CYTOKINE AND HORMONE MODULATION OF HLA-DR MOLECULES IN BREAST CARCINOMA CELL LINES

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Cytokine and Hormone Modulation of HLA-DR Molecules in Breast Carcinoma Cell Lines

By

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ABSTRACT

Cytokines and steroidal hormones modulate HLA-DR expression on breast cancer cell lines (BCCL). This study compared the individual and combined quantitative effects of IFN-γ, IL-4, TGF-β1, and 17β-estradiol on HLA-DR expression on 8 human breast carcinoma cell lines (MCF-7, T47D, BT-474, BT-20, MDA MB 157, MDA MB 231, MDA MB 468, SKBR3) through the use of monoclonal antibodies and flow cytometry and CELISA.

Due to problems with the reconstitution solvents, we were unable to determine the effects of estradiol treatment on HLA-DR expression. However, comparison of constitutive and IFN-γ-induced HLA-DR expression in medium containing or depleted of estrogen illustrated that media components, particularly estrogen, significantly modulated HLA-DR expression on BCCL.

We report distinct patterns in cytokine modulation of HLA-DR expression when co-cultured in estrogen-depleted media. Although IFN-γ increased HLA-DR expression on all cell lines, addition of IL-4 selectively increased HLA-DR expression on MCF-7 and SKBR3, and addition of TGF-β1 selectively increased expression on BT-20 and SKBR3. Furthermore, analysis of TGF-β1 sensitivity through detection of phosphorylated Smad2 and Smad3 proteins (P-Smad2, P-Smad3) by Western blotting showed differential expression of P-Smad proteins in BCCL, as both BT-20 and SKBR3 expressed P-Smad3 but not P-Smad2. Since P-Smad3 is thought to inhibit CIITA in most cell types, we suggest that TGF-β1 mediated up-regulation of HLA-DR on breast cancer cell lines may be mediated through a Smad-independent pathway.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

AF-1, -2:	Transcription-activating function-1 or -2
APC:	Antigen presenting cell
APS:	Ammonium persulphate
β_2M:	Beta-2 microglobulin
BCCL:	Breast cancer cell line
BSA:	Bovine serum albumin
cAMP:	Cyclic adenosine monophosphate
CBP:	CREB-binding protein
CDK7:	Cyclin dependent kinases 7
CDK9:	Cyclin dependent kinases 9
CELISA:	Cell enzyme-linked immunosorbant assay
CIITA:	Class II transactivator
CLIP:	Class II-associated invariant chain peptide
CREB:	cAMP responsive element binding protein
DBD:	DNA-binding domain
DC:	Dendritic cell
DEPC:	Diethyl procarbonate
dH_2O:	Distilled water
DMSO:	Dimethylsulfoxide
DNA:	Deoxyribonucleic acid
dNTPs:	Deoxynucleotide triphosphates
E_2:	Estrogen
EDTA:	Ethylenediaminetetraacetic acid
EGF:	Epidermal growth factor
ERα:	Estrogen receptor-alpha
ERβ:	Estrogen receptor-beta
ERE:	Estrogen responsive element
ERK1/2:	Extracellular-signal-regulated kinase
FCS:	Fetal calf serum
γc:	Common gamma-chain
GAPDH:	Glyceraldehyde phosphate dehydrogenase
GAS:	Gamma activating sequence
HLA:	Human leukocyte antigen
HSP:	Heat shock protein
IFN-β:	Interferon-beta
IFN-γ:	Interferon-gamma
IHW:	International histocompatibility workshop
Ii:	Invariant chain
IL-4:	Interleukin-4
IL-4R:	Interleukin-4 receptor
IMDM:	Iscove's modified Dulbecco's medium

IRF:	Interferon regulatory factor
IRS-1, -2:	Insulin receptor substrate-1, -2
JAK:	Janus activating kinase
JNK:	c-Jun amino-terminal kinase
LBD:	Ligand-binding domain
LCR:	Locus control region
LRR:	Leucine rich region
mAb:	Monoclonal antibody
MAPK:	Mitogen activated protein kinase
MFI:	Mean fluorescence intensity
MHC:	Major histocompatibility complex
MIIC:	MHC class II compartment
mRNA:	Messenger ribonucleic acid
NFκB:	Nuclear factor kappa B
NF-Y:	Nuclear factor binding to the Y box
OBF-1:	Octamer binding factor 1
OBS:	Octamer-binding site
OCT-1, -2:	Octamer binding transcription factor-1 or -2
OD:	Optical density
PBS:	Phosphate buffered saline
PCAF:	p300/CBP-associated factor
PCR:	Polymerase chain reaction
PgR:	Progesterone receptor
PI3K:	Phosphoinositide 3-kinase
pI, pII, pIII, pIV:	*MHC2TA* gene promoters 1-4
Pol II:	RNA polymerase II
PLL:	Poly-L-lysine
p-TEFb:	Positive transcription elongation factor b
RFX:	Regulatory factor X
RFXANK:	RFX-associated protein with ankyrin repeats
RFXAP:	RFX-associated protein
RNA:	Ribonucleic acid
RT-PCR:	Reverse transcriptase polymerase chain reaction
SBE:	Smad-binding element
SDS:	Sodium dodecyl sulphate
SOSC-1:	Suppressor of cytokine signaling-1
SRC-1:	Steroid receptor coactivator 1
STAT:	Signal transducer and activator of transcription
$TAF_{II}32$, 70:	TATA binding protein associated factor 32 or 70
TCR:	T cell receptor
TEC:	Thymic epithelial cell
TFIIB:	TATA box binding protein-associated transcription factor IIB
TGF-β:	Transforming growth factor-beta
TM:	Transmembrane

Tx:	Treatment
USF-1:	Upstream stimulatory factor 1
X2BP:	X2-binding protein

LIST OF APPENDICES

Chapter 1: Introduction

1.1. Major Histocompatibility Complex (MHC)

The major histocompatibility complex (MHC), also referred to as human leukocyte antigen (HLA) complex, is a collection of highly polymorphic genes that encode cell surface glycoproteins essential in the induction and regulation of adaptive immune responses. HLA proteins are divided into three groups: HLA class I, class II, and class III. HLA class I (HLA-A, -B, -C) and class II (HLA-DR, -DP, -DQ) molecules are vital in adaptive immune responses by virtue of their ability to bind and present antigenic peptides to $CD8^+$ and $CD4^+$ T cells respectively. HLA molecules on thymic epithelial cells (TEC) are also crucial for positive selection, negative selection, and maturation of T cells in the thymus. This ensures survival of T cells that carry T cell receptors (TCR) capable of recognizing self-MHC molecules, while eliminating autoreactive T cells (for review see [1]).

While MHC class I molecules are present on most nucleated cells, constitutive expression of MHC class II molecules is largely restricted to antigen presenting cells (APCs), namely dendritic cells (DC), B cells, and macrophages. MHC Class II expression is regulated on these cells in a maturation-dependent manner, such that MHC expression on DC increases with maturation, whereas B cells lose MHC expression when they mature to plasma cells [2-4]. In addition, TEC and activated T cells also express HLA class II, and expression may be induced on most other MHC negative cell types by various stimuli, such as interferon gamma (IFN-γ).

1.1.1. Nomenclature of HLA Class II Molecules

Nomenclature of HLA class II molecules consists of three letters: the first (D) indicates the class, the second (M, O, P, Q, or R) the family, and the third (α or β) the chain. Individual genes are numbered, and the numerous allelic variants of these genes are noted by a number preceded by an asterisk. For example, HLA-DRB1*0401 stands for allelic variant 0401 of gene 1, which encodes the β chain of a class II molecule belonging to the R family [5].

1.1.2. Structure of HLA Class II Molecules

HLA class II genes are located within approximately 4 Mb of DNA on the distal part of chromosome 6p21.3 and encode the polymorphic HLA-DR, HLA-DP, and HLA-DQ proteins. Each HLA class II molecule is a heterodimer, composed of two transmembrane glycoproteins, α (34 kDa) and β (29 kDa), which associate through non-covalent interactions (Figure 1.1).

Each chain has four domains: the peptide-binding domain (α_1 or β_1), the immunoglobulin-like domain (α_2 or β_2), the transmembrane region, and the cytoplasmic tail. The peptide binding groove of HLA class II molecules consists of two parts. The floor consists of a rather flat antiparallel β-pleated sheet composed by both α and β chains, while both walls are coiled into α-helix formations. The peptide-binding groove is formed between the α1 and β1 domains [5] and remains open-ended to accommodate longer peptides [6]. Peptides bind in an extended conformation and are held by a series of hydrogen bonds between the peptide backbone and conserved amino acid side chains

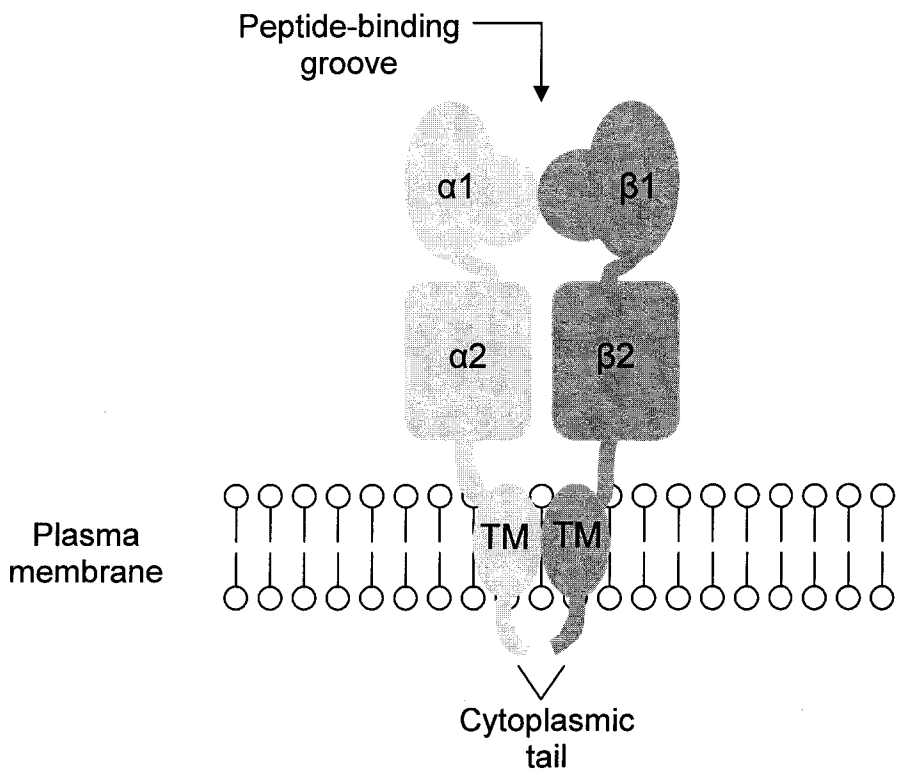


Figure 1.1. Structure of HLA class II molecules. Each of the Class II α and β chains has four domains: the peptide-binding domain (formed by α1 and β1), the immunoglobulin-like domain (α2 or β2), the transmembrane region (TM), and the cytoplasmic tail. Adapted from [5].

lining the groove. There is an extreme degree of variation concentrated on amino acid residues shaping the peptide-binding site, leading to a high degree of peptide-binding specificity.

