(5):859-69.

184. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, et al. Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. Endocr Rev. 2001 Apr;22(2):153-83.

185. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. Physiol Rev. 1999 Jan;79(1):143-80.

186. Chow CW, Rincon M, Cavanagh J, Dickens M, Davis RJ. Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway. Science. 1997 Nov 28;278(5343):1638-41.

187. Rossomando AJ, Payne DM, Weber MJ, Sturgill TW. Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. Proc Natl Acad Sci U S A. 1989 Sep;86(18):6940-3.

188. Dhillon AS, Hagan S, Rath O, Kolch W. MAP kinase signalling pathways in cancer. Oncogene. 2007 May 14;26(22):3279-90.

189. Niault TS, Baccarini M. Targets of raf in tumorigenesis. Carcinogenesis. 2010 Jul;31(7):1165-74.

190. Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, et al. Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature. 1993 Jul 15;364(6434):249-52.

191. Chadee DN, Kyriakis JM. MLK3 is required for mitogen activation of B-raf, ERK and cell proliferation. Nat Cell Biol. 2004 Aug;6(8):770-6.

192. Seger R, Krebs EG. The MAPK signaling cascade. FASEB J. 1995 Jun;9(9):726-35.

193. Angel P, Karin M. The role of jun, fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta. 1991 Dec 10;1072(2-3):129-57.

194. Whitmarsh AJ, Shore P, Sharrocks AD, Davis RJ. Integration of MAP kinase signal transduction pathways at the serum response element. Science. 1995 Jul 21;269(5222):403-7.

195. Monje P, Marinissen MJ, Gutkind JS. Phosphorylation of the carboxyl-terminal transactivation domain of c-fos by extracellular signal-regulated kinase mediates the transcriptional activation of AP-1 and cellular transformation induced by platelet-derived growth factor. Mol Cell Biol. 2003 Oct;23(19):7030-43.

196. Murphy LO, Smith S, Chen RH, Fingar DC, Blenis J. Molecular interpretation of ERK signal duration by immediate early gene products. Nat Cell Biol. 2002 Aug;4(8):556-64.

197. Bogoyevitch MA, Ngoei KR, Zhao TT, Yeap YY, Ng DC. C-jun N-terminal kinase (JNK) signaling: Recent advances and challenges. Biochim Biophys Acta. 2010 Mar;1804(3):463-75.

198. Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Cell Biol. 2007 Apr;19(2):142-9.

199. Coso OA, Chiariello M, Kalinec G, Kyriakis JM, Woodgett J, Gutkind JS. Transforming G protein-coupled receptors potently activate JNK (SAPK). evidence for a divergence from the tyrosine kinase signaling pathway. J Biol Chem. 1995 Mar 10;270(10):5620-4.

200. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell. 2000 Oct 13;103(2):239-52.

201. Lisnock J, Griffin P, Calaycay J, Frantz B, Parsons J, O'Keefe SJ, et al. Activation of JNK3 alpha 1 requires both MKK4 and MKK7: Kinetic characterization of in vitro phosphorylated JNK3 alpha 1. Biochemistry. 2000 Mar 21;39(11):3141-8.

202. Turjanski AG, Vaque JP, Gutkind JS. MAP kinases and the control of nuclear events. Oncogene. 2007 May 14;26(22):3240-53.

203. Martins I, Deshayes F, Baton F, Forget A, Ciechomska I, Sylla K, et al. Pathologic expression of MHC class II is driven by mitogen-activated protein kinases. Eur J Immunol. 2007 Mar;37(3):788-97.

204. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev. 2001 Apr;81(2):807-69.

205. Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, et al. MAP kinases. Chem Rev. 2001 Aug;101(8):2449-76.

206. Waetzig GH, Seegert D, Rosenstiel P, Nikolaus S, Schreiber S. p38 mitogen-activated protein kinase is activated and linked to TNF-alpha signaling in inflammatory bowel disease. J Immunol. 2002 May 15;168(10):5342-51.

207. Cuschieri J, Maier RV. Mitogen-activated protein kinase (MAPK). Crit Care Med. 2005 Dec;33(12 Suppl):S417-9.

208. Rubinfeld H, Seger R. The ERK cascade: A prototype of MAPK signaling. Mol Biotechnol. 2005 Oct;31(2):151-74.

209. Whitmarsh AJ, Davis RJ. Role of mitogen-activated protein kinase kinase 4 in cancer. Oncogene. 2007 May 14;26(22):3172-84.

210. Ma W, Lim W, Gee K, Aucoin S, Nandan D, Kozlowski M, et al. The p38 mitogen-activated kinase pathway regulates the human interleukin-10 promoter via the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages. J Biol Chem. 2001 Apr 27;276(17):13664-74.

211. Yang SH, Sharrocks AD, Whitmarsh AJ. Transcriptional regulation by the MAP kinase signaling cascades. Gene. 2003 Nov 27;320:3-21.

212. Casals C, Barrachina M, Serra M, Lloberas J, Celada A. Lipopolysaccharide up-regulates MHC class II expression on dendritic cells through an AP-1 enhancer without affecting the levels of CIITA. J Immunol. 2007 May 15;178(10):6307-15.

213. Adamski J, Benveniste EN. 17beta-estradiol activation of the c-jun N-terminal kinase pathway leads to down-regulation of class II major histocompatibility complex expression. Mol Endocrinol. 2005 Jan;19(1):113-24.

214. Voong LN, Slater AR, Kratovac S, Cressman DE. Mitogen-activated protein kinase ERK1/2 regulates the class II transactivator. J Biol Chem. 2008 Apr 4;283(14):9031-9.

215. Lapidus RG, Ferguson AT, Ottaviano YL, Parl FF, Smith HS, Weitzman SA, et al. Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. Clin Cancer Res. 1996 May;2(5):805-10.

216. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet. 2002 Jun;3(6):415-28.

217. Keen JC, Davidson NE. The biology of breast carcinoma. Cancer. 2003 Feb 1;97(3 Suppl):825-33.

218. Brinkman JA, El-Ashry D. ER re-expression and re-sensitization to endocrine therapies in ER-negative breast cancers. J Mammary Gland Biol Neoplasia. 2009 Mar;14(1):67-78.

219. Thomas G. MAP kinase by any other name smells just as sweet. Cell. 1992 Jan 10;68(1):3-6.

220. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: Receptor heterodimerization in development and cancer. EMBO J. 2000 Jul 3;19(13):3159-67.

221. DiGiovanna MP, Chu P, Davison TL, Howe CL, Carter D, Claus EB, et al. Active signaling by HER-2/neu in a subpopulation of HER-2/neu-overexpressing ductal carcinoma in situ: Clinicopathological correlates. Cancer Res. 2002 Nov 15;62(22):6667-73.

222. Sharma AK, Horgan K, Douglas-Jones A, McClelland R, Gee J, Nicholson R. Dual immunocytochemical analysis of oestrogen and epidermal growth factor receptors in human breast cancer. Br J Cancer. 1994 Jun;69(6):1032-7.

223. Sharma AK, Horgan K, McClelland RA, Douglas-Jones AG, Van Agthoven T, Dorssers LC, et al. A dual immunocytochemical assay for oestrogen and epidermal growth factor receptors in tumour cell lines. Histochem J. 1994 Apr;26(4):306-10.

224. van Agthoven T, Timmermans M, Foekens JA, Dorssers LC, Henzen-Logmans SC. Differential expression of estrogen, progesterone, and epidermal growth factor receptors in

normal, benign, and malignant human breast tissues using dual staining immunohistochemistry. Am J Pathol. 1994 Jun;144(6):1238-46.

225. Bayliss J, Hilger A, Vishnu P, Diehl K, El-Ashry D. Reversal of the estrogen receptor negative phenotype in breast cancer and restoration of antiestrogen response. Clin Cancer Res. 2007 Dec 1;13(23):7029-36.

226. Oh AS, Lorant LA, Holloway JN, Miller DL, Kern FG, El-Ashry D. Hyperactivation of MAPK induces loss of ERalpha expression in breast cancer cells. Mol Endocrinol. 2001 Aug;15(8):1344-59.

227. Creighton CJ, Hilger AM, Murthy S, Rae JM, Chinnaiyan AM, El-Ashry D. Activation of mitogen-activated protein kinase in estrogen receptor alpha-positive breast cancer cells in vitro induces an in vivo molecular phenotype of estrogen receptor alpha-negative human breast tumors. Cancer Res. 2006 Apr 1;66(7):3903-11.

228. Rexhepaj E, Brennan DJ, Holloway P, Kay EW, McCann AH, Landberg G, et al. Novel image analysis approach for quantifying expression of nuclear proteins assessed by immunohistochemistry: Application to measurement of oestrogen and progesterone receptor levels in breast cancer. Breast Cancer Res. 2008;10(5):R89.

229. Bai Z, Gust R. Breast cancer, estrogen receptor and ligands. Arch Pharm (Weinheim). 2009 Mar;342(3):133-49.

230. Collaborative Group on Hormonal Factors in Breast Cancer. Menarche, menopause, and breast cancer risk: Individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies. Lancet Oncol. 2012 Nov;13(11):1141-51.

231. Jonat W, Pritchard KI, Sainsbury R, Klijn JG. Trends in endocrine therapy and chemotherapy for early breast cancer: A focus on the premenopausal patient. J Cancer Res Clin Oncol. 2006 May;132(5):275-86.

232. Yamaguchi Y. Microenvironmental regulation of estrogen signals in breast cancer. Breast Cancer. 2007;14(2):175-81.

233. Albanito L, Sisci D, Aquila S, Brunelli E, Vivacqua A, Madeo A, et al. Epidermal growth factor induces G protein-coupled receptor 30 expression in estrogen receptor-negative breast cancer cells. Endocrinology. 2008 Aug;149(8):3799-808.