1.1.3. HLA-DR Polymorphism

Both α and β chains may contribute to HLA-DR polymorphism. Currently, the HLA-DR α-chain has only three DRA alleles (DRA*0101, DRA*010201, and DRA*010202) [7] offering limited polymorphism, while there are 394 HLA-DRB1 alleles, 41 HLA-DRB3 alleles, 13 HLA-DRB4 alleles, and 18 HLA-DRB5 alleles [7]. Thus, most HLA-DR polymorphism is derived from DRB genes.

The complete set of HLA alleles present on each chromosome is referred to as the HLA haplotype, and the number of HLA-DRB genes expressed by an individual depends on the haplotype inherited. These haplotypes include DRB1*15 and 16 expressed in association with DRB5; DRB1*03, 11, 12, 13, and 14 with DRB3; DRB1*04, 07, and 09 with DRB4; and DRB1*01, 10 and 08, which are not in association with any other DRB-expressed genes [8]. Thus, assuming heterozygosity, an individual can express as many as 4 HLA-DR allotypes.

1.2. MHC Class II Antigen Presentation Pathway

Alpha and β chains of MHC class II molecules are rapidly synthesized within the endoplasmic reticulum. There they associate with the invariant chain (Ii), which acts as a chaperone to ensure efficient folding of newly synthesized α and β subunits. The Ii forms

trimers, with each subunit non-covalently binding to an α:β class II heterodimer, forming a large nonameric structure. The Ii binds to HLA class II molecules such that part of the polypeptide chain, the class II-associated invariant chain peptide (CLIP), occupies the peptide-binding groove [9] preventing loading of endogenous peptides within the endoplasmic reticulum [10].

Ii also directs assembled heterodimers out of the endoplasmic reticulum, through the Golgi apparatus, and into primary lysosomes which fuse with early endosomes to form the MHC class II compartment (MIIC) [11]. The cytoplasmic tail of Ii contains a dileucine motif that directs class II / Ii chain complexes to localize within the endocytic pathway [12]. It is within the MIIC that the majority of peptide loading will occur [13] as class II molecules encounter antigenic peptides generated by proteolytic degradation of endocytosed exogenous proteins [14].

Ii is degraded within the MIIC by lysosomal proteases, such as cathepsin L and S [15-17]. However the CLIP fragment is protected from degradation by being deeply embedded in the class II structure, and therefore continues to occupy the peptide - binding groove. The exchange of CLIP for antigenic peptides is facilitated by the low pH of lysosomal MIICs [18]. Additional support is supplied by HLA-DM, a specialized lysosomal chaperone encoded by HLA-DMA and HLA-DMB genes in the HLA locus [19]. HLA-DM functions as a "peptide editor," releasing CLIP and unstable, low-affinity binding peptides from the peptide–binding groove, while retaining stable, high-affinity bound peptides [20-24]. HLA-DM also stabilizes empty class II molecules that would

otherwise aggregate into nonfunctional complexes [21, 25]. Most HLA-DM heterodimers reside in the MIICs with very low amounts detected on the cell surface [26, 27].

HLA-DO, a heterodimer composed of gene products of HLA-DNα and HLA-DOβ [28, 29], is a negative regulator of HLA-DM. HLA-DO is predominantly expressed in B cells, and regulates HLA-DM mediated CLIP release and formation of class II–peptide antigenic complexes [30].

1.3. Regulation of HLA Class II Gene Expression

Expression of HLA class II and related genes is regulated mainly at the level of transcription and is characterized by the presence of a conserved sequence of cis-acting elements within the promoter region. These elements include the W (or S), X, X2 and Y boxes, and are collectively known as the S-X-Y module [31, 32].

Transcription of HLA class II genes requires the assembly of a highly stable macromolecular nucleoprotein complex that binds on the S-X-Y module. This complex, referred to as the enhanceosome, consists of regulatory factor X (RFX), X2-binding protein (X2BP) / cyclic adenosine monophosphate (cAMP) response element binding protein, and nuclear factor Y (NF-Y) multimeric factors [33] (Figure 1.2).

The transcription factors that compose the enhanceosome are well characterized. The X box is bound by RFX, a trimeric complex composed of RFX5, RFXANK (RFX-B) and RFXAP [34-37]. The downstream X2 box is recognized by X2BP, a complex that includes cAMP response element binding protein (CREB) [38]. The trimeric NF-Y complex, composed of NF-YA, NF-YB, and NF-YC, binds to the Y box [39]. The *in vivo*

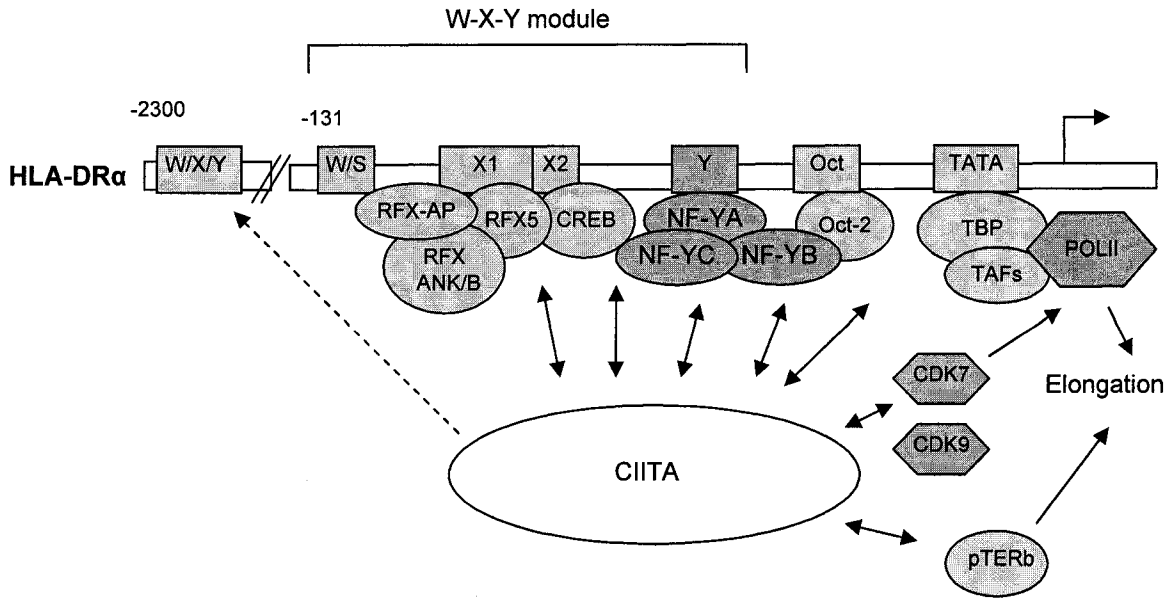


Figure 1.2. CIITA interaction with MHC enhanceosome on the HLA-DRα promoter. MHC Class II promoters share a common set of *cis*-acting elements, the W/S, X1, X2, and Y boxes (S-X-Y module) which are bound by constitutive and ubiquitous transcription factors forming the multiprotein MHC Class II enhanceosome complex. The enhanceosome provides an appropriate interaction surface for recruitment of CIITA. CIITA associates with an array of TAFs and elongation factors which lead to initiation of mRNA synthesis. Upstream of the proximal promoter is a recently discovered inverted S-X-Y module, known as the locus control region, which also associates with CIITA and RFX. Adapted from [33].

W-box-binding protein remains unclear, although a number of proteins including RFX have been shown to bind the W box *in vitro* [40].

The DRα promoter also includes an ATGCAAAT octamer-binding site (OBS). In B cells this site is bound by transcription factors Oct-1 and Oct-2 [41]. Octamer binding factor 1 (OBF-1) binds to Oct-1 and Oct-2, increasing their ability to activate transcription [42, 43].

The transcription factors described above are ubiquitously and constitutively expressed, but fail to induce MHC class II expression on their own. Instead they form the enhanceosome complex which provides an appropriate interaction surface for recruitment of the class II transactivator (CIITA) [31, 44, 45].

CIITA is believed to activate transcription by recruiting various components of the basal transcription machinery via its amino-terminal activation domains. CIITA can interact with general transcription factors TFIIB (TATA binding protein-associated transcription factor IIB), $TAF_{II}32$, and $TAF_{II}70$, which promote transcription initiation [46, 47]; TIIH and p-TEFb (positive elongation factor b) which enhance promoter clearance and transcription elongation [47, 48]; and CREB-binding protein (CBP) involved in chromatin remodeling [49, 50]. These transcription factors are not mutually exclusive, thus several factors may activate transcription simultaneously. Recent studies also show that CIITA interacts with the cyclin-dependent kinases 7 (CDK7) and CDK9 and enhances their ability to phosphorylate the carboxy-terminal domain of RNA polymerase II (Pol II), thereby initiating promoter clearance and mRNA synthesis [51].

1.3.1. Class II Transactivator

CIITA functions as a non-DNA binding coactivator that exerts its activity through multiple protein-protein interactions with the enhanceosome complex [45, 52, 53]. Binding of CIITA to the enhanceosome requires stereospecific alignment and stringent spacing, such that a 1-2 bp change in the S-X distance is detrimental for CIITA recruitment and function [54, 55]. Although multiple interactions between CIITA and the proximal S-X-Y module are essential for MHC Class II expression, basal and induced class II gene expression is also regulated by a putative Locus Control Region (LCR) that lies approximately 2.3 kb upstream of the HLA-DRα gene in humans [56].

In contrast to the relative constitutive expression of DNA-binding factors composing the HLA enhanceosome complex, expression of the *MHC2TA* gene encoding CIITA is tightly regulated. In most situations, the expression pattern of *MHC2TA* largely parallels MHC class II expression with CIITA dictating the extent to which MHC class II genes are expressed (for review see [57]). CIITA is thus referred to as the "master regulator" of MHC class II genes.

In addition, CIITA is important in the regulation of several other genes containing S-X-Y boxes within their promoters. These include genes encoding accessory proteins required for MHC class II restricted antigen presentation [58], such as Ii [59], HLA-DM [60, 61], and HLA-DO [15, 60, 62-64]. Expression of these proteins is essential for MHC class II complex assembly and translocation to the cell surface [23, 65, 66]. Reports also indicate that CIITA contributes, albeit to a lesser extent, to expression of classical MHC class I and β_2 microglobulin (β_2M) genes via interaction with a region showing homology

to the S-X-Y module within the MHC class I promoter and β_2M promoter [67-69]. Thus, CIITA either partly or completely controls multiple genes involved in antigen presentation.

1.3.1.1. Structure of CIITA

CIITA protein contains four domains (Figure 1.3). The amino-terminal contains a region rich in acidic amino acids (residues 1-125) which has transactivation properties, possibly achieved by providing surfaces for interaction with the histone acetylase CBP and RFXANK [44, 49, 50]. Downstream of this acidic region is a domain rich in proline, serine, and threonine (residues 126-336), containing multiple phosphorylation sites. The midsection of the protein contains a GTP-binding domain (residues 337-702), involved in protein self-association and is important in nuclear import [70-72]. Finally, the carboxy-terminus contains a leucine-rich (LRR) domain (residues 930-1130) that affects nuclear translocation and self-association [73, 74]. In total, three regions of CIITA are implicated in nuclear localization: the carboxy-terminus [75], the GTP-binding motif [76], and the LLR [73].

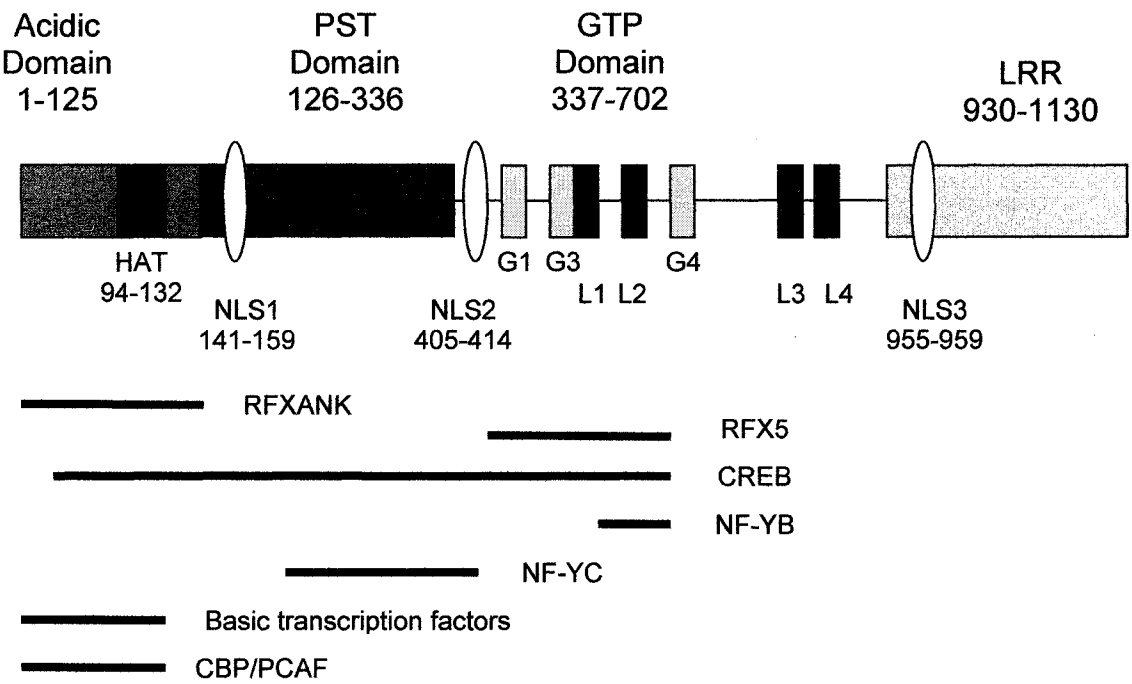


Figure 1.3. Structure and functional regions of CIITA protein. CIITA contains an acidic domain (red), a P/S/T domain (blue), three nuclear localization domains (NLS), and a LRR (pink). The G1, G3, and G4 domains (gray boxes) define a GTP-binding domain. The four LXXL sites (green boxes) are thought to be involved in protein-protein interactions. Bars underneath schematic indicate regions of CIITA found to interact with transcription factors and coactivators. Adapted from [31] and [77].

1.3.1.2. Modulation of CIITA Expression

Since enhanceosome components are ubiquitously expressed, differences in MHC class II expression between cell lines, during differentiation, and induced by cytokines is due to differences in CIITA expression. CIITA transcription may be up-regulated by IFN-γ [59, 78, 79], lipopolysaccharide [80], and interleukin-4 (IL-4) [80]. However, both constitutive and IFN-γ-induced CIITA expressions are frequently silenced in tumor cells of various origins.

CIITA is also negatively regulated by several factors, such as interleukin-1-beta (IL-1β) [81], IFN-β [82], tumor necrosis factor-alpha (TNFα) [83], IL-10 [84], nitric oxide [85], transforming growth factor-beta (TGF-β) [86-88], prostaglandins [89], and statins [90, 91]. In addition, many human pathogens down-regulate MHC class II expression and evade the immune system by inhibiting CIITA expression [92-94].

1.3.2. MHC2TA Promoters

Transcriptional activation of the *MHC2TA* gene encoding CIITA is controlled by four independent promoters (I-IV). These promoters are distributed over approximately 12 kb, but do not share any sequence homology and are not co-regulated. Immediately downstream of each promoter exists a unique exon 1 that is spliced with the remaining shared exons to form three different types of CIITA (I, III, and IV) (Figure 1.4). Translation of CIITA mRNA may begin in exon 2; however, types I and III CIITA mRNA contain translational start sites in exon 1 that give rise to distinct mRNA transcripts encoding CIITA molecules with different amino-terminal protein sequences

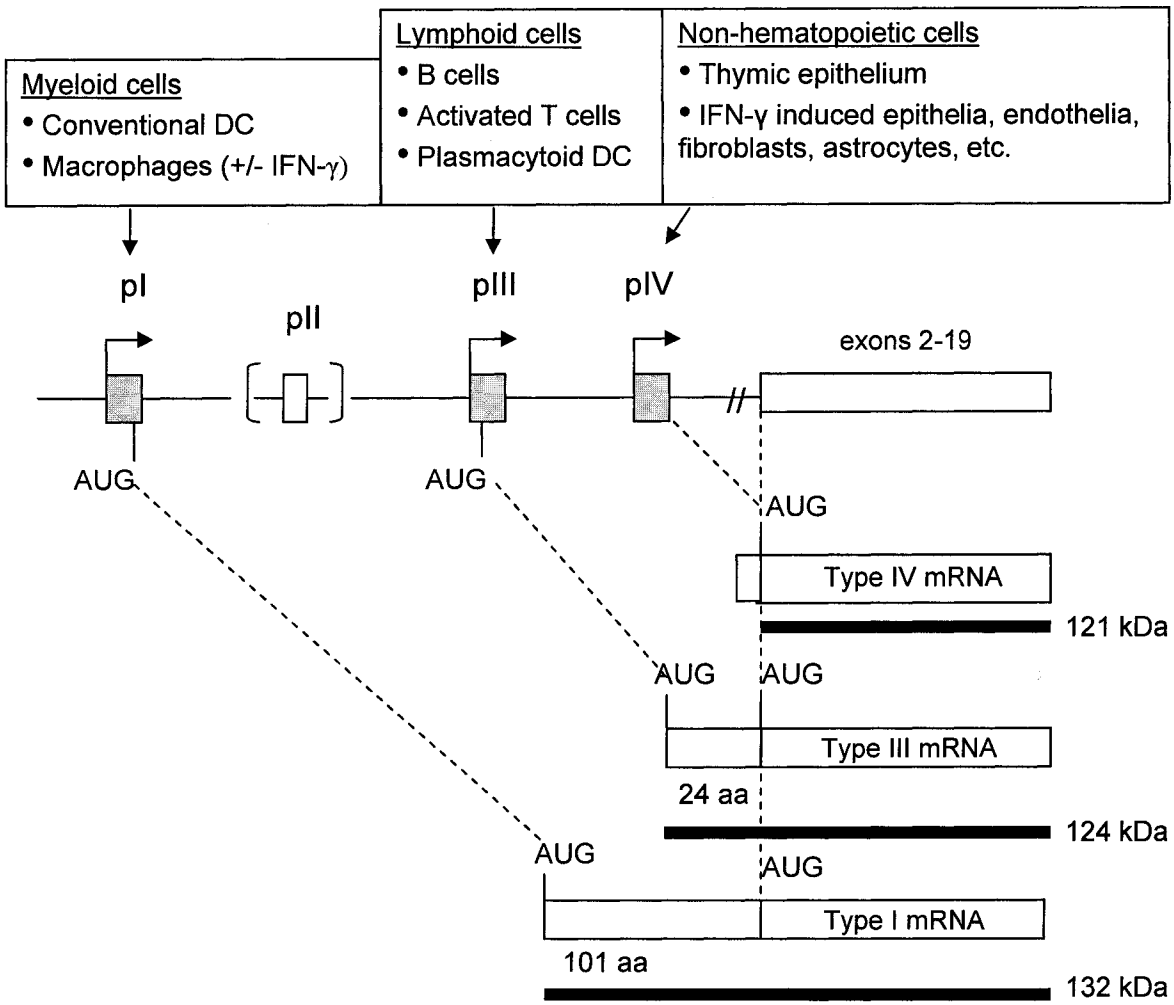


Figure 1.4. Expression of the *MHC2TA* gene. Four promoters (pI, pII, pIII, pIV) have been identified in humans. Usage of these promoters leads to the splicing (dashed lines) of alternative first exons (shaded boxes) to a shared second exon, forming three types of CIITA mRNA that encode three different protein isoforms (black bars). These proteins differ only at their amino-terminal ends. The boundary between the alternative first exons and the shared second exon is indicated by a vertical line. pII displays very low transcriptional activity and its significance is unknown. Adapted from [95].

[96]. These three isoforms of CIITA (121, 124, and 132 kDa) are all known to exist *in vivo* [97].

Promoters I, III, and IV are highly conserved between the human and mouse genome, while promoter II has only been found in the human genome and its function remains unknown. Promoter I is active in myeloid cells (conventional DC and IFN-γ activated macrophages); promoter III is active in lymphoid cells (B-1 and B-2 cells, activated T cells, and plasmacytoid DC) [96]; and promoter IV is mainly responsible for induction by IFN-γ in non-bone marrow-derived cells (endothelial, epithelial, fibroblasts, and astrocytes) [98], but is constitutively expressed in TEC [99].

1.4. Cytokine Modulation of HLA-Class II Expression

1.4.1. Interferon-gamma (IFN-γ)

Induction of MHC class II molecules by IFN-γ is CIITA-dependent [59, 78, 79]. IFN-γ binds to the IFN-γ-receptor and induces synthesis of CIITA through promoter IV in most cell types [81, 96, 98-100]. IFN-γ may also contribute to CIITA induction via promoter III in human fibroblasts and B cells [100-102].

IFN-γ induction via pIV is dependent on three cis-acting sequences, a gamma activating sequence (GAS) element, an interferon regulatory factor (IRF) binding site, and an E box, which all function in synergy (Figure 1.5). Interaction of IFN-γ with its receptor activates Janus activating kinases (JAK) 1 and JAK2, which leads to phosphorylation, dimerization, and nuclear import of signal transducer and activator of transcription 1 (STAT-1). STAT-1 binds cooperatively to the GAS sequences only in the