234. Edwards DP. Regulation of signal transduction pathways by estrogen and progesterone. Annu Rev Physiol. 2005;67:335-76.

235. Ellmann S, Sticht H, Thiel F, Beckmann MW, Strick R, Strissel PL. Estrogen and progesterone receptors: From molecular structures to clinical targets. Cell Mol Life Sci. 2009 Aug;66(15):2405-26.

236. Gosden JR, Middleton PG, Rout D. Localization of the human oestrogen receptor gene to chromosome 6q24----q27 by in situ hybridization. Cytogenet Cell Genet. 1986;43(3-4):218-20.

237. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, et al. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab. 1997 Dec;82(12):4258-65.

238. Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, et al. Estrogen receptors: How do they signal and what are their targets. Physiol Rev. 2007 Jul;87(3):905-31.

239. Tora L, White J, Brou C, Tasset D, Webster N, Scheer E, et al. The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell. 1989 Nov 3;59(3):477-87.

240. Picard D, Kumar V, Chambon P, Yamamoto KR. Signal transduction by steroid hormones: Nuclear localization is differentially regulated in estrogen and glucocorticoid receptors. Cell Regul. 1990 Feb;1(3):291-9.

241. Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, et al. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. EMBO J. 1999 Sep 1;18(17):4608-18.

242. Denger S, Reid G, Kos M, Flouriot G, Parsch D, Brand H, et al. ERalpha gene expression in human primary osteoblasts: Evidence for the expression of two receptor proteins. Mol Endocrinol. 2001 Dec;15(12):2064-77.

243. Flouriot G, Brand H, Denger S, Metivier R, Kos M, Reid G, et al. Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. EMBO J. 2000 Sep 1;19(17):4688-700.

244. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF. Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. Biochem Biophys Res Commun. 2005 Nov 4;336(4):1023-7.

245. Penot G, Le Peron C, Merot Y, Grimaud-Fanouillere E, Ferriere F, Boujrad N, et al. The human estrogen receptor-alpha isoform hERalpha46 antagonizes the proliferative influence of hERalpha66 in MCF7 breast cancer cells. Endocrinology. 2005 Dec;146(12):5474-84.

246. Lewandowski S, Kalita K, Kaczmarek L. Estrogen receptor beta. potential functional significance of a variety of mRNA isoforms. FEBS Lett. 2002 Jul 31;524(1-3):1-5.

247. Moore JT, McKee DD, Slentz-Kesler K, Moore LB, Jones SA, Horne EL, et al. Cloning and characterization of human estrogen receptor beta isoforms. Biochem Biophys Res Commun. 1998 Jun 9;247(1):75-8.

248. Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y, et al. Molecular cloning and characterization of human estrogen receptor betacx: A potential inhibitor ofestrogen action in human. Nucleic Acids Res. 1998 Aug 1;26(15):3505-12.

249. Zhao C, Matthews J, Tujague M, Wan J, Strom A, Toresson G, et al. Estrogen receptor beta2 negatively regulates the transactivation of estrogen receptor alpha in human breast cancer cells. Cancer Res. 2007 Apr 15;67(8):3955-62.

250. Owman C, Blay P, Nilsson C, Lolait SJ. Cloning of human cDNA encoding a novel heptahelix receptor expressed in burkitt's lymphoma and widely distributed in brain and peripheral tissues. Biochem Biophys Res Commun. 1996 Nov 12;228(2):285-92.

251. O'Dowd BF, Nguyen T, Marchese A, Cheng R, Lynch KR, Heng HH, et al. Discovery of three novel G-protein-coupled receptor genes. Genomics. 1998 Jan 15;47(2):310-3.

252. Feng Y, Gregor P. Cloning of a novel member of the G protein-coupled receptor family related to peptide receptors. Biochem Biophys Res Commun. 1997 Feb 24;231(3):651-4.

253. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology. 2005 Feb;146(2):624-32.

254. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science. 2005 Mar 11;307(5715):1625-30.

255. Thomas C, Gustafsson JA. The different roles of ER subtypes in cancer biology and therapy. Nat Rev Cancer. 2011 Jul 22;11(8):597-608.

256. Filardo EJ, Quinn JA, Bland KI, Frackelton AR,Jr. Estrogen-induced activation of erk-1 and erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol. 2000 Oct;14(10):1649-60.

257. Kanda N, Watanabe S. 17beta-estradiol inhibits oxidative stress-induced apoptosis in keratinocytes by promoting bcl-2 expression. J Invest Dermatol. 2003 Dec;121(6):1500-9.

258. Razandi M, Pedram A, Park ST, Levin ER. Proximal events in signaling by plasma membrane estrogen receptors. J Biol Chem. 2003 Jan 24;278(4):2701-12.

259. Klein-Hitpass L, Ryffel GU, Heitlinger E, Cato AC. A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor. Nucleic Acids Res. 1988 Jan 25;16(2):647-63.

260. Kumar V, Chambon P. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell. 1988 Oct 7;55(1):145-56.

261. Chen D, Pace PE, Coombes RC, Ali S. Phosphorylation of human estrogen receptor alpha by protein kinase A regulates dimerization. Mol Cell Biol. 1999 Feb;19(2):1002-15.

262. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell. 1998 Dec 23;95(7):927-37.

263. Tzukerman M, Zhang XK, Hermann T, Wills KN, Graupner G, Pfahl M. The human estrogen receptor has transcriptional activator and repressor functions in the absence of ligand. New Biol. 1990 Jul;2(7):613-20.

264. Hayashi S, Yamaguchi Y. Estrogen signaling pathway and hormonal therapy. Breast Cancer. 2008;15(4):256-61.

265. Hall JM, Couse JF, Korach KS. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem. 2001 Oct 5;276(40):36869-72.

266. Skandalis SS, Afratis N, Smirlaki G, Nikitovic D, Theocharis AD, Tzanakakis GN, et al. Cross-talk between estradiol receptor and EGFR/IGF-IR signaling pathways in estrogen-responsive breast cancers: Focus on the role and impact of proteoglycans. Matrix Biol. 2013 Sep 21.

267. Marino M, Galluzzo P, Ascenzi P. Estrogen signaling multiple pathways to impact gene transcription. Curr Genomics. 2006;7(8):497-508.

268. Faupel-Badger JM, Duggan MA, Sherman ME, Garcia-Closas M, Yang XR, Lissowska J, et al. Prolactin receptor expression and breast cancer: Relationships with tumor characteristics among pre- and post-menopausal women in a population-based case-control study from poland. Horm Cancer. 2013 Nov 19.

269. Schroeder MD, Symowicz J, Schuler LA. PRL modulates cell cycle regulators in mammary tumor epithelial cells. Mol Endocrinol. 2002 Jan;16(1):45-57.

270. Gonzalez L, Zambrano A, Lazaro-Trueba I, Lopez E, Gonzalez JJ, Martin-Perez J, et al. Activation of the unliganded estrogen receptor by prolactin in breast cancer cells. Oncogene. 2009 Mar 12;28(10):1298-308.

271. Miller KK, Al-Rayyan N, Ivanova MM, Mattingly KA, Ripp SL, Klinge CM, et al. DHEA metabolites activate estrogen receptors alpha and beta. Steroids. 2013 Jan;78(1):15-25.

272. Ding L, Yan J, Zhu J, Zhong H, Lu Q, Wang Z, et al. Ligand-independent activation of estrogen receptor alpha by XBP-1. Nucleic Acids Res. 2003 Sep 15;31(18):5266-74.

273. Rowan BG, Weigel NL, O'Malley BW. Phosphorylation of steroid receptor coactivator-1. identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. J Biol Chem. 2000 Feb 11;275(6):4475-83.

274. Font de Mora J, Brown M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. Mol Cell Biol. 2000 Jul;20(14):5041-7.

275. Yamashita H, Nishio M, Kobayashi S, Ando Y, Sugiura H, Zhang Z, et al. Phosphorylation of estrogen receptor alpha serine 167 is predictive of response to endocrine therapy and increases postrelapse survival in metastatic breast cancer. Breast Cancer Res. 2005;7(5):R753-64.

276. Norman AW, Mizwicki MT, Norman DP. Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. Nat Rev Drug Discov. 2004 Jan;3(1):27-41.

277. Pietras RJ, Marquez-Garban DC. Membrane-associated estrogen receptor signaling pathways in human cancers. Clin Cancer Res. 2007 Aug 15;13(16):4672-6.

278. Song RX, Barnes CJ, Zhang Z, Bao Y, Kumar R, Santen RJ. The role of shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. Proc Natl Acad Sci U S A. 2004 Feb 17;101(7):2076-81.

279. Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER. ERs associate with and regulate the production of caveolin: Implications for signaling and cellular actions. Mol Endocrinol. 2002 Jan;16(1):100-15.

280. Maggiolini M, Picard D. The unfolding stories of GPR30, a new membrane-bound estrogen receptor. J Endocrinol. 2010 Feb;204(2):105-14.

281. Saito S, Foegh ML, Motomura N, Lou H, Kent K, Ramwell PW. Estradiol inhibits allograft-inducible major histocompatibility complex class II antigen expression and transplant arteriosclerosis in the absence of immunosuppression. Transplantation. 1998 Dec 15;66(11):1424-31.

282. Lou H, Kodama T, Zhao YJ, Maurice P, Wang YN, Katz N, et al. Inhibition of transplant coronary arteriosclerosis in rabbits by chronic estradiol treatment is associated with abolition of MHC class II antigen expression. Circulation. 1996 Dec 15;94(12):3355-61.

283. Saito S, Motomura N, Lou H, Ramwell PW, Foegh ML. Specific effects of estrogen on growth factor and major histocompatibility complex class II antigen expression in rat aortic allograft. J Thorac Cardiovasc Surg. 1997 Nov;114(5):803,9; discussion 809-10.

284. Cenci S, Toraldo G, Weitzmann MN, Roggia C, Gao Y, Qian WP, et al. Estrogen deficiency induces bone loss by increasing T cell proliferation and lifespan through IFN-gamma-induced class II transactivator. Proc Natl Acad Sci U S A. 2003 Sep 2;100(18):10405-10.

285. Adamski J, Benveniste EN. 17beta-estradiol activation of the c-jun N-terminal kinase pathway leads to down-regulation of class II major histocompatibility complex expression. Mol Endocrinol. 2005 Jan;19(1):113-24.

286. Jiang SY, Jordan VC. Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst. 1992 Apr 15;84(8):580-91.

287. Levenson AS, Jordan VC. Transfection of human estrogen receptor (ER) cDNA into ER-negative mammalian cell lines. J Steroid Biochem Mol Biol. 1994 Dec;51(5-6):229-39.

288. Soule HD, Vazguez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst. 1973 Nov;51(5):1409-16.

289. Keydar I, Chen L, Karby S, Weiss FR, Delarea J, Radu M, et al. Establishment and characterization of a cell line of human breast carcinoma origin. Eur J Cancer. 1979 May;15(5):659-70.

290. Lasfargues EY, Coutinho WG, Redfield ES. Isolation of two human tumor epithelial cell lines from solid breast carcinomas. J Natl Cancer Inst. 1978 Oct;61(4):967-78.

291. Cailleau R, Olive M, Cruciger QV. Long-term human breast carcinoma cell lines of metastatic origin: Preliminary characterization. In Vitro. 1978 Nov;14(11):911-5.

292. Trempe GL. Human breast cancer in culture. Recent Results Cancer Res. 1976;(57)(57):33-41.

293. Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. Breast Cancer Res. 2011 Aug 12;13(4):215.

294. Burdall SE, Hanby AM, Lansdown MR, Speirs V. Breast cancer cell lines: Friend or foe? Breast Cancer Res. 2003;5(2):89-95.

295. Lampson LA, Levy R. Two populations of ia-like molecules on a human B cell line. J Immunol. 1980 Jul;125(1):293-9.

296. Camacho-Carvajal MM, Klingler S, Schnappauf F, Hake SB, Steimle V. Importance of class II transactivator leucine-rich repeats for dominant-negative function and nucleo-cytoplasmic transport. Int Immunol. 2004 Jan;16(1):65-75.

297. Nandan D, Reiner NE. TGF-beta attenuates the class II transactivator and reveals an accessory pathway of IFN-gamma action. J Immunol. 1997 Feb 1;158(3):1095-101.

298. Chang CH, Flavell RA. Class II transactivator regulates the expression of multiple genes involved in antigen presentation. J Exp Med. 1995 Feb 1;181(2):765-7.

299. Chin KC, Mao C, Skinner C, Riley JL, Wright KL, Moreno CS, et al. Molecular analysis of G1B and G3A IFN gamma mutants reveals that defects in CIITA or RFX result in defective class II MHC and ii gene induction. Immunity. 1994 Nov;1(8):687-97.

300. Jazaeri O, Shupnik MA, Jazaeri AA, Rice LW. Expression of estrogen receptor alpha mRNA and protein variants in human endometrial carcinoma. Gynecol Oncol. 1999 Jul;74(1):38-47.

301. Gottwald E, Muller O, Polten A. Semiquantitative reverse transcription-polymerase chain reaction with the agilent 2100 bioanalyzer. Electrophoresis. 2001 Oct;22(18):4016-22.

302. Newman RA, Ormerod MG, Greaves MF. The presence of HLA-DR antigens on lactating human breast epithelium and milk fat globule membranes. Clin Exp Immunol. 1980 Sep;41(3):478-86.

303. Sedlak J, Speiser P, Zeillinger R, Krugluger W, Wiltschke C, Kubista E, et al. Cytokine (IFN-alpha, IFN-gamma, IL-1-alpha, TNF-alpha)-induced modulation of HLA cell surface expression in human breast cancer cell lines. Neoplasma. 1992;39(5):269-72.

304. Tzortzakaki E, Spilianakis C, Zika E, Kretsovali A, Papamatheakis J. Steroid receptor coactivator 1 links the steroid and interferon gamma response pathways. Mol Endocrinol. 2003 Dec;17(12):2509-18.

305. Donaghue C, Westley BR, May FE. Selective promoter usage of the human estrogen receptor-alpha gene and its regulation by estrogen. Mol Endocrinol. 1999 Nov;13(11):1934-50.

306. Santen RJ, Song RX, Zhang Z, Kumar R, Jeng MH, Masamura A, et al. Long-term estradiol deprivation in breast cancer cells up-regulates growth factor signaling and enhances estrogen sensitivity. Endocr Relat Cancer. 2005 Jul;12 Suppl 1:S61-73.

307. Abraham GE, Odell WD, Swerdloff RS, Hopper K. Simultaneous radioimmunoassay of plasma FSH, LH, progesterone, 17-hydroxyprogesterone, and estradiol-17 beta during the menstrual cycle. J Clin Endocrinol Metab. 1972 Feb;34(2):312-8.

308. Ranella A, Vassiliadis S, Mastora C, Valentina M, Dionyssopoulou E, Athanassakis I. Constitutive intracellular expression of human leukocyte antigen (HLA)-DO and HLA-DR but not HLA-DM in trophoblast cells. Hum Immunol. 2005 Jan;66(1):43-55.

309. Morris P, Shaman J, Attaya M, Amaya M, Goodman S, Bergman C, et al. An essential role for HLA-DM in antigen presentation by class II major histocompatibility molecules. Nature. 1994 Apr 7;368(6471):551-4.

310. Tora L, Mullick A, Metzger D, Ponglikitmongkol M, Park I, Chambon P. The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. EMBO J. 1989 Jul;8(7):1981-6.

311. Sommer A, Hoffmann J, Lichtner RB, Schneider MR, Parczyk K. Studies on the development of resistance to the pure antiestrogen faslodex in three human breast cancer cell lines. J Steroid Biochem Mol Biol. 2003 May;85(1):33-47.

312. Aka JA, Lin SX. Comparison of functional proteomic analyses of human breast cancer cell lines T47D and MCF7. PLoS One. 2012;7(2):e31532.

313. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology. 1997 Mar;138(3):863-70.

314. Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lakins J, Lupu R. Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. Oncol Rep. 2000 Jan-Feb;7(1):157-67.

315. Adamski J, Ma Z, Nozell S, Benveniste EN. 17beta-estradiol inhibits class II major histocompatibility complex (MHC) expression: Influence on histone modifications and cbp recruitment to the class II MHC promoter. Mol Endocrinol. 2004 Aug;18(8):1963-74.

316. Segars JH, Driggers PH. Estrogen action and cytoplasmic signaling cascades. part I: Membrane-associated signaling complexes. Trends Endocrinol Metab. 2002 Oct;13(8):349-54.

317. Ma C, Zuo W, Wang X, Wei L, Guo Q, Song X. Lapatinib inhibits the activation of NF-kappaB through reducing phosphorylation of IkappaB-alpha in breast cancer cells. Oncol Rep. 2013 Feb;29(2):812-8.

318. Ruan SQ, Wang ZH, Wang SW, Fu ZX, Xu KL, Li DB, et al. Heregulin-beta1-induced GPR30 upregulation promotes the migration and invasion potential of SkBr3 breast cancer cells via ErbB2/ErbB3-MAPK/ERK pathway. Biochem Biophys Res Commun. 2012 Apr 6;420(2):385-90.

319. Martins I, Sylla K, Deshayes F, Lauriol J, Ghislin S, Dieu-Nosjean MC, et al. Coexpression of major histocompatibility complex class II with chemokines and nuclear NFkappaB p50 in melanoma: A rational for their association with poor prognosis. Melanoma Res. 2009 Aug;19(4):226-37.

320. Notas G, Kampa M, Pelekanou V, Castanas E. Interplay of estrogen receptors and GPR30 for the regulation of early membrane initiated transcriptional effects: A pharmacological approach. Steroids. 2012 Aug;77(10):943-50.

321. Zhang L, Wu YD, Li P, Tu J, Niu YL, Xu CM, et al. Effects of cyclooxygenase-2 on human esophageal squamous cell carcinoma. World J Gastroenterol. 2011 Nov 7;17(41):4572-80.

322. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A. 2011 Mar 29;108(13):5354-9.

323. Brocke P, Garbi N, Momburg F, Hammerling GJ. HLA-DM, HLA-DO and tapasin: Functional similarities and differences. Curr Opin Immunol. 2002 Feb;14(1):22-9.

324. Reich M, Zou F, Sienczyk M, Oleksyszyn J, Boehm BO, Burster T. Invariant chain processing is independent of cathepsin variation between primary human B cells/dendritic cells and B-lymphoblastoid cells. Cell Immunol. 2011;269(2):96-103.

325. Catalfamo M, Serradell L, Roura-Mir C, Kolkowski E, Sospedra M, Vives-Pi M, et al. HLA-DM and invariant chain are expressed by thyroid follicular cells, enabling the expression of compact DR molecules. Int Immunol. 1999 Feb;11(2):269-77.

326. Muntasell A, Carrascal M, Alvarez I, Serradell L, van Veelen P, Verreck FA, et al. Dissection of the HLA-DR4 peptide repertoire in endocrine epithelial cells: Strong influence of invariant chain and HLA-DM expression on the nature of ligands. J Immunol. 2004 Jul 15;173(2):1085-93.

327. Spurrell DR, Oldford SA, Frost T, Larsen B, Codner D, Edgecombe A, et al. Discordant expression of HLA class II-associated co-chaperones and HLA-DRB alleles in cultured fibroblast-like synoviocytes. Hum Immunol. 2004 Dec;65(12):1516-29.

328. Long X, Nephew KP. Fulvestrant (ICI 182,780)-dependent interacting proteins mediate immobilization and degradation of estrogen receptor-alpha. J Biol Chem. 2006 Apr 7;281(14):9607-15.