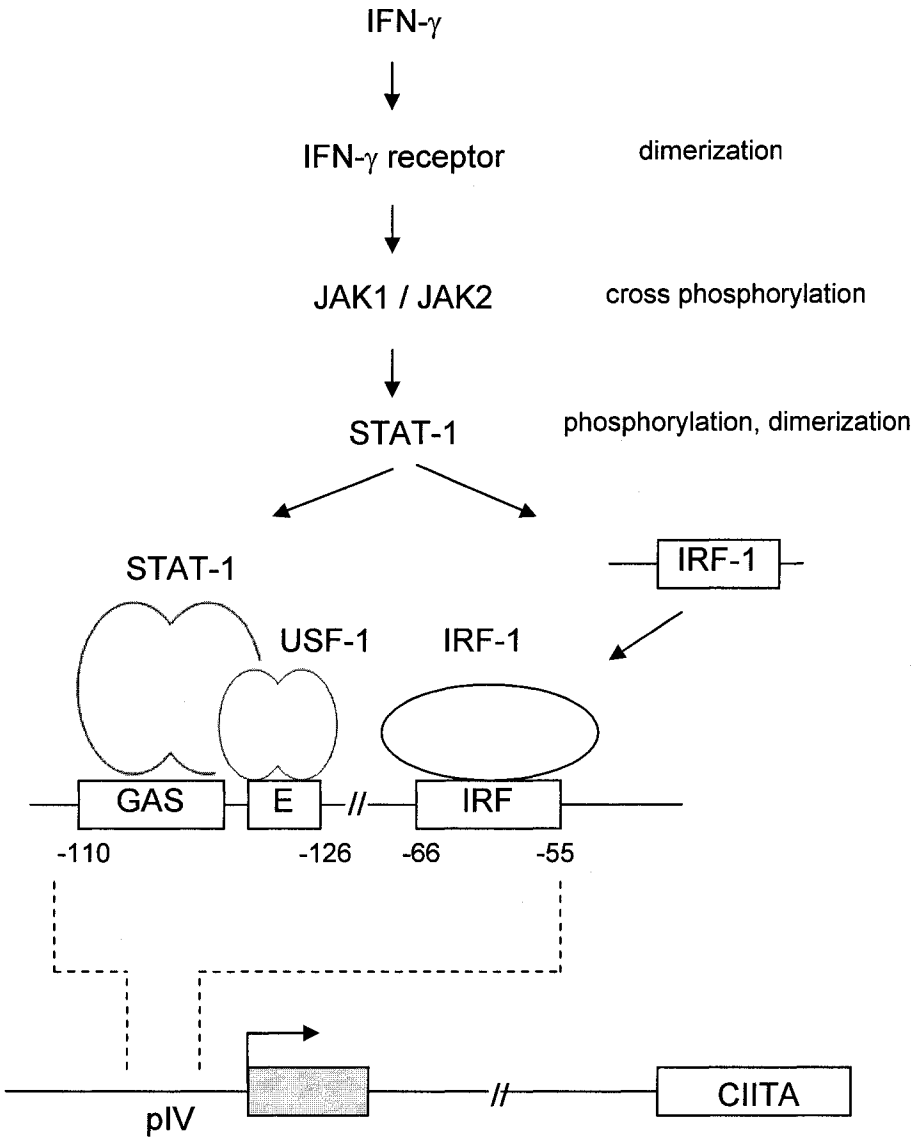


Figure 1.5. Signal transduction cascade mediating IFN-γ induction of HLA class II. JAK1 and JAK2 kinases become activated following interaction of IFN-γ with its receptor. This leads to phosphorylation, dimerization, and nuclear import of STAT-1. STAT-1 binds cooperatively with USF-1 to the GAS/E box motif present in pIV of the *MHC2TA* gene. STAT-1 also activates IRF-1, which collectively with STAT-1 and USF-1 leads to expression of CIITA and induction of MHC class II. Adapted from [57].

presence of a ubiquitously expressed transcription factor, upstream stimulatory factor 1 (USF-1), which binds to the adjacent E box motif present in pIV of the *MHCT2A* gene [99]. STAT-1 also activates expression of IRF-1, which binds to the IRF sequence [100]. Activation of pIV by STAT-1, USF-1, and IRF-1 leads to expression of CIITA and subsequent induction of MHC class II expression.

In contrast to the IFN-γ response activated through pIV, pIII is activated directly by IFN-γ-induced phosphorylation of STAT-1 [100, 101]. Thus, cooperation between STAT-1 and IRF-1 is not necessary for induction of CIITA through pIII, but may explain the enhanced response of pIV to IFN-γ. Activation of STAT-1 is an immediate response and does not require protein synthesis, while activation of IRF-1 is a secondary response that requires protein synthesis and prior activaction of other molecules, including STAT-1 [100]. Therefore, STAT-1 mediates a faster IFN-γ response via pIII, while IRF-1 mediates a slower response via pIV [100].

IFN-γ-induced gene activation is generally a transient event. Suppressor of cytokine signaling -1 (SOCS-1) is induced by IFN-γ and this protein negatively regulates the IFN-γ signal transduction pathway by binding to JAK2 and inhibiting its kinase activity [103, 104]. SOCS-1 can also suppress IFN-γ-activated expression of pIV of the MHC2TA gene [84]. Hypermethylation and silencing of specific SOCS genes has been reported in breast and ovarian cancers, and may augment cytokine responses in these tissues [105].

1.4.2. Interleukin-4 (IL-4)

IL-4 is a pleiotrophic cytokine typically produced by mast cells, basophils, and T_H2 lymphocytes [106, 107], but also produced by tumor infiltrating lymphocytes in breast cancer [108, 109]. IL-4 regulates differentiation of naïve T lymphocytes, promotes proliferation and immunoglobulin class-switching in B cells, and controls growth and differentiation of other hematopoietic cells (for review see [110] and [111]). These actions are mediated via a multiunit transmembrane receptor, the IL-4 receptor (IL-4R) [112, 113].

The IL-4R is composed of two subunits, the first being the ligand-binding chain, IL-4Rα. The second subunit, in hematopoietic cells, is typically the γ-common chain (γc), first identified as a component of the IL-2R [114, 115], and forms type I IL-4Rs. However, recent studies indicate that in nonhematopoietic cells the IL-13Rα' chain is the predominant accessory chain of the IL-4R complex [116, 117], forming type II IL-4Rs. This agrees with other reports that the α chain is necessary for IL-4 activity but IL-4R may also act independently of γc chain [118, 119]. Breast tumor cells are reported to express type II IL-4Rs [120, 121].

The IL-4R lacks intrinsic kinase activity; therefore it requires receptor-associated kinases for the initiation of signal transduction. Binding of IL-4 to the IL-4R activates JAK1 and JAK3 [122], which phosphorylate the cytoplasmic domain of the receptor as well as downstream signaling molecules. Two main pathways are activated in this manner, the insulin-receptor substrate-1 (IRS-1) / IRS-2 pathway and the STAT-6 pathway [123, 124]. Both of these pathways are thought to communicate with several

signaling pathways, including the phosphoinositol-3-kinase (PI3K), Ras/mitogen activated protein kinase (MAPK), and AKT pathways (for review see [111, 125]).

Studies indicate that IL-4-mediated signaling by STAT-6 (Figure 1.6) plays a role in the transcription activation of MHC class II. IL-4 has been shown to increase MHC class II antigen expression on B cells [126-128], human endometrial adenocarcinoma cell lines [129], and to selectively increase HLA-DR and HLA-DP antigens on human monocytes [130]. However, IL-4-induced cell surface expression of both MHC class II antigens and IL-4 receptor are completely abrogated in lymphocytes from STAT-6-knockout mice [131, 132], suggesting that such effects are STAT-6-dependent. Furthermore, transfection of STAT-6c, a dominant negative form of STAT-6 lacking the functionally critical SH2 domain residues, prevents cells from expressing MHC class II by inhibiting endogenous STAT-6 dimer formation [133].

IL-4 may also up-regulate MHC class I, class II, β_2M, and tumor associated antigens on human melanoma cells and human renal cell carcinomas, and these effects are enhanced by combination with IFN-γ [134, 135]. A similar additive effect of IL-4 and IFN-γ on HLA-DR expression in melanoma and breast carcinoma cells was reported by Obiri et al. [136], while contrasting reports indicate IL-4 suppresses IFN-γ-induction of CIITA and MHC class II expression in microglial cells, astrocytes, and monocytes [84, 130, 137-139]. It is currently unknown if IL-4-mediated induction of MHC on cancer cells is also STAT-6-dependent. Nevertheless, the combined augmentation of MHC class II by IFN-γ and IL-4 is of particular interest as these cytokines are generally known to

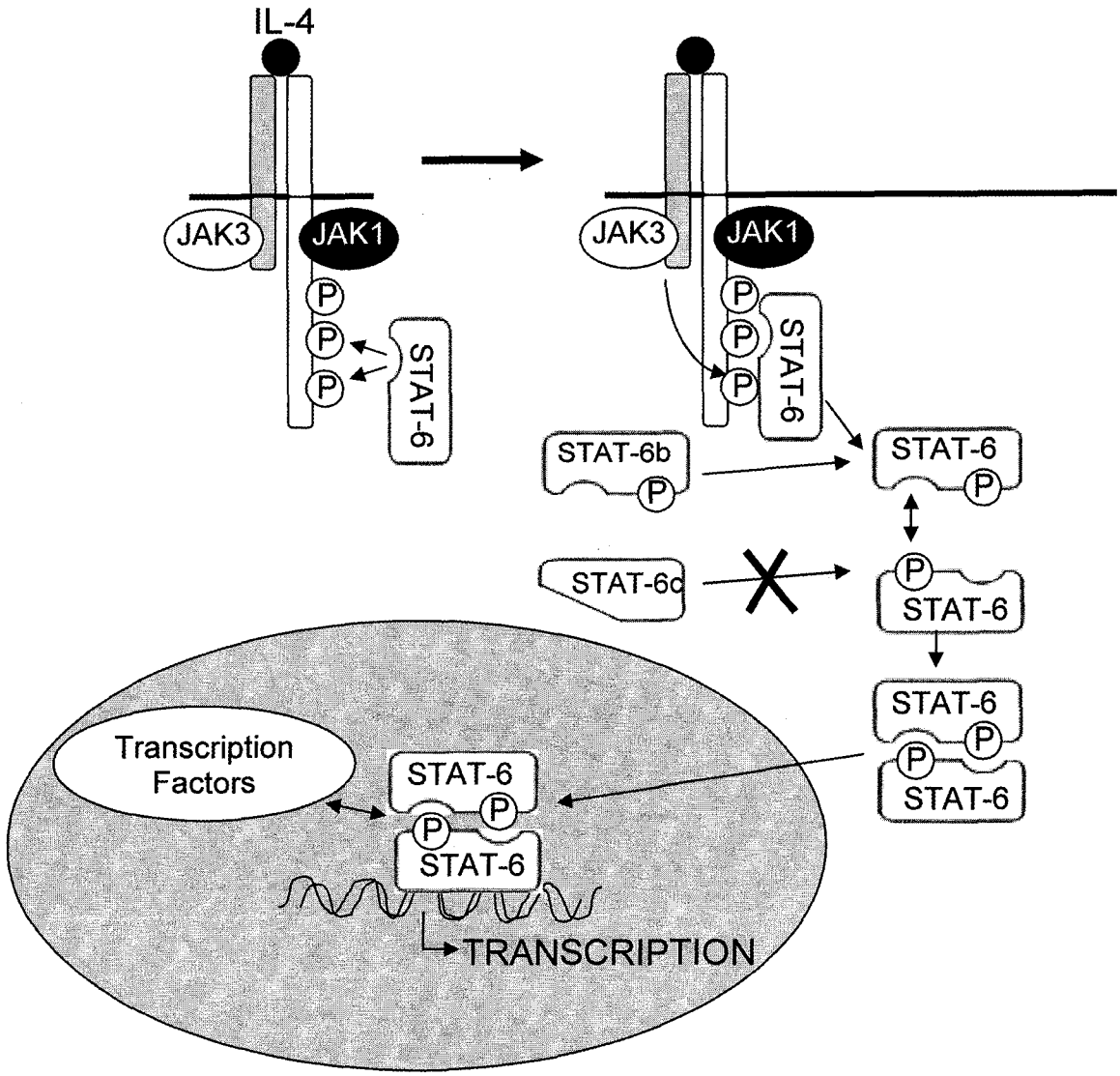


Figure 1.6. IL-4 receptor signaling through the STAT-6 pathway. IL-4 engagement of the IL-4R results in the activation of JAK1 and JAK3 and phosphorylation of specific tyrosine residues in the receptor cytoplasmic region. STAT-6 binds to the phosphorylated receptor by a highly conserved SH2 domain and becomes phosphorylated. Phosphorylated STAT-6 disengages from the IL-4Rα cytoplasmic tail, forms homodimers with a second P-STAT-6, and translocates to the nucleus where it activates transcription of responsive genes in cooperation with other transcription factors. Alternatively spliced forms of STAT-6 have deletion in the amino-terminal (STAT-6b) or SH2 regions (STAT-6c) and regulate STAT-6 signaling. The exact mechanism by which STAT-6 activates transcription is still being determined. Adapted from [111].

have antagonist effects, such as during the differentiation of naïve $CD4^+$ T cells into T_H1 and T_H2 subsets.