329. Robertson JF. ICI 182,780 (fulvestrant)--the first oestrogen receptor down-regulator--current clinical data. Br J Cancer. 2001 Nov;85 Suppl 2:11-4.

330. Lam L, Hu X, Aktary Z, Andrews DW, Pasdar M. Tamoxifen and ICI 182,780 increase bcl-2 levels and inhibit growth of breast carcinoma cells by modulating PI3K/AKT, ERK and IGF-1R pathways independent of ERalpha. Breast Cancer Res Treat. 2009 Dec;118(3):605-21.

331. Westerheide SD, Louis-Plence P, Ping D, He XF, Boss JM. HLA-DMA and HLA-DMB gene expression functions through the conserved S-X-Y region. J Immunol. 1997 May 15;158(10):4812-21.

332. Morris AC, Beresford GW, Mooney MR, Boss JM. Kinetics of a gamma interferon response: Expression and assembly of CIITA promoter IV and inhibition by methylation. Mol Cell Biol. 2002 Jul;22(13):4781-91.

333. Li Y, Birnbaumer L, Teng CT. Regulation of ERRalpha gene expression by estrogen receptor agonists and antagonists in SKBR3 breast cancer cells: Differential molecular mechanisms mediated by g protein-coupled receptor GPR30/GPER-1. Mol Endocrinol. 2010 May;24(5):969-80.

334. Rohn WM, Lee YJ, Benveniste EN. Regulation of class II MHC expression. Crit Rev Immunol. 1996;16(3):311-30.

335. Johnson MD, Westley BR, May FE. Oestrogenic activity of tamoxifen and its metabolites on gene regulation and cell proliferation in MCF-7 breast cancer cells. Br J Cancer. 1989 May;59(5):727-38.

336. Kisanga ER, Gjerde J, Guerrieri-Gonzaga A, Pigatto F, Pesci-Feltri A, Robertson C, et al. Tamoxifen and metabolite concentrations in serum and breast cancer tissue during three dose regimens in a randomized preoperative trial. Clin Cancer Res. 2004 Apr 1;10(7):2336-43.

337. Bulun SE, Price TM, Aitken J, Mahendroo MS, Simpson ER. A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcription. J Clin Endocrinol Metab. 1993 Dec;77(6):1622-8.

338. Shahrokh K, Cheatham TE,3rd, Yost GS. Conformational dynamics of CYP3A4 demonstrate the important role of Arg212 coupled with the opening of ingress, egress and solvent channels to dehydrogenation of 4-hydroxy-tamoxifen. Biochim Biophys Acta. 2012 Oct;1820(10):1605-17.

339. Robertson JF, Harrison M. Fulvestrant: Pharmacokinetics and pharmacology. Br J Cancer. 2004 Mar;90 Suppl 1:S7-10.

340. Giamarchi C, Chailleux C, Callige M, Rochaix P, Trouche D, Richard-Foy H. Two antiestrogens affect differently chromatin remodeling of trefoil factor 1 (pS2) gene and the fate of estrogen receptor in MCF7 cells. Biochim Biophys Acta. 2002 Oct 11;1578(1-3):12-20.

341. Nalbandian G, Paharkova-Vatchkova V, Mao A, Nale S, Kovats S. The selective estrogen receptor modulators, tamoxifen and raloxifene, impair dendritic cell differentiation and activation. J Immunol. 2005 Aug 15;175(4):2666-75.

342. Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E, et al. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J. 1996 Mar 15;15(6):1292-300.

343. Krawczyk M, Reith W. Regulation of MHC class II expression, a unique regulatory system identified by the study of a primary immunodeficiency disease. Tissue Antigens. 2006 Mar;67(3):183-97.

344. Shi B, Vinyals A, Alia P, Broceno C, Chen F, Adrover M, et al. Differential expression of MHC class II molecules in highly metastatic breast cancer cells is mediated by the regulation of the CIITA transcription implication of CIITA in tumor and metastasis development. Int J Biochem Cell Biol. 2006;38(4):544-62.

345. Piskurich JF, Linhoff MW, Wang Y, Ting JP. Two distinct gamma interferon-inducible promoters of the major histocompatibility complex class II transactivator gene are differentially regulated by STAT1, interferon regulatory factor 1, and transforming growth factor beta. Mol Cell Biol. 1999 Jan;19(1):431-40.

346. Ahmed CM, Johnson HM. IFN-gamma and its receptor subunit IFNGR1 are recruited to the IFN-gamma-activated sequence element at the promoter site of IFN-gamma-activated genes: Evidence of transactivational activity in IFNGR1. J Immunol. 2006 Jul 1;177(1):315-21.

347. Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, et al. Estrogen receptors: How do they signal and what are their targets. Physiol Rev. 2007 Jul;87(3):905-31.

348. Tsuno T, Mejido J, Zhao T, Schmeisser H, Morrow A, Zoon KC. IRF9 is a key factor for eliciting the antiproliferative activity of IFN-alpha. J Immunother. 2009 Oct;32(8):803-16.

349. O'Donnell PW, Haque A, Klemsz MJ, Kaplan MH, Blum JS. Cutting edge: Induction of the antigen-processing enzyme IFN-gamma-inducible lysosomal thiol reductase in melanoma cells is STAT1-dependent but CIITA-independent. J Immunol. 2004 Jul 15;173(2):731-5.

350. Radosevich M, Song Z, Gorga JC, Ksander B, Ono SJ. Epigenetic silencing of the CIITA gene and posttranscriptional regulation of class II MHC genes in ocular melanoma cells. Invest Ophthalmol Vis Sci. 2004 Sep;45(9):3185-95.

351. Holling TM, Schooten E, Langerak AW, van den Elsen PJ. Regulation of MHC class II expression in human T-cell malignancies. Blood. 2004 Feb 15;103(4):1438-44.

352. Radosevich M, Jager M, Ono SJ. Inhibition of MHC class II gene expression in uveal melanoma cells is due to methylation of the CIITA gene or an upstream activator. Exp Mol Pathol. 2007 Feb;82(1):68-76.

353. Shi B, Vinyals A, Alia P, Broceno C, Chen F, Adrover M, et al. Differential expression of MHC class II molecules in highly metastatic breast cancer cells is mediated by the regulation of the CIITA transcription implication of CIITA in tumor and metastasis development. Int J Biochem Cell Biol. 2006;38(4):544-62.

354. Morimoto Y, Toyota M, Satoh A, Murai M, Mita H, Suzuki H, et al. Inactivation of class II transactivator by DNA methylation and histone deacetylation associated with absence of HLA-DR induction by interferon-gamma in haematopoietic tumour cells. Br J Cancer. 2004 Feb 23;90(4):844-52.

355. Collingwood TN, Urnov FD, Wolffe AP. Nuclear receptors: Coactivators, corepressors and chromatin remodeling in the control of transcription. J Mol Endocrinol. 1999 Dec;23(3):255-75.

356. Barnes CJ, Vadlamudi RK, Kumar R. Novel estrogen receptor coregulators and signaling molecules in human diseases. Cell Mol Life Sci. 2004 Feb;61(3):281-91.

357. McDonnell DP, Norris JD. Connections and regulation of the human estrogen receptor. Science. 2002 May 31;296(5573):1642-4.

358. Tzortzakaki E, Spilianakis C, Zika E, Kretsovali A, Papamatheakis J. Steroid receptor coactivator 1 links the steroid and interferon gamma response pathways. Mol Endocrinol. 2003 Dec;17(12):2509-18.

359. Zhang JJ, Vinkemeier U, Gu W, Chakravarti D, Horvath CM, Darnell JE,Jr. Two contact regions between Stat1 and CBP/p300 in interferon gamma signaling. Proc Natl Acad Sci U S A. 1996 Dec 24;93(26):15092-6.

360. Zika E, Fauquier L, Vandel L, Ting JP. Interplay among coactivator-associated arginine methyltransferase 1, CBP, and CIITA in IFN-gamma-inducible MHC-II gene expression. Proc Natl Acad Sci U S A. 2005 Nov 8;102(45):16321-6.

361. Kovalchuk O, Tryndyak VP, Montgomery B, Boyko A, Kutanzi K, Zemp F, et al. Estrogen-induced rat breast carcinogenesis is characterized by alterations in DNA methylation, histone modifications and aberrant microRNA expression. Cell Cycle. 2007 Aug 15;6(16):2010-8.

362. Ginter T, Bier C, Knauer SK, Sughra K, Hildebrand D, Munz T, et al. Histone deacetylase inhibitors block IFNgamma-induced STAT1 phosphorylation. Cell Signal. 2012 Jul;24(7):1453-60.

363. Stender JD, Kim K, Charn TH, Komm B, Chang KC, Kraus WL, et al. Genome-wide analysis of estrogen receptor alpha DNA binding and tethering mechanisms identifies Runx1 as a novel tethering factor in receptor-mediated transcriptional activation. Mol Cell Biol. 2010 Aug;30(16):3943-55.

364. Connett JM, Hunt SR, Hickerson SM, Wu SJ, Doherty GM. Localization of IFN-gamma-activated Stat1 and IFN regulatory factors 1 and 2 in breast cancer cells. J Interferon Cytokine Res. 2003 Nov;23(11):621-30.

365. Bouker KB, Skaar TC, Riggins RB, Harburger DS, Fernandez DR, Zwart A, et al. Interferon regulatory factor-1 (IRF-1) exhibits tumor suppressor activities in breast cancer associated with caspase activation and induction of apoptosis. Carcinogenesis. 2005 Sep;26(9):1527-35.

366. Zhu Y, Singh B, Hewitt S, Liu A, Gomez B, Wang A, et al. Expression patterns among interferon regulatory factor-1, human X-box binding protein-1, nuclear factor kappa B, nucleophosmin, estrogen receptor-alpha and progesterone receptor proteins in breast cancer tissue microarrays. Int J Oncol. 2006 Jan;28(1):67-76.