1.4.3. Transforming Growth Factor-Beta (TGF-β)

TGF-β was first identified and named for its ability to stimulate the proliferation and transformation of mesenchymal cells [140]. It is now known as a ubiquitous and essential regulator of cellular and physiological processes including proliferation, differentiation, migration, cell survival, angiogenesis, and immunosurveillance (for review see [141]).

Three TGF-β isoforms are expressed in mammals (TGF-β1,-β2, -β3) and each is encoded by a unique gene and expressed in a tissue-specific and developmentally regulated fashion. Of these, TGF-β1 is the most abundant and universally expressed isoform [141].

TGF-β is secreted into the extracellular matrix as a latent protein complex bound to a latency-associated-protein and one of four isoforms of the TGF-β binding protein. Activation of TGF-β is required for biological activity, which occurs through poorly understood mechanisms, likely involving proteolytic processing of associated proteins and release of the TGF-β ligand. Once activated, TGF-β regulates cellular processes by binding to three high-affinity surface receptors: TβRI, TβRII, and TβRIII [142]. Where expressed, TβRIII is the most abundant TGF-β receptor and classically functions by binding TGF-β ligand and transferring it to signaling receptors, TβRI and TβRII [143]. In

this manner, recent reports suggest that TβRIII plays an important role in regulating TGF-β signaling [144].

TβRI and TβRII contain serine / threonine protein kinases in their intracellular domains, and TβRI initiates intracellular signaling by phosphorylating the Smad family of proteins (depicted in Figure 1.7). The importance of this pathway in cancer development is underscored by the observation that Smad2 and Smad4 are deleted or mutated in some cancer cell lines and tumors [145-147]. Aside from the Smad-dependent signaling pathway, Smad-independent signaling may also occur through MAPK signaling pathways [148, 149], Rho guanosine triphophatases [150], PI3K / AKT [151], and protein phosphate 2A [152]. The precise molecular mechanism by which TGF-β signals through these alternative pathways has not been established.

Reports indicate that TGF-β markedly attenuates IFN-γ-induced CIITA expression by inhibition of MHC2TA transcription in various cell types, including astroglioma, fibrosarcoma, epithelial, monocyte, synovial, microglial, and B cells [84, 87, 88, 101, 153]. This inhibition occurs through suppression of both CIITA pIII and pIV [101, 153]. Surprisingly however, TGF-β does not affect IFN-γ-induced phosphorylation of JAK1, JAK2 or STAT-1, nor does it interfere with binding of STAT-1, USF-1 or IRF-1 to pIV of the MHC2TA gene [86, 154]. Thus, TGFβ inhibits IFN-γ-induced Class II expression without affecting the individual components of the IFN-γ signaling pathway.

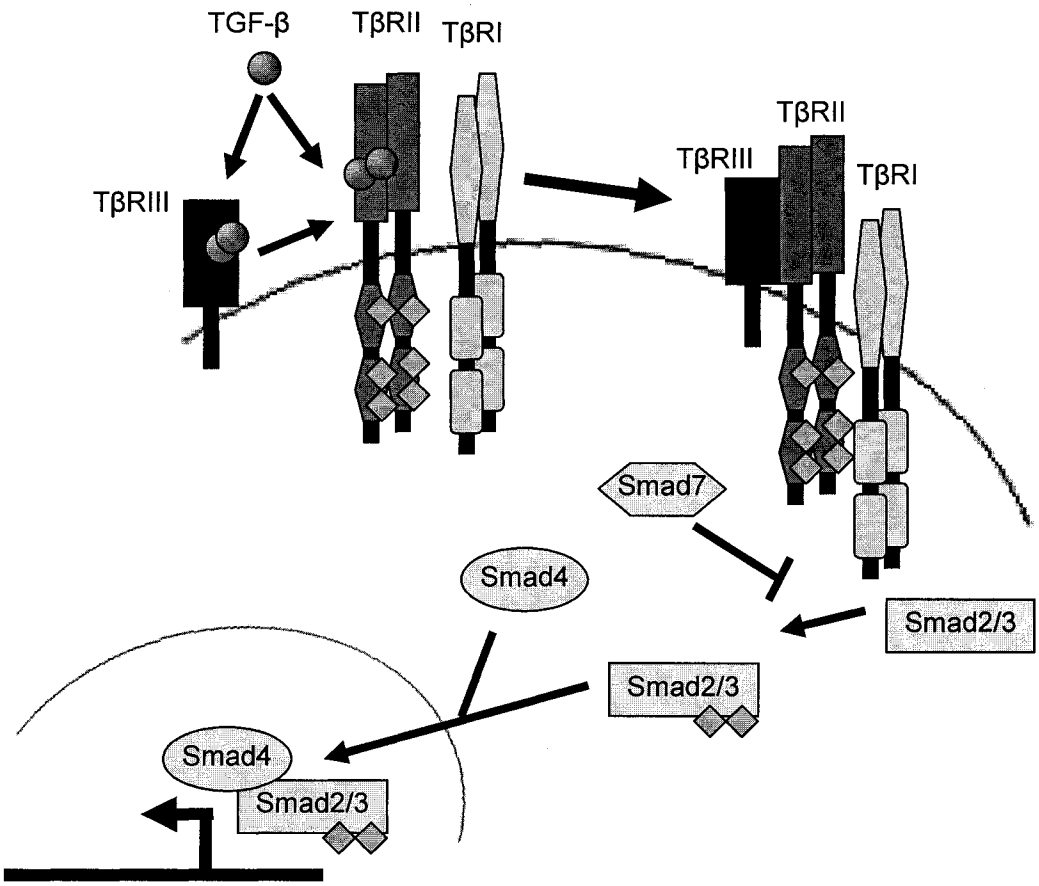


Figure 1.7. Transforming growth factor-β signaling through the SMAD pathway. TGF-β binds TβRII directly or through TβRIII, inducing association of TβRII with TβRI. The TβRII kinase is constitutively active and activates TβRI by phosphorylation of serine and threonine residues [155], which propagates the signal by phosphorylation of Smad2 or Smad3. Phosphorylated Smad2 or Smad3 associate with Smad4, a common partner for all receptor activated Smads, forming a heteromeric complex that translocates to the nucleus and regulates gene transcription in a cell-specific manner [156, 157]. Smad6 and Smad7 are inhibitory Smads that block TGF-β signaling by preventing the activation of Smad2 or Smad3 by TβRI [158]. Adapted from [141] and [159].

1.5. Estrogen and Estrogen Receptors

Approximately 60% of breast cancer patients have hormone-dependent breast cancer, which expresses estrogen receptors and requires estrogen for growth [160]. However, the proportion of patients with hormone-sensitive tumors is higher among postmenopausal women [160]. Studies illustrate that breast cancer development and progression are directly influenced by steroid hormones, particularly estrogen, via its interaction with specific target cell promoters (reviewed by [161]). Studies also indicate that prolonged stimulation of breast ductal epithelium by estrogen contributes to an increase in cell proliferation and an increase in cell survival (reviewed by [162]). Furthermore, the risk of developing breast cancer is enhanced in individuals experiencing early menarche or late menopause as these increase the exposure time to endogenous ovarian hormones [163].

Estradiol, the most biologically active estrogen, is synthesized primarily from androgens by the enzyme aromatase in the ovaries of premenopausal woman [164, 165]. This enzyme is also present in various tissues, including adipose tissue, skin, brain, bone, and placenta. It has been demonstrated that despite low serum levels, certain breast carcinomas display high concentrations of estrogen [166, 167] due to local synthesis and increased expression of intratumoral aromatase [168, 169]. This suggests that the estrogen responsible for breast cancer development may not be circulating estrogen, but rather that produced locally.

Estrogen typically exerts its effects through the estrogen receptor (ER) [170]. Free estrogens passively diffuse across the cell membrane and are preferentially retained in

target cells through formation of a high affinity complex with nuclear ERs. ER variant proteins lacking the nuclear localization signals are also known to occur, causing them to remain in the cytoplasm or cell membrane [171-173].

Two forms of the human ER gene exist, the first identified was ERα [174], and the second was ERβ [175]. ERβ was originally cloned from the rat prostate [176], but has since been identified in mice [177], and in the human thymus, spleen, ovaries, and testis [175]. Low levels are also detected in normal and carcinoma human breast tissue [178, 179]. However, the function and potential role of ERβ in cancer progression is presently unclear.

In the absence of ligand, ER exists as a monomer bound by heat shock proteins (HSPs) [180, 181]. Ligand binding activates the receptor, dissociating the HSPs, causing a conformational change in the receptor, phosphorylation of distinct serine / threonine residues [182], and nuclear translocation. Activated receptors dimerize, interact with a variety of coregulatory molecules forming a receptor complex that acts as a transcription factor by binding to estrogen-responsive elements (ERE) located in the promoter region of target genes. The classical ERE is a palindromic sequence composed of two inverted hexanucleotide repeats [180]. Ligand-bound ERα and/or ERβ may bind to EREs as homo- or heterodimers to alter gene transcription [180, 181] (Figure 1.8A). Coactivators and corepressors may modulate this classical pathway of ER transcriptional activation by interacting with basal transcription machinery to either help unwind or repress the chromatin structure [183-189].

In contrast to ERβ, ERα has been extensively studied. Protein levels of ERα and the ER-regulated progesterone receptor are elevated in premalignant and malignant breast lesions as opposed to normal tissue, and both receptors currently serve as predictive and prognostic factors in the clinical management of breast cancer [190]. Consequently, inhibition of ERα has become one of the major strategies for the prevention and treatment of breast cancer [191, 192]. ERα expression in breast tumor is considered a good prognostic indicator, identifying patients with a lower risk of relapse, better overall survival [193], and more likely to respond to antiestrogen therapy.

The ERα protein is highly homologous between species, and several important domains (A-F) have been identified [194] (Figure 1.8B). The ligand-binding domain resides in the carboxy-terminal (region E) and contains an estrogen-inducible transcription-activating function, known as AF-2 [195, 196]. A second transcription-activating function (AF-1) is localized in the amino-terminal (A/B region) and is believed to be constitutively active in a cell- and promoter-specific manner [197]. Between AF-1and AF-2 is the centrally located DNA-binding domain (DBD) (region C) and hinge region (region D). The DBD interacts with EREs [170, 192]. The flexible hinge region allows rotation of the DBD to accommodate bindings to DNA response elements and interaction with HSPs. The D region also contains a putative nuclear localization sequence [198].