367. Bouker KB, Skaar TC, Riggins RB, Harburger DS, Fernandez DR, Zwart A, et al. Interferon regulatory factor-1 (IRF-1) exhibits tumor suppressor activities in breast cancer associated with caspase activation and induction of apoptosis. Carcinogenesis. 2005 Sep;26(9):1527-35.

368. Ning Y, Riggins RB, Mulla JE, Chung H, Zwart A, Clarke R. IFNgamma restores breast cancer sensitivity to fulvestrant by regulating STAT1, IFN regulatory factor 1, NF-kappaB, BCL2 family members, and signaling to caspase-dependent apoptosis. Mol Cancer Ther. 2010 May;9(5):1274-85.

369. Bouker KB, Skaar TC, Fernandez DR, O'Brien KA, Riggins RB, Cao D, et al. Interferon regulatory factor-1 mediates the proapoptotic but not cell cycle arrest effects of the steroidal antiestrogen ICI 182,780 (faslodex, fulvestrant). Cancer Res. 2004 Jun 1;64(11):4030-9.

370. Klar D, Hammerling GJ. Induction of assembly of MHC class I heavy chains with beta 2microglobulin by interferon-gamma. EMBO J. 1989 Feb;8(2):475-81.

371. Drew PD, Franzoso G, Becker KG, Bours V, Carlson LM, Siebenlist U, et al. NF kappa B and interferon regulatory factor 1 physically interact and synergistically induce major histocompatibility class I gene expression. J Interferon Cytokine Res. 1995 Dec;15(12):1037-45.

372. Vallejo AN, Pease LR. The locus-specific enhancer activity of the class I major histocompatibility complex interferon-responsive element is associated with a gamma-interferon (IFN)-inducible factor distinct from STAT1alpha, p48, and IFN regulatory factor-1. J Biol Chem. 1996 Nov 22;271(47):29813-21.

373. Zhou F. Molecular mechanisms of IFN-gamma to up-regulate MHC class I antigen processing and presentation. Int Rev Immunol. 2009;28(3-4):239-60.

374. Yeh YT, Ou-Yang F, Chen IF, Yang SF, Su JH, Hou MF, et al. Altered p-JAK1 expression is associated with estrogen receptor status in breast infiltrating ductal carcinoma. Oncol Rep. 2007 Jan;17(1):35-9.

375. Gupta N, Grebhardt S, Mayer D. Janus kinase 2--a novel negative regulator of estrogen receptor alpha function. Cell Signal. 2012 Jan;24(1):151-61.

376. Leung KC, Doyle N, Ballesteros M, Sjogren K, Watts CK, Low TH, et al. Estrogen inhibits GH signaling by suppressing GH-induced JAK2 phosphorylation, an effect mediated by SOCS-2. Proc Natl Acad Sci U S A. 2003 Feb 4;100(3):1016-21.

377. Manthey D, Behl C. From structural biochemistry to expression profiling: Neuroprotective activities of estrogen. Neuroscience. 2006;138(3):845-50.

378. Coughlan N, Thillainadesan G, Andrews J, Isovic M, Torchia J. Beta-estradiol-dependent activation of the JAK/STAT pathway requires p/CIP and CARM1. Biochim Biophys Acta. 2013 Jun;1833(6):1463-75.

379. Dai R, Phillips RA, Karpuzoglu E, Khan D, Ahmed SA. Estrogen regulates transcription factors STAT-1 and NF-kappaB to promote inducible nitric oxide synthase and inflammatory responses. J Immunol. 2009 Dec 1;183(11):6998-7005.

380. Truax AD, Thakkar M, Greer SF. Dysregulated recruitment of the histone methyltransferase EZH2 to the class II transactivator (CIITA) promoter IV in breast cancer cells. PLoS One. 2012;7(4):e36013.

381. Kanda N, Watanabe S. 17beta-estradiol inhibits the production of interferon-induced protein of 10 kDa by human keratinocytes. J Invest Dermatol. 2003 Mar;120(3):411-9.

382. Holloway JN, Murthy S, El-Ashry D. A cytoplasmic substrate of mitogen-activated protein kinase is responsible for estrogen receptor-alpha down-regulation in breast cancer cells: The role of nuclear factor-kappaB. Mol Endocrinol. 2004 Jun;18(6):1396-410.

383. Vo AT, Millis RM. Epigenetics and breast cancers. Obstet Gynecol Int. 2012;2012:602720.

384. Yao Y, Xu Q, Kwon MJ, Matta R, Liu Y, Hong SC, et al. ERK and p38 MAPK signaling pathways negatively regulate CIITA gene expression in dendritic cells and macrophages. J Immunol. 2006 Jul 1;177(1):70-6.

385. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, et al. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem. 1998 Jul 17;273(29):18623-32.

386. Lampson LA, Levy R. Two populations of ia-like molecules on a human B cell line. J Immunol. 1980 Jul;125(1):293-9.

387. Landsverk OJ, Ottesen AH, Berg-Larsen A, Appel S, Bakke O. Differential regulation of MHC II and invariant chain expression during maturation of monocyte-derived dendritic cells. J Leukoc Biol. 2012 May;91(5):729-37.

388. Wood P, Mulay V, Darabi M, Chan KC, Heeren J, Pol A, et al. Ras/mitogen-activated protein kinase (MAPK) signaling modulates protein stability and cell surface expression of scavenger receptor SR-BI. J Biol Chem. 2011 Jul 1;286(26):23077-92.

389. Hedley ML, Urban RG, Strominger JL. Assembly and peptide binding of major histocompatibility complex class II heterodimers in an in vitro translation system. Proc Natl Acad Sci U S A. 1994 Oct 25;91(22):10479-83.

390. Robertson SE, Setty SR, Sitaram A, Marks MS, Lewis RE, Chou MM. Extracellular signal-regulated kinase regulates clathrin-independent endosomal trafficking. Mol Biol Cell. 2006 Feb;17(2):645-57.

391. Bocelli-Tyndall C, Zajac P, Di Maggio N, Trella E, Benvenuto F, Iezzi G, et al. Fibroblast growth factor 2 and platelet-derived growth factor, but not platelet lysate, induce proliferation-dependent, functional class II major histocompatibility complex antigen in human mesenchymal stem cells. Arthritis Rheum. 2010 Dec;62(12):3815-25.

392. Reith W, LeibundGut-Landmann S, Waldburger JM. Regulation of MHC class II gene expression by the class II transactivator. Nat Rev Immunol. 2005 Oct;5(10):793-806.

393. Walseng E, Bakke O, Roche PA. Major histocompatibility complex class II-peptide complexes internalize using a clathrin- and dynamin-independent endocytosis pathway. J Biol Chem. 2008 May 23;283(21):14717-27.

394. Inoue M, Mimura K, Izawa S, Shiraishi K, Inoue A, Shiba S, et al. Expression of MHC class I on breast cancer cells correlates inversely with HER2 expression. Oncoimmunology. 2012 Oct 1;1(7):1104-10.

395. Maruyama T, Mimura K, Sato E, Watanabe M, Mizukami Y, Kawaguchi Y, et al. Inverse correlation of HER2 with MHC class I expression on oesophageal squamous cell carcinoma. Br J Cancer. 2010 Aug 10;103(4):552-9.

396. Herrmann F, Lehr HA, Drexler I, Sutter G, Hengstler J, Wollscheid U, et al. HER-2/neu-mediated regulation of components of the MHC class I antigen-processing pathway. Cancer Res. 2004 Jan 1;64(1):215-20.

397. Luo W, Sharif TR, Houghton PJ, Sharif M. CGP 41251 and tamoxifen selectively inhibit mitogen-activated protein kinase activation and c-fos phosphoprotein induction by substance P in human astrocytoma cells. Cell Growth Differ. 1997 Nov;8(11):1225-40.

398. Park S, Kim B, Kim J, Won KJ, Lee S, Kwon S, et al. Tamoxifen induces vasorelaxation via inhibition of mitogen-activated protein kinase in rat aortic smooth muscle. J Vet Med Sci. 2003 Nov;65(11):1155-60.

399. Amos SM, Duong CP, Westwood JA, Ritchie DS, Junghans RP, Darcy PK, et al. Autoimmunity associated with immunotherapy of cancer. Blood. 2011 Jul 21;118(3):499-509.

400. Amos SM, Duong CP, Westwood JA, Ritchie DS, Junghans RP, Darcy PK, et al. Autoimmunity associated with immunotherapy of cancer. Blood. 2011 Jul 21;118(3):499-509.

401. Kochenderfer JN, Wilson WH, Janik JE, Dudley ME, Stetler-Stevenson M, Feldman SA, et al. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. Blood. 2010 Nov 18;116(20):4099-102.

402. Chamuleau ME, Ossenkoppele GJ, van de Loosdrecht AA. MHC class II molecules in tumour immunology: Prognostic marker and target for immune modulation. Immunobiology. 2006;211(6-8):619-25.

403. van der Stoep N, Biesta P, Quinten E, van den Elsen PJ. Lack of IFN-gamma-mediated induction of the class II transactivator (CIITA) through promoter methylation is predominantly found in developmental tumor cell lines. Int J Cancer. 2002 Feb 1;97(4):501-7.

404. De Lerma Barbaro A, De Ambrosis A, Banelli B, Li Pira G, Aresu O, Romani M, et al. Methylation of CIITA promoter IV causes loss of HLA-II inducibility by IFN-gamma in promyelocytic cells. Int Immunol. 2008 Nov;20(11):1457-66.

405. Naves R, Lennon AM, Barbieri G, Reyes L, Puga G, Salas L, et al. MHC class II-deficient tumor cell lines with a defective expression of the class II transactivator. Int Immunol. 2002 May;14(5):481-91.

406. Le Roy E, Muhlethaler-Mottet A, Davrinche C, Mach B, Davignon JL. Escape of human cytomegalovirus from HLA-DR-restricted CD4(+) T-cell response is mediated by repression of gamma interferon-induced class II transactivator expression. J Virol. 1999 Aug;73(8):6582-9.

407. Wojciechowski W, DeSanctis J, Skamene E, Radzioch D. Attenuation of MHC class II expression in macrophages infected with mycobacterium bovis bacillus calmette-guerin involves class II transactivator and depends on the Nramp1 gene. J Immunol. 1999 Sep 1;163(5):2688-96.

408. Wojciechowski W, DeSanctis J, Skamene E, Radzioch D. Attenuation of MHC class II expression in macrophages infected with mycobacterium bovis bacillus calmette-guerin involves class II transactivator and depends on the Nramp1 gene. J Immunol. 1999 Sep 1;163(5):2688-96.

409. Ostmeier H, Fuchs B, Otto F, Mawick R, Lippold A, Krieg V, et al. Prognostic immunohistochemical markers of primary human melanomas. Br J Dermatol. 2001 Aug;145(2):203-9.

410. Concha A, Ruiz-Cabello F, Cabrera T, Nogales F, Collado A, Garrido F. Different patterns of HLA-DR antigen expression in normal epithelium, hyperplastic and neoplastic malignant lesions of the breast. Eur J Immunogenet. 1995 Aug;22(4):299-310.

411. Wang HY, Peng G, Guo Z, Shevach EM, Wang RF. Recognition of a new ARTC1 peptide ligand uniquely expressed in tumor cells by antigen-specific CD4+ regulatory T cells. J Immunol. 2005 Mar 1;174(5):2661-70.

412. Jacobs JF, Nierkens S, Figdor CG, de Vries IJ, Adema GJ. Regulatory T cells in melanoma: The final hurdle towards effective immunotherapy? Lancet Oncol. 2012 Jan;13(1):e32-42.

413. Panelli MC, Wang E, Shen S, Schluter SF, Bernstein RM, Hersh EM, et al. Interferon gamma (IFNgamma) gene transfer of an EMT6 tumor that is poorly responsive to IFNgamma stimulation: Increase in tumor immunogenicity is accompanied by induction of a mouse class II transactivator and class II MHC. Cancer Immunol Immunother. 1996 Feb;42(2):99-107.

414. Armstrong TD, Clements VK, Martin BK, Ting JP, Ostrand-Rosenberg S. Major histocompatibility complex class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor-specific immunity. Proc Natl Acad Sci U S A. 1997 Jun 24;94(13):6886-91.

415. Clements VK, Baskar S, Armstrong TD, Ostrand-Rosenberg S. Invariant chain alters the malignant phenotype of MHC class II+ tumor cells. J Immunol. 1992 Oct 1;149(7):2391-6.

416. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Iwashige H, Aridome K, et al. Invariant chain expression in gastric cancer. Cancer Lett. 2001 Jul 10;168(1):87-91.

417. Xu M, Qiu G, Jiang Z, von Hofe E, Humphreys RE. Genetic modulation of tumor antigen presentation. Trends Biotechnol. 2000 Apr;18(4):167-72.

418. Ostrand-Rosenberg S. Tumor immunotherapy: The tumor cell as an antigen-presenting cell. Curr Opin Immunol. 1994 Oct;6(5):722-7.

419. Callahan MJ, Nagymanyoki Z, Bonome T, Johnson ME, Litkouhi B, Sullivan EH, et al. Increased HLA-DMB expression in the tumor epithelium is associated with increased CTL infiltration and improved prognosis in advanced-stage serous ovarian cancer. Clin Cancer Res. 2008 Dec 1;14(23):7667-73.

420. Chaturvedi P, Hengeveld R, Zechel MA, Lee-Chan E, Singh B. The functional role of class II-associated invariant chain peptide (CLIP) in its ability to variably modulate immune responses. Int Immunol. 2000 Jun;12(6):757-65.

421. van Luijn MM, van den Ancker W, Chamuleau ME, Zevenbergen A, Westers TM, Ossenkoppele GJ, et al. Absence of class II-associated invariant chain peptide on leukemic blasts of patients promotes activation of autologous leukemia-reactive CD4+ T cells. Cancer Res. 2011 Apr 1;71(7):2507-17.

422. Martin BK, Frelinger JG, Ting JP. Combination gene therapy with CD86 and the MHC class II transactivator in the control of lung tumor growth. J Immunol. 1999 Jun 1;162(11):6663-70.

423. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001 Sep 11;98(19):10869-74.

424. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001 Sep 11;98(19):10869-74.

425. Nahta R, Esteva FJ. HER-2-targeted therapy: Lessons learned and future directions. Clin Cancer Res. 2003 Nov 1;9(14):5078-84.

426. Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, et al. Basal-like and triple-negative breast cancers: A critical review with an emphasis on the implications for pathologists and oncologists. Mod Pathol. 2011 Feb;24(2):157-67.

427. Jones C, Payne J, Wells D, Delhanty JD, Lakhani SR, Kortenkamp A. Comparative genomic hybridization reveals extensive variation among different MCF-7 cell stocks. Cancer Genet Cytogenet. 2000 Mar;117(2):153-8.

428. Kim TK, Eberwine JH. Mammalian cell transfection: The present and the future. Anal Bioanal Chem. 2010 Aug;397(8):3173-8.

429. Kushner PJ, Hort E, Shine J, Baxter JD, Greene GL. Construction of cell lines that express high levels of the human estrogen receptor and are killed by estrogens. Mol Endocrinol. 1990 Oct;4(10):1465-73.

430. Gadalla SE, Alexandraki A, Lindstrom MS, Nister M, Ericsson C. Uncoupling of the ERalpha regulated morphological phenotype from the cancer stem cell phenotype in human breast cancer cell lines. Biochem Biophys Res Commun. 2011 Feb 25;405(4):581-7.

431. Zhang H, Lin Y, Xiao Y, Wang SM, Liu XX, Wang SM. Stable transfection of estrogen receptor-alpha suppresses expression of cyclooxygenase-2 and vascular endothelial growth factor-C in MDA-MB-231 breast cancer cells. Chin Med J (Engl). 2010 Aug 5;123(15):1989-94.

432. Gould SJ, Subramani S. Firefly luciferase as a tool in molecular and cell biology. Anal Biochem. 1988 Nov 15;175(1):5-13.

433. Bronstein I, Fortin J, Stanley PE, Stewart GS, Kricka LJ. Chemiluminescent and bioluminescent reporter gene assays. Anal Biochem. 1994 Jun;219(2):169-81.

434. Tworoger SS, Hankinson SE. Prolactin and breast cancer risk. Cancer Lett. 2006 Nov 18;243(2):160-9.

435. Arendt LM, Evans LC, Rugowski DE, Garcia-Barchino MJ, Rui H, Schuler LA. Ovarian hormones are not required for PRL-induced mammary tumorigenesis, but estrogen enhances neoplastic processes. J Endocrinol. 2009 Oct;203(1):99-110.

436. Rasmussen LM, Frederiksen KS, Din N, Galsgaard E, Christensen L, Berchtold MW, et al. Prolactin and oestrogen synergistically regulate gene expression and proliferation of breast cancer cells. Endocr Relat Cancer. 2010 Aug 16;17(3):809-22.

437. Gonzalez L, Zambrano A, Lazaro-Trueba I, Lopez E, Gonzalez JJ, Martin-Perez J, et al. Activation of the unliganded estrogen receptor by prolactin in breast cancer cells. Oncogene. 2009 Mar 12;28(10):1298-308.

438. Damiano JS, Wasserman E. Molecular pathways: Blockade of the PRLR signaling pathway as a novel antihormonal approach for the treatment of breast and prostate cancer. Clin Cancer Res. 2013 Apr 1;19(7):1644-50.

439. Connett JM, Badri L, Giordano TJ, Connett WC, Doherty GM. Interferon regulatory factor 1 (IRF-1) and IRF-2 expression in breast cancer tissue microarrays. J Interferon Cytokine Res. 2005 Oct;25(10):587-94.

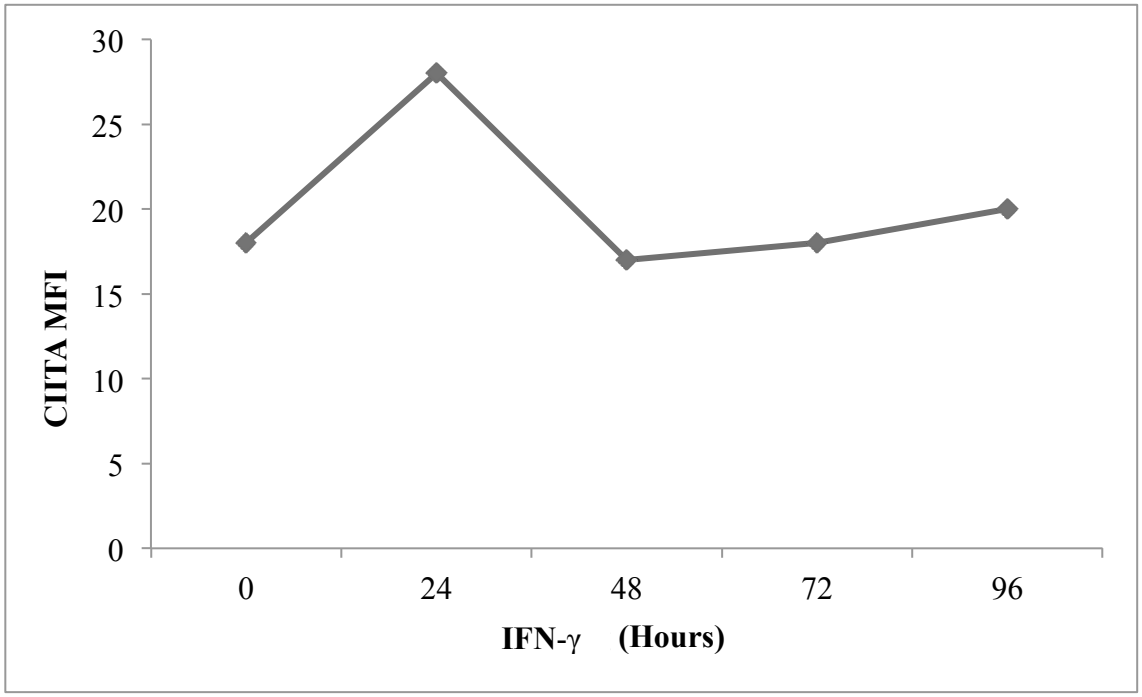
440. Xi H, Eason DD, Ghosh D, Dovhey S, Wright KL, Blanck G. Co-occupancy of the interferon regulatory element of the class II transactivator (CIITA) type IV promoter by interferon regulatory factors 1 and 2. Oncogene. 1999 Oct 21;18(43):5889-903.