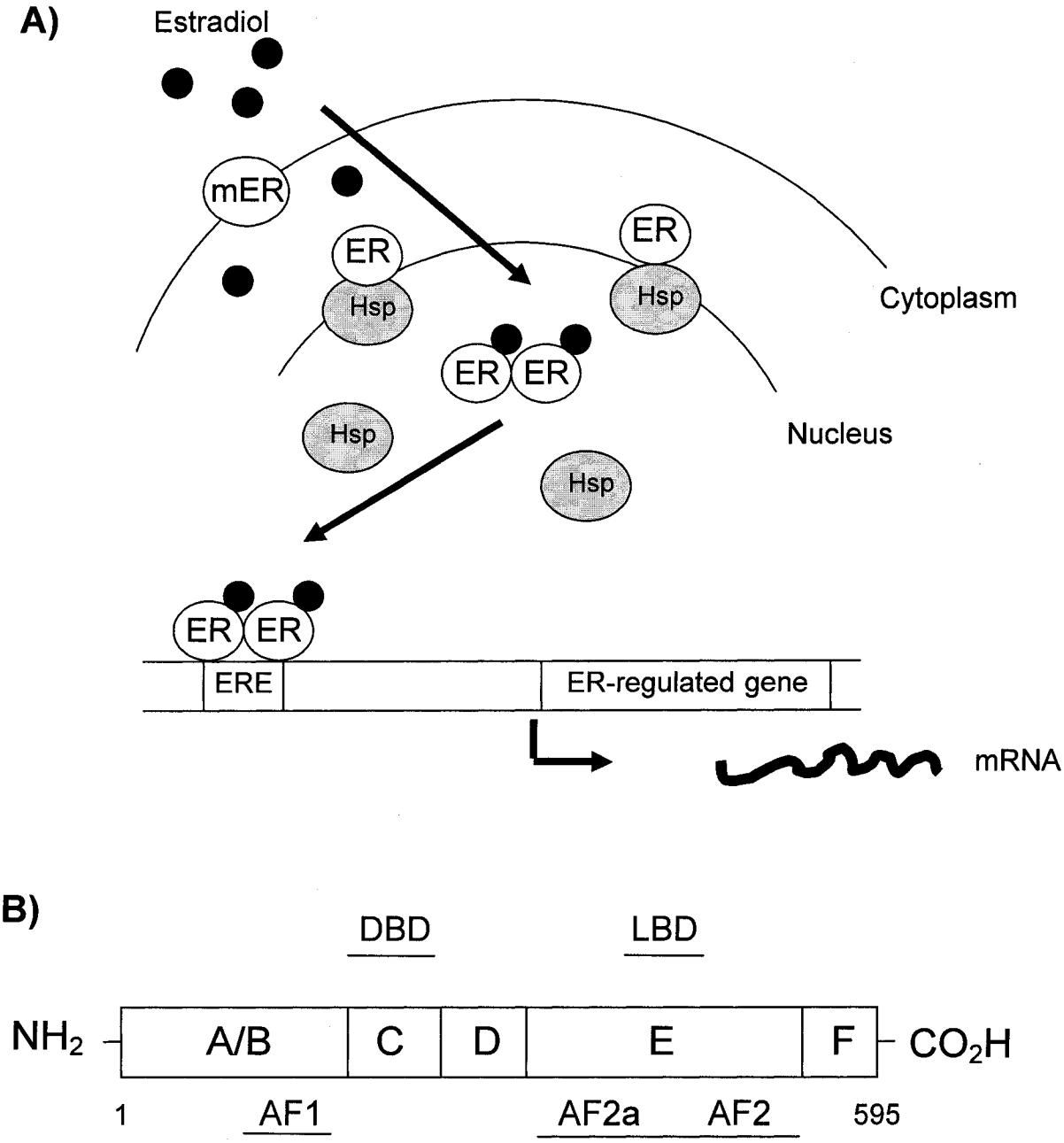


Figure 1.8. Estrogen receptor structure and signaling pathway. A) Estrogen signaling through the estrogen receptor. B) Functional domains of ERα. AF1, AF2, and AF2a function in transcriptional activation, DBD functions in DNA-binding, and LBD functions in ligand-binding. Adapted from [162].

1.5.1. Estrogen and MHC Class II Expression

17β-estradiol has been shown to have immunomodulatory effects on MHC class II expression. In the early 1990s, investigators showed estrogen-mediated down-regulation of MHC class II expression on breast carcinoma epithelial cells and leukocytes [199, 200]. Furthermore, using different animal models it has repeatedly been shown that estrogen treatment of rodents receiving tissue transplants leads to better graft survival, accompanied by a significant reduction or complete abolishment of MHC class II on the allograft vasculature [201-203].

More recently, ovariectomy has been shown to up-regulate IFN-γ-induced CIITA expression on macrophages [204]. ER-deficient mice also demonstrate increased stimulation of $CD4^+$ T cells by splenic macrophages [205], suggesting that estrogen suppresses MHC class II expression. In addition, 17β-estradiol has been shown to down-regulate constitutive and IFN-γ-induced class II expression on astrocytes, fibrosarcoma cells, macrophages, and brain endothelial cells [206]. 17β-estradiol stimulation also results in ERα association with the MHC class II promoter [207]. These inhibitory effects are not mediated by changes in IFN-γ signaling components, or CIITA mRNA, or CIITA protein levels, but by inhibition of chromatin remodeling and recruitment of transcription factors to the class II promoter [206].

Reports indicate that association of CIITA with the class II MHC promoter correlates with H3 and H4 histone acetylation, and that histone acetylation is necessary for MHA class II transcription [56, 208]. 17β-estradiol attenuates H3 and H4 histone acetylation, as well as CBP recruitment to the class II promoter, thereby inhibiting

histone acetylation and ultimately inhibiting the chromatin conformation involved in MHC gene transcription [206].

Through the use of multiple pharmacological inhibitors and blocking of the classical estrogen-signaling pathway, Adamski and Beneveniste [207] demonstrated that estradiol-mediated inhibition of MHC class II occurs through activation of a nonclassical signaling pathway, the c-Jun N-terminal kinase (JNK) pathway. Of further interest is the fact that the JNK signaling pathway is primarily activated by cytokines and cellular stress [209]. Moreover, increasing evidence suggests that other pathways, aside form the JNK pathway, can also phosphorylate and/or activate the ER. These pathways include PI3K / AKT, ERK1/2 (extracellular-signal-regulated kinase) MAPK, p38 MAPK, and SMAD signaling pathways, which may also become activated by cytokines.

1.6. Hypothesis

Previous studies in our laboratory and by others showed that HLA class II expression on synovial fibroblasts and carcinoma cell lines is modulated by treatment with IFN-γ [210-213]. Furthermore, generic HLA class II expression induced by IFN-γ is further up-regulated by cytokines such as IL-4 on human melanomas, renal cell carcinomas, and breast carcinomas [134-136], or down-regulated by cytokines such as TGF-β on astrocytes, monocytes, microglial cells, fibrosarcoma cells, epithelial cells, melanoma cells, and synovial cells [84, 87, 88]. More recently, Sharon A. Oldford in our laboratory showed that HLA-DR allotypic expression on breast carcinoma *in situ* is significantly associated with reduced ERα, reduced PgR, and reduced diagnosis age,

suggesting estrogen may be implicated (S.O., unpublished data). Her study also suggests that allotypic expression is influenced by the *in situ* cytokine milieu, particularly IFN-γ, IL-4 and TGF-β. Based on these studies, we hypothesized that ERα, 17β-estradiol, and cytokines IFN-γ, IL-4 and TGF-β1 differentially modulate allotypic HLA-DR expression on breast carcinoma cell lines.

1.7. Objectives

1) To examine the effects of 17β-estradiol on constitutive and IFN-γ-induced HLA-DR expression on ER+ and ER- breast cancer cell lines.

2) To investigate the individual and combined effects of IFN-γ, IL-4, and TGF-β1 treatments on HLA-DR allelic expression on breast cancer cell lines and correlate these results with ERα classification.

As this study evolved, we found that TGF-β1 treatment mediated differential effects on HLA-DR expression on breast cancer cell lines. Therefore, we extended the study to include a third objective:

3) To examine breast cancer cell lines for differences in TGF-β1 sensitivity and particular components of the Smad signaling pathway to explain cell line specific effects of TGF-β1 on HLA-DR expression.

Chapter 2: Materials and Methods

2.1. Cell cultures

Breast carcinoma cell lines BT-20, BT-474, MCF-7, MDA MB 157, MDA MB-231, MDA MB 468, SKBR3, and T47D (Table 2.1) were generous gifts from Dr. Shou-Ching Tang and Dr. Alan Pater (Memorial University of Newfoundland, St. John's, NL). These cell lines were maintained as adherent cultures in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Burlington, ON) supplemented with 10% heat-inactivated FCS (fetal calf serum) (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin G sodium (Invitrogen), 100 μg/ml streptomycin sulfate (Invitrogen), and 0.25 μg/ml amphotericin B as Fungizone® in 0.85% saline (Invitrogen). Cultures were incubated at 37°C in a 7% CO_2 atmosphere in 25 cm^2 cell culture flasks (Corning Incorporated, Corning NY). The medium was refreshed every 3 days.

Non-adherent B cell lines COX, VAVY, SAVC, MT14B, CB6B, SLE-005, MGAR, and PLH (Table 2.2) were used as positive controls throughout this study. B cells were grown as suspension cultures in 25 cm^2 cell culture flasks and maintained at 3.0 x10^5 cells/ml in IMDM under similar culture conditions as above.

2.2. Estrogen-Depleted Medium

Standard medium components often contain steroidal estrogens within FCS and non-steroidal estrogen-like isomers in the form of the standard pH indicator phenol red [214, 215]. Phenol red has been shown to simulate estrogenic activity through

Table 2.1. Description of human breast carcinoma cell lines used in this study

Breast Cancer Cell Line	ATCC [a] Identification	Type of Cancer [a]	HLA-DR type [b]	ERα status [c]	PgR status [c]
BT-20	HTB-19	Adenocarcinoma	DRβ1*0404, DRβ4, DRβ1*1301, DRβ3*01	-	-
BT-474	HTB-20	Invasive Ductal Carcinoma	DRβ1*0401, DRβ4, DRβ1*15, DRβ5	+	+
MCF-7	HTB-22	Adenocarcinoma	DRβ1*03, DRβ3*0202, DRβ1*15, DRβ5	+	+
MDA MB 157	HTB-24	Medullary Carcinoma	DRβ1*0401, DRβ4, DRβ1*15, DRβ5	-	-
MDA MB 231	HTB-26	Adenocarcinoma	DRβ1*07, DRβ4, DRβ1*13, DRβ3*0202	-	-
MDA MB 468	HTB-132	Adenocarcinoma	DRβ1*0102, DRβ1*07, DRβ4	-	-
SKBR3	HTB-30	Adenocarcinoma	DRβ1*07, DRβ4, DRβ1*1302, DRβ3*0302	-	-
T47D	HTB-133	Ductal Carcinoma	DRβ1*0102	+	+

[a] American Type Culture Collection (Manassas, VA)
[b] Previously determined by A.D. Edgecombe 2002 [210] by DNA typing using commercially available kits.
[c] Lacroix and Leclercq 2004 [216].