441. Joyce MM, Burghardt JR, Burghardt RC, Hooper RN, Jaeger LA, Spencer TE, et al. Pig conceptuses increase uterine interferon-regulatory factor 1 (IRF1), but restrict expression to stroma through estrogen-induced IRF2 in luminal epithelium. Biol Reprod. 2007 Aug;77(2):292-302.

Appendices

Appendix 1 Kinetics of IFN-γ effect on CIITA expression in VC5.

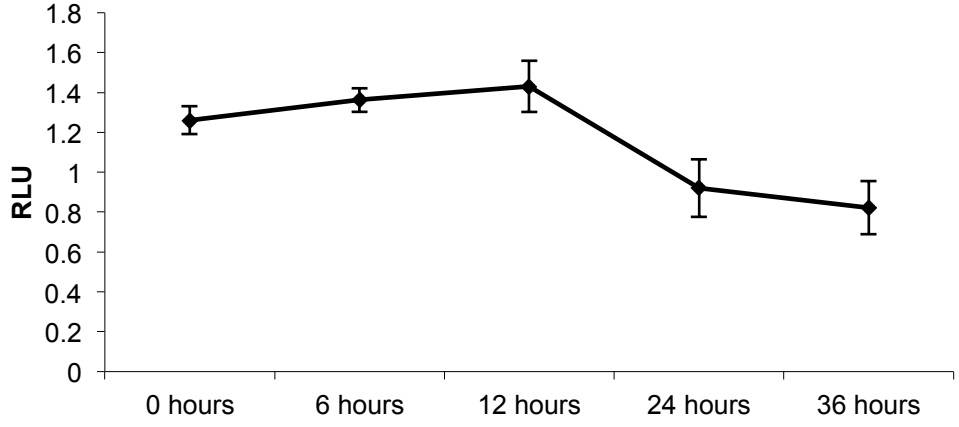
Cells were cultured in the presence of IFN-γ (100 Units/ml) for various times and assayed for CIITA expression using CIITA #21 antibody and intracellular flow cytometry. The graph represents one experiment.


30
25
20
15
10
5
0
CIITA MFI
0
24
48
72
96
IFN-γ (Hours)

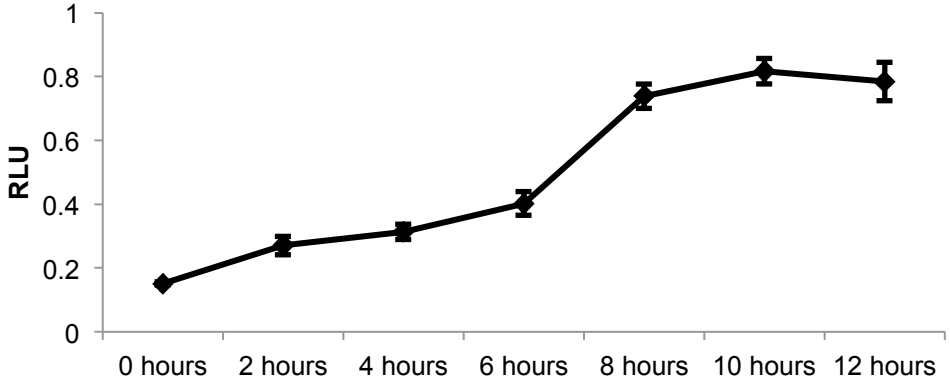
Appendix 2 Kinetics for CIITA promoter activity in VC5 cells.

Cells were cultured in the presence of IFN-γ (100) Units/ml for various time points and assayed for A) pIII CIITA activity using pIII CIITA luciferase construct B) pIV CIITA activity using pIV CIITA luciferase construct. CIITA promoter activity is expressed in the form of raw data (relative light unit (RLU)) and is a representative of one experiment.

A


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
RLU
0 hours
6 hours
12 hours
24 hours
36 hours

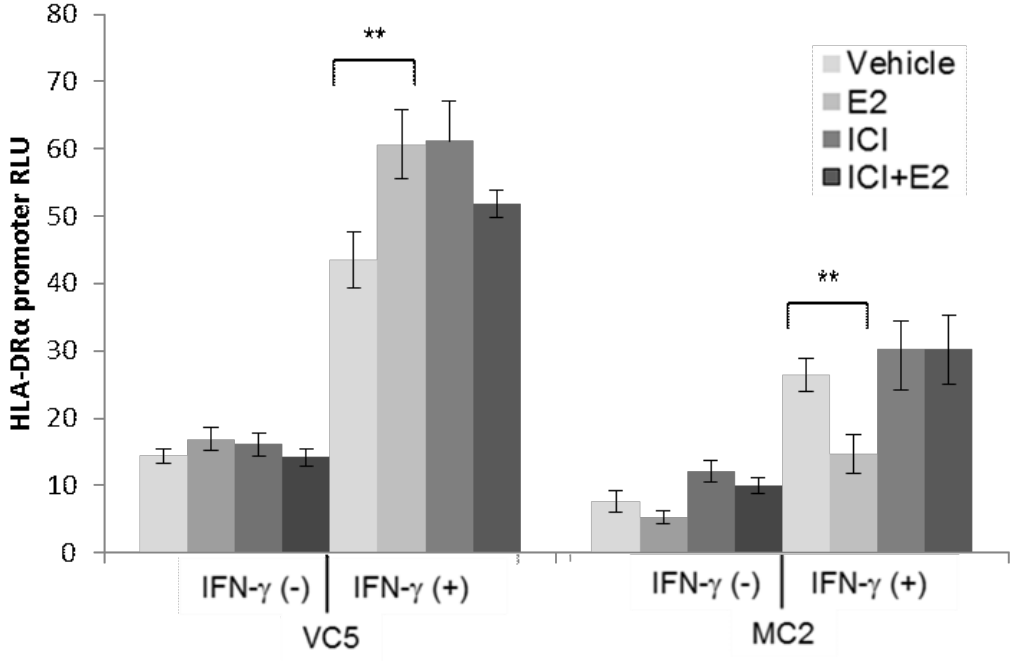
B


1
0.8
0.6
0.4
0.2
0
RLU
0 hours
2 hours
4 hours
6 hours
8 hours
10 hours
12 hours

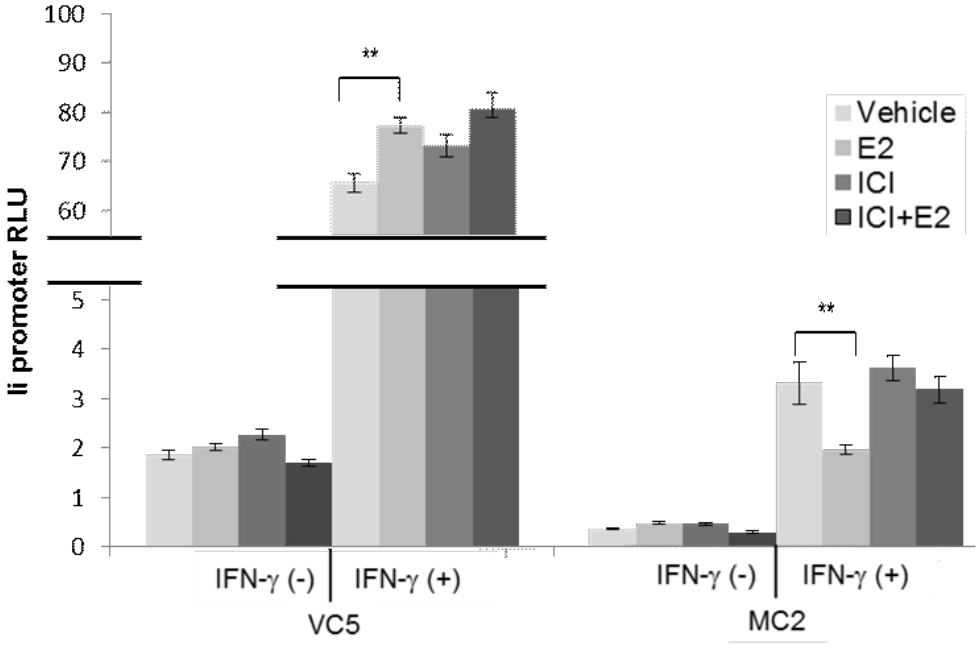
Appendix 3 E_2-ER signaling pathway interferes with HLA-DRA and invariant chain promoter activity in MC2.

VC5 and MC2 cells were cultured in estrogen-depleted media overnight followed by transfection with (A) HLA-DRA luciferase constructs and (B) Ii luciferase constructs. On the subsequent day, the cells were treated with vehicle (ethanol), E_2 (10^{-9} M) and /or Fulvestrant (ICI; 10^{-6} M), and stimulated or not with IFN-γ (100 U/ml) for 24 hr. Error bars represent the mean ± standard errors of the mean (SEM) of 5 replicates (**$p<0.01$). Promoter activity is expressed in the form of raw data (relative light unit (RLU)) and is a representative of one experiment.

A

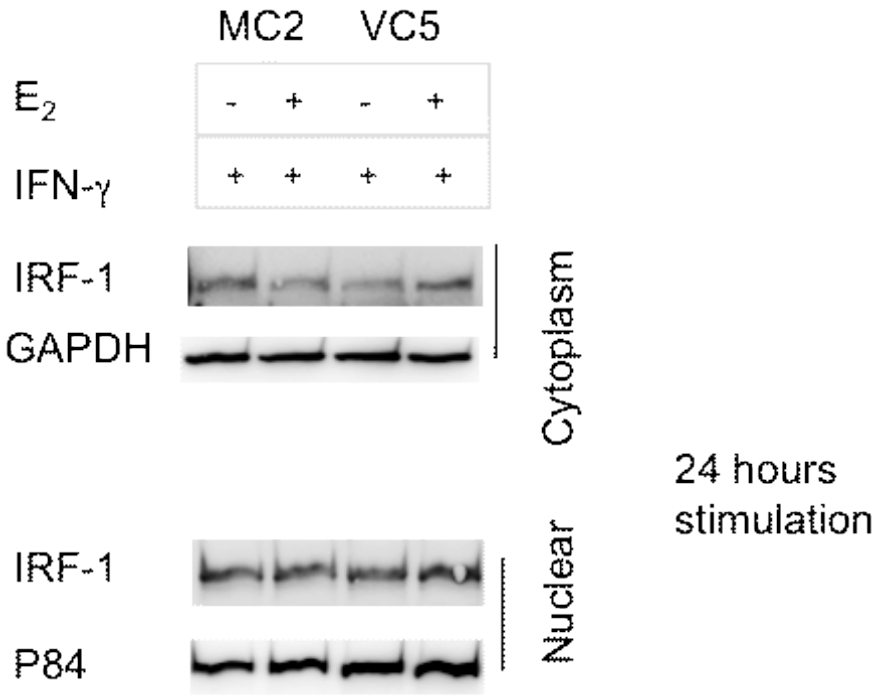

HLA-DRα promoter RLU
80
70
60
50
40
30
20
10
0
**
**
Vehicle
E2
ICI
ICI+E2
IFN-γ (-)
IFN-γ (+)
IFN-γ (-)
IFN-γ (+)
VC5
MC2

B


Ii promoter RLU
100
90
80
70
60
5
4
3
2
1
0
**
**
Vehicle
E2
ICI
ICI+E2
IFN-γ (-)
IFN-γ (+)
IFN-γ (-)
IFN-γ (+)
VC5
MC2

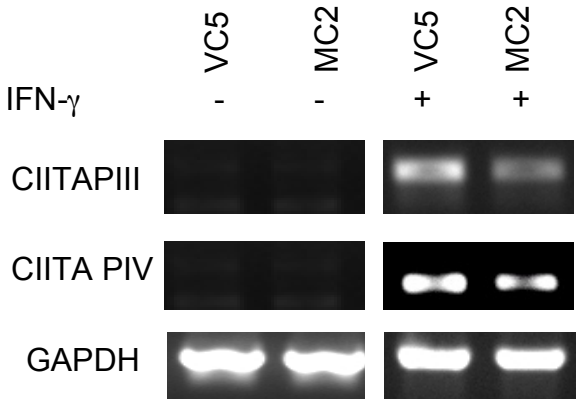
Appendix 4 IRF1 is significantly reduced in MC2 compared to VC5.

VC5 and MC2 were treated with vehicle (ethanol), E_2 (10^{-9} M) stimulated or not with IFN-γ (100 Units/ml) for 24 hr. Cytoplasmic and nuclear lysates were prepared and subjected to immunoblotting probed with IRF1 antibody, GAPDH and/or P84 antibodies as a loading control. Figure represents one experiment.


MC2
VC5
E2
- + - +
IFN-γ
+ + + +
IRF-1
GAPDH
Cytoplasm
24 hours
stimulation
IRF-1
P84
Nuclear

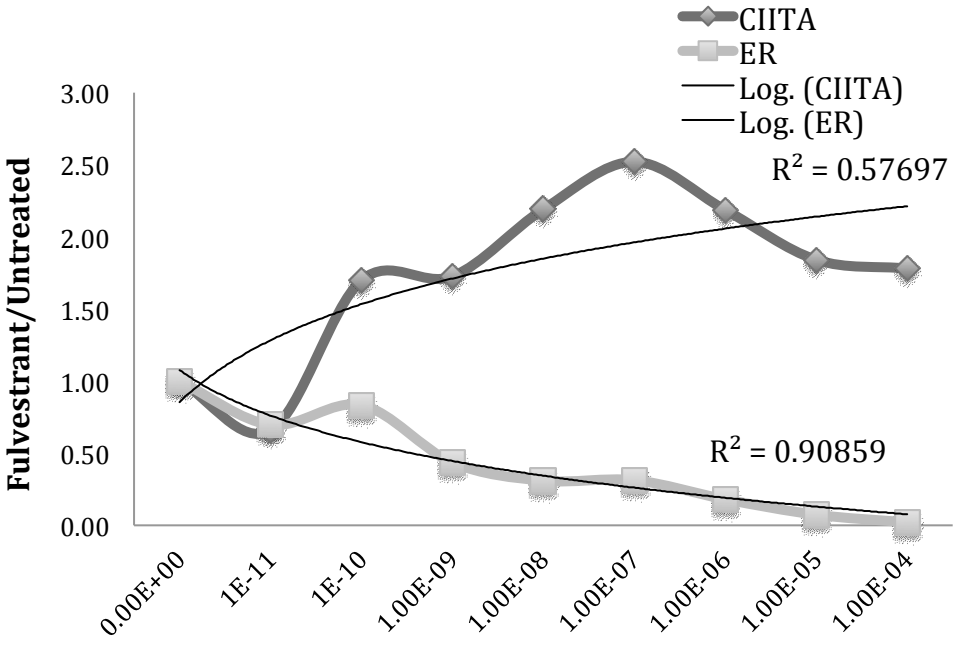
Appendix 5 IFN-γ inducible CIITA expressions is downregulated in MC2 at the mRNA level.

VC5 and MC2 cells were cultured in estrogen-depleted medium and stimulated with IFN-γ (100 Units/ml) for 12 hr. Total RNA was isolated and subjected to RT-PCR analysis using primers specific for pIII and pIV CIITA. Figure represents one experiment.


VC5
MC2
VC5
MC2
IFN-γ
-
-
+
+
CIITAPIII
CIITA PIV
GAPDH

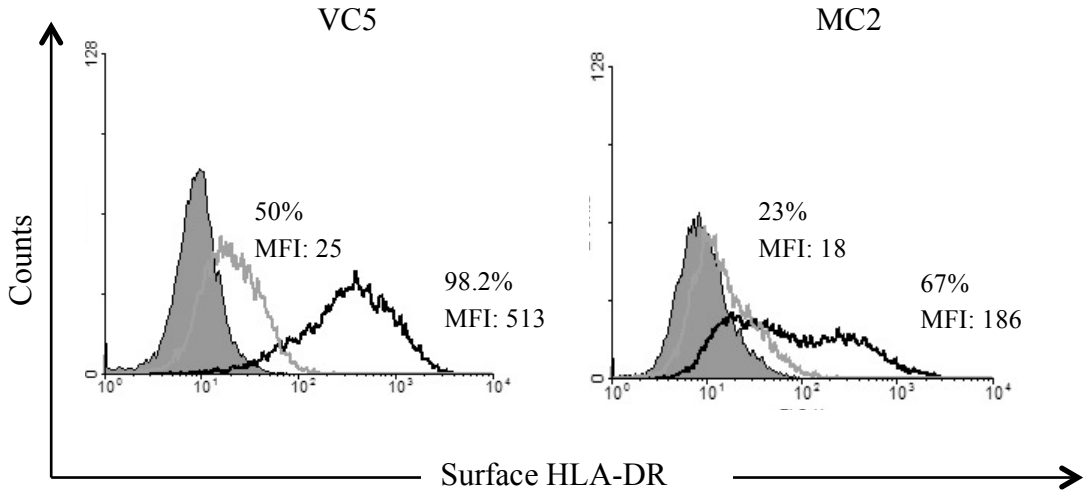
Appendix 6 Fulvestrant induces CIITA expression in a dose dependent manner.

MCF-7 were pre-treated with increasing concentration of Fulvestrant (ICI) as indicated, followed by IFN-γ (100 Units/ml) stimulation. Whole cell RIPA lysates were prepared and subjected to immunoblotting using ERα antibody (HC-20) and CIITA antibody (#21). CIITA and ERα were normalized to GAPDH and the band intensity after normalization is presented in the line graph in the form of the ratio of ICI/vehicle. Figure represents one experiment.


CIITA
ER
Log. (CIITA)
Log. (ER)
R² = 0.57697
R² = 0.90859
Fulvestrant/Untreated
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.00E+00
1E-11
1E-10
1.00E-09
1.00E-08
1.00E-07
1.00E-06
1.00E-05
1.00E-04

Appendix 7 Effect of MEK inhibitor, U0126, on HLA-DR expression in VC5 and MC2 cells.

VC5 and MC2 cells were treated with 10 μM U0126 or vehicle (DMS0) and stimulated or not with IFN-γ (100 Units/ml) for 72 hours. HLA-DR surface expression (detected by L243) was analysed by flow cytometry. Shaded histogram = isotype control, black line = DMSO, grey line = U0126. Figure represents one experiment.


VC5
MC2
Counts
128
0
50%
MFI: 25
98.2%
MFI: 513
23%
MFI: 18
67%
MFI: 186
Surface HLA-DR