Table 2.2. B cell controls used in this study

B Cell Line	IHW [a] Identification	HLA-DR Type
COX	IHW 9022	DRβ1*0301, DRβ3*0101
VAVY	IHW 9023	DRβ1*0301, DRβ3*0101
SAVC	IHW 9034	DRβ1*0401, DRβ4*0101
MT14B	IHW 9098	DRβ1*0404, DRβ4*0101
CB6B	IHW 9060	DRβ1*1301, DRβ3*0202
SLE-005	IHW 9059	DRβ1*13, DRβ3*0301
MGAR	IHW 9014	DRβ1*1501, DRβ5*0101
PLH	IHW 9047	DRβ1*0701, DRβ4*0101

[a] 11th International Histocompatibility Workshop

competitive binding to the estrogen receptor, stimulation of the growth rate of human breast cancer (MCF-7) cells, and elevation of progesterone receptor levels in these cells [217]. For some experiments where it was necessary to exclude exogenous steroids, adherent breast cancer cell lines were cultured in Phenol Red-free IMDM medium supplemented with 10% inactivated charcoal-dextran treated FCS (HyClone, Logan, UT), for a maximum of 5 days before assays. Estrogen-depleted (E_2-depleted) medium was further supplemented with L-glutamine, penicillin G sodium, streptomycin sulfate, and amphotericin B as before. Cell lines could not be maintained in such medium as studies indicate that ER+ breast cancer cells lose hormone responsiveness if maintained for prolonged periods in an estrogen-free environment [218].

2.3. Harvesting of Breast Cancer Cell Lines

Cultures were harvested once 80-100% confluent, as determined by phase contrast microscopy. Harvesting was accomplished by aspiration of medium from culture flasks and incubation with 0.25% trypsin (Invitrogen) diluted in phosphate buffered saline (PBS) for a maximum of 5 minutes at 37°C or until cells were no longer adherent. An equivalent volume of medium was added to quench trypsin activity before cell suspensions were transferred to 15 ml centrifuge tubes. Cells were centrifuged at 290 x g for 7 minutes at 7°C, after which supernatant was decanted, and pellet re-suspended in 5 mls media. Cells were then washed as before, re-suspended in 5 ml medium, and counted using a hemocytometer and phase contrast microscopy. A volume containing 3.0 x 10^5

cells was re-plated into 25 cm^2 cell culture flasks in a final volume of 7 ml medium to maintain cultures. Medium was refreshed every 3 days.

B cells were maintained in standard medium at 3.0 x 10^5 cells/ml. Cells were removed and replaced with fresh medium every 3 days.

2.4. Cytokine and Hormone Treatment of Breast Cancer Cell Lines

To determine the effects of cytokine and hormone treatments on HLA-DR expression, BCCL were treated with either 100 units/ml of human recombinant IFN-γ (BD Pharmingen), or 500 units/ml of human recombinant IL-4 (BD Pharmingen), or 10 ng/ml of human recombinant TGF-β1 (Chemicon International), or 10^{-9}M 17β-estradiol (E-2257, Sigma), or combinations of the above treatments. Twenty-four hours prior to cytokine treatment, cell lines were subcultured as described above in 25 cm^2 tissue culture flasks at 4.0 x 10^5 in 7 ml of medium or in 6 well plates at 1.5 x 10^5 in 2 ml of medium.

At the time of treatment, standard medium was aspirated from cells and replaced with either standard medium or E_2-depleted medium containing the respective cytokine(s) and/or hormone of interest. To identify constitutive gene expression, one flask/well received medium without addition of cytokine or hormone. Cell cultures were incubated for 96 hours (previously determined to be the optimal incubation period by A. Edgecombe [210]), after which HLA-DR expression was assessed by either flow cytometry or CELISA.

2.5. Flow Cytometry

2.5.1. Cell Surface Protein Expression

Flow cytometry was used to detect cell surface expression of HLA-DR antigens. Adherent cell cultures were harvested using 0.25% trypsin as previously described and aliquots of non-adherent B cell lines were used as positive controls (Table 2.4). Cells were washed in medium, re-suspended in an appropriate volume of FACS buffer to give 3.0 x 10^6 cells/ml, and 50 µl of cell suspension was added to each 5 ml polystyrene round-bottom tube (Falcon, Becton Dickson Bioscience). FACS buffer contained 0.2% fetal bovine serum (Invitrogen) and 0.02% sodium azide (BDH Chemicals, Poole, England) in PBS. Twenty-five microlitres of primary mAb (monoclonal antibody), diluted to a predetermined concentration (Table 2.3) in FACS buffer, was also added to each tube and mixture was incubated in the dark for 30 minutes at 4°C.

Following this incubation, cells were washed twice with 2 ml FACS buffer and centrifuged at 453 x g for 5 minutes. After the second wash, supernatants were decanted and tubes were blotted on gauze before 25 µl of secondary antibody, goat anti-mouse immunoglobulin-G labeled with phycoerythrin flurochrome (Jackson ImmunoResearch) diluted 1/40 in FACS buffer, was added to each tube. Following another 30 minute incubation in the dark at 4°C, cells were washed twice in FACS buffer as before, and finally, 150 µl 1% paraformaldehyde in PBS was added to each tube. Cells were stored in the dark at 4°C until analysis was done using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) and CellquestPro Software (Becton-Dickinson).

Table 2.3. Primary antibodies used to detect expression of HLA–DR allelic products by flow cytometry and CELISA.

Antibody	Isotype	Specificity	Concentration		Reference/Source
			Flow	CELISA	
L243 (Supernatant)	Mouse IgG2a	pan HLA-DR	2.4 μg/ml	2.4 μg/ml	Lampson and Levy 1980 [219]
NFLD.D1 (Purified)	Mouse IgG1	pan DR4	39 μg/ml	50 μg/ml	Drover et al. 1994 [220]
NFLD.D7 (Supernatant)	Mouse IgG1	DR4, DR15, DR16, DRβ3	Undiluted	Undiluted	Drover et al. 1994 [220]
NFLD.D10 (Purified)	Mouse IgG1	DR1, DR4 except β1*0402, DR9, DR10, DR14, DR15, DRβ5*0201	2.5 μg/ml	1.0 μg/ml	Drover et al. 1994 [220]
NFLD.M1 (Supernatant)	Mouse IgG1	DR4, DR8, DR52	Undiluted	Undiluted	Drover et al. 1985 [221], Fu et al. 1995 [222]
UK8.1 (Supernatant)	Mouse IgG2b	DR3, DR11, DR13, DR14 except β1*1404	1/200	NT	Bodmer et al. 1985 [223], 11th International Histocompatibility Workshop
TAL8.1	Mouse IgG2b	DR3, DR6, DR11, DR13	50 μg/ml	50 μg/ml	Research Diagnostics Inc. (Flanders, NJ)
7.3.19.1 (Supernatant)	Mouse IgG2b	DR3, DRβ3	1/100	1/25000	Koning et al. 1984 [224], 11th International Histocompatibility Workshop
SFR16.DR7G (Supernatant)	Rat IgG2b	pan DR7	1/25	1/20	Radka et al. 1984 [225], 11th International Histocompatibility Workshop
359-13F10 (Supernatant)	Mouse IgG1	pan DR4	Undiluted	1/2	Radka et al. 1984 [225]

NT: Not Tested.

Table 2.4. Binding [a] of HLA-DR specific monoclonal antibodies to cell surface HLA-DR on B cell lines.

Antibody	B Cell Line							
	COX β1*0301 β3*0101	**VAVY** β1*0301 β3*0101	**SAVC** β1*0401 β4*0101	**MT14B** β1*0404 β4*0101	**CB6B** β1*1301 β3*0202	**SLE-005** β1*13	**MGAR** β1*1501 β5*0101	**PLH** β1*0701 β4*0101
L243 [b] All DR	[c] 5633.1	1090.0	6824.5	3387.2	1480.9	3254.8	3722.1	2245.7
NFLD.D1 DR4	-	-	3026.6	1380.1	-	-	-	-
NFLD.D7 DR4, DR15, DRβ3	[d] +	-	6293.1	-	[d] +	-	2791.0	-
NFLD.D10 DR1, DR4, R15	-	-	6242.5	4489.3	-	-	2661.5	-
NFLD.M1 DR4, DRβ3	[d] +	-	1407.5	[d] +	1588.1	-	187.4	-
UK8.1 DR3, DR13	1570.5	-	-	-	-	-	-	-
TAL8.1 DR3, DR13	-	30.4	-	-	-	27.8	-	-
7.3.19.1 DR3, DRβ3	8527.4	752.2	-	-	1745.5	-	-	-
SFR16.DR7G DR7	-	-	-	-		-	-	1370.7
359-13F10 DR4	-	-	3262.6	2876.6		-	-	-

[a] Antibody binding was determined by flow cytometry. Data are the average of at least two separate experiments.
[b] HLA-DR type recognized by the antibody. Antibodies may recognize more HLA-DR types than listed (see Table 2.3).
[c] Mean fluorescence intensity with the background subtracted.
[d] Bindings previously reported by A. E. Edgecombe [210] through flow cytometry analysis.

2.5.2. Interpretation of Flow Cytometry Results

HLA-DR expression was determined as:

$$\text{Mean Fluorescence Intensity (MFI)}_{\text{Test}} - \text{MFI}_{\text{Negative Control}}$$

or as the degree of HLA-DR expression determined as:

$$\frac{\text{MFI}_{\text{Test}}}{\text{MFI}_{\text{Negative Control}}}$$

Values twice the backgrounds were considered positive. When two or more experiments were conducted, data were averaged and standard deviations were calculated.

2.6. Cell Enzyme Linked Immunosorbant Assay (CELISA)

2.6.1. Cell Surface Protein Expression

In addition to flow cytometry, CELISA was preformed to determine cell surface HLA-DR antigen expression. Adherent cell cultures were harvested using 0.25% trypsin as previously described and non-adherent B cell lines were used as positive controls. Appropriate volumes of cells were removed and centrifuged at 290 x g for 7 minutes at 7°C, after which cells were re-suspended in 2 ml 0.5% bovine serum albumin (BSA) (Sigma Aldrich) in PBS, and centrifuged at 453 x g for 6 minutes at 7°C. Supernatants were decanted, and cells re-suspended in appropriate volume of 0.5% BSA to give 2.5 x 10^6 cells/ml. Ten microliters of cell suspension and 10 µl primary mAb, diluted to a predetermined concentration using 0.5% BSA (Table 2.3), was then added to each well of

polyvinyl chloride treated 96-well U-bottom flexible plates (Becton Dickinson, Franklin Lakes, NJ) before 1 hour incubation at room temperature.

Cells were then washed three times with 100 µl of 0.5% BSA and centrifuged at 387 x g for 6 minutes at 7°C. Following each wash, supernatants were decanted and plates blotted on paper towel. After the third wash, 50 µl of secondary antibody, goat anti-mouse immunoglobulin-G specific for Fcγ chains and labeled with horseradish peroxidase (HRP) (Jackson ImmunoResearch), diluted 1/5000 in 2% BSA, was added to each well and incubated for 1 hour at room temperature. At this time, 75 µl of 1X poly-L-lysine (PLL) (Sigma) in PBS was added to each well of the reading plates, PRO-BIND polystyrene flat bottom plates (Becton Dickinson).

Following this incubation, cells were washed three times with 0.5% BSA as before, and PLL was removed from the reading plates. One hundred microliters of 1X PBS was then added to each well of the flex plates, and cells were transferred from a flex plate to a reading plate. Once transferred, cells were centrifuged at 248 x g for 6 min at 7°C, supernatants decanted, and cells incubated in the dark for 30 minutes at room temperature with 50 µl of substrate. The substrate was prepared using 4.9 ml 0.1M Citric Acid, 5.1 ml 0.2M Phosphate, 10 ml dH_2O, 8 mg orthrophenylenediamine (Sigma), and 8 µl 30% hydrogen peroxide (BDH). The reaction was stopped by adding 50 µl of 2.5 N hydrogen sulphate in dH_2O per well and the plates were read at 490 nm using a 3550 BioRad Microplate Reader (Bio-Rad, Richmond, CA) and Microman software (BioRad).

2.6.2. Interpretation of CELISA results

HLA-DR expression was determined as:

$$\text{Optical Density (OD)}_{\text{Test}} - \text{OD}_{\text{Background}}$$

or as a ratio of:

$$\frac{\text{OD}_{\text{Test}}}{\text{OD}_{\text{background}}}$$

Values less than 2 were considered negative. CELISA assays were performed in triplicate wells, allowing averages and standard deviations to be determined within each assay. When experiments were preformed more than once, data were averaged and standard deviations were determined between experiments. Student two-tailed t-tests were used to determine differences in HLA-DR expression. Differences were considered significant if $p < 0.05$.

2.7. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

2.7.1. RNA extraction

Culture supernatant was aspirated from adherent breast cancer cell lines which had been cultured for 96 hours in 25 cm^2 tissue culture flasks containing either untreated standard media or untreated estrogen-depleted medium, and cells were washed once with 5 ml PBS. PBS was then aspirated and cells detached from culture flasks by addition of 1 ml TRIZOL reagent (Invitrogen). Once detached, cells were transferred to a 1.5 ml Eppendorf tube and incubated for 4 minutes at room temperature to allow cell lysis. Cells

were then centrifuged at 16,000 x g for 10 min at room temperature. Following centrifugation, supernatant was transferred to a fresh 1.5 ml Eppendorf tube, and 0.2 ml chloroform (Sigma) added and shaken vigorously for 30 seconds. Mixtures were then centrifuged at 16,000 x g for 15 minutes at 4°C. After this incubation, the clear aqueous phase containing the RNA was removed and an equivalent volume of isopropanol (Sigma) was added. RNA was precipitated overnight at -20 °C.

RNA was centrifuged at 16,000 x g for 15 minutes at 4°C, after which the supernatant was decanted and the pellet washed with 1 ml 75% ethanol dissolved in DEPC (diethyl procarbonate) water. Following centrifugation at 13,714 x g for 5 minutes at room temperature, the supernatant was removed, the pellet was allowed to air dry for 5 minutes, and RNA was re-suspended in 20-50 µl DEPC water depending on pellet size. RNA was quantified using UV spectrophotometry. A A_{260}/A_{280} ratio of approximately 1.8 was used to estimate RNA purity. The following calculation was used to measure RNA concentration:

$(A_{260} - A_{320})$ x (40 µg/ml) x (reciprocal of dilution) = concentration RNA (µg/ml)

2.7.2. DNase Treatment of RNA

All RNA extracts were subjected to DNase treatment (Ambion, Austin, TX) to remove any DNA contamination. 0.1% volume of DNA-free 10X DNase Buffer and 1 µl of DNA-free DNase was added to RNA samples, mixed gently, and incubated in a 37°C water bath for 30 minutes. After incubation, 0.1% total volume of DNase Inactivation

Reagent was added to the RNA preparation and incubated for 2 minutes at room temperature. Samples were then centrifuged at 13,714 x g for 1 min at room temperature to pellet the DNase Inactivation Reagent. Following centrifugation, supernatants were removed and RNA was transferred to a 1.5 ml Eppendorf tube. To prevent ribonuclease activity, 0.5 µl of RNasine inhibitor (Promega, Madison, WI) was added to RNA samples before storage at -70^{0}C.

2.7.3. cDNA synthesis

cDNA was synthesized from DNase treated RNA samples using a First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech UK limited, Little Chaifont Buckinghamshire, England). 1 µg of RNA was diluted in a total volume of 8 µl DEPC water in 0.2 ml thin walled micro tubes (Gordon Technologies Inc., Mississauga, ON) and incubated at 65°C for 10 minutes to denature the RNA. Following this incubation, samples were quickly placed on ice. PCR reaction mixture consisting of 5 µl Bulk First-Strand Reaction Mix, 1 µl Not I-d $(T)_{18}$ primer diluted 1/25 in DEPC water, and 1 µl of DTT (dithiothreitol) solution were added to each tube of denatured RNA. Samples were then incubated at 37°C for 1 hour, and 70°C for 10 minute in a Biometra T-Gradient (Montreal Biotech Inc., Kirkland, PQ). cDNA was stored at –70°C until needed.

2.7.4. RT-PCR primers

Primers used to analyze mRNA transcription of IFN-γ, IL-4, and TGF-β were currently available in the laboratory, and had been previously synthesized by Invitrogen from sequences acquired from published references (Table 2.5). β-actin primers were kind gifts from Dr. Laura Gillespie and Dr. Gary Paterno (Memorial University of Newfoundland, St. John's, NL). ERα and GAPDH (glyceraldehyde phosphate dehydrogenase) primer sequences were acquired from published references and entered into the *Amplify* program (Engels, 1993) to assess primer affinity to the gene of interest, as well as product size and possible primer dimer formations. ERα and GAPDH primer sequences were also analyzed using *OligoTech Analysis* software (Wilsonville, OR) to determine G+C content and melting temperature. Selection of ERα and GAPDH primers were based on the above analysis and were synthesized by Invitrogen.

2.7.5. PCR Amplification

For each reaction, 1 μl of cDNA or RNA was added to one well of a 96 well 0.2 ml thin walled polypropylene PCR microplate (BioCan Scientific Inc., Gordon Technologies) in combination with a PCR reaction mix consisting of PCR buffer (Invitrogen) containing 200 mM Tris-HCl buffer (pH 8.4), 500 mM KCl, 10 mM dNTP (deoxyribonucleotide triphosphates) (Invitrogen), 50 mM $MgCl_2$ (Invitrogen), Taq DNA polymerase (Invitrogen), 1 μl of both forward and reverse primers, and DNA-free RNA-free water in a final volume of 50 μl. Concentration of primers, and quantity of $MgCl_2$,

Table 2.5. Primers used in this study to detect cytokine and ERα transcription by RT-PCR.

Primer	Sequence (5'-3')	Size	Reference/Source
ERα sense	*GCTGCAAGGCCTTCTTCAA*	550	Jazaeri et al. 1999 [226]
ERα antisense	TCATCAGGATCTCTAGCCAG		
IFN-γ sense	AGTTATATCTTGGCTTTTCA	356	Kotake et al. 1996 [227]
IFN-γ antisense	ACCGAATAATTAGTCAGCTT		
IL-4 sense	CCTCTGTTCTTCCTGCTAGCATGTGCC	373	Kotake et al. 1996 [227]
IL-4 antisense	CCAACGTACTCTGGTTGGCTTCCTTCA		
TGF-β1 sense	GCCCTGGACACCAACTATTGC	161	Marrogi et al. 1997 [228]
TGF-β1 antisense	AGGCTCCAAATGTAGGGGCAGG		
βactin sense	ATCTGGCACCACACCTTCTACAATGAGCTGCG	837	Paterno et al. 1998 [229]
βactin antisense	CGTCATACTCCTGCTTGCTGATCCACATCTGC		
GAPDH sense	TGACCTTGCCCACAGCCTTG	443	Gottwald et al. 2001 [230]
GAPDH antisense	CATCACCATCTTCCAGGAGCG		

Taq polymerase, and DNA-free RNA-free water varied depending of the gene of interest (Table 2.6). RNA samples were run simultaneously to ensure that RNA used in cDNA synthesis did not contain DNA contamination. PCR microplates were covered with domed caps, placed in a Biomed T-Gradient, and cDNA amplified using the respective 35-cycle PCR protocol (Table 2.7).

2.7.6. Electrophoresis of PCR Products

Five microliters of each PCR reaction was mixed with 1 µl of loading buffer and loaded onto a 1.5% agarose gel. Gels were composed of 0.6 g agarose (Invitrogen) dissolved in 40 ml 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. To access product size, gels were also loaded with 3 µl of 100 bp DNA ladder (Invitrogen) mixed with loading buffer. PCR products were electrophoresed at 120V for 20-30 minutes in a Mini Sub DNA Cell electrophoresis chamber (Bio-Rad) containing 0.5X TBE buffer. Once separated, PCR products were visualized and photographed under UV light using Eagle Eye II Still Video system (Stratagene, La Jolla, CA), or ChemiGenius bio-imaging system (SYNGENE, Frederick, MD).

Table 2.6. PCR reaction mixtures used in cDNA amplification.

PCR mix	β-actin (20 pM) (μl)	GAPDH (20 pM) (μl)	IL-2 (10 pM) (μl)	IL-4 (10 pM) (μl)	TGFβ (10 pM) (μl)	IFN-γ (10 pM) (μl)	ERα (20 pM) (μl)
10X Buffer	5	5	5	5	5	5	5
DNTP	1	1	1	1	1	1	1
$MgCl_2$	1.5	2	2	1.5	2	2	2
Primer +	1	1	1	1	1	1	1
Primer -	1	1	1	1	1	1	1
Taq DNA polymerase	0.2	0.2	0.25	0.25	0.25	0.25	0.25
H_2O	39.3	38.8	39	39.3	39	39	38.75

Table 2.7. PCR cycling conditions used to amplify gene transcription

Primer	PCR conditions
IFN-γ	94°C for 1 min, 55°C for 1 min, 72°C for 1 min for 35 cycles.
IL-4	94°C for 1 min, 65°C for 1 min, 72°C for 1 min for 35 cycles. Final extension at 72°C for 5 min.
TGF-β1	94°C for 1 min, 65°C for 1 min, 72°C for 1 min for 35 cycles. Final extension at 72°C for 5 min
ERα	95°C for 2 min, 95°C for 2 min, 51°C for 45 sec, 72°C for 45 sec for 35 cycles. Final extension at 72°C for 10 min

2.7.7. Interpretation of RT-PCR Results

The density of gene transcription was normalized to GAPDH according to the following calculation:

$$\text{Value} = \frac{\text{Test}_{(\text{cytokine or ER}\alpha)} - \text{Water}_{(\text{cytokine or ER}\alpha)}}{\text{Test}_{(\text{GAPDH})} - \text{Water}_{(\text{GAPDH})}} \times 100\%$$

2.8. Western Blots

2.8.1. Preparation of Cell Lysates

Western blots were used to assess breast cancer cell lines responsive to TGF-β1 stimulation. Adherent breast cancer cell lines were harvested using trypsin, plated in E_2-depleted medium at a density of 1.5 x 10^5 cells/well in 6 well plates and placed at 37°C in a 7% CO_2 incubator overnight. Medium was then replaced with fresh E_2-depleted medium and cells were incubated for 96 hours. Following this incubation, ligand stimulations were performed by addition of 5 ng/ml TGF-β1 (Chemicon) for 1 hour. Control wells were left untreated. Cells were placed on ice and washed twice with 2 ml cold PBS. After complete removal of PBS, cells were incubated on ice for 2 minutes with 75 µl cold Ripa lysis buffer (0.5 ml 10X PBS, 0.5 ml Triton X 100, 0.5 ml 10X Roche complete EDTA-free (ethylenediaminetetraacetic acid) protease inhibitor cocktail (Roche, Mississauga, ON), 250 µl 1M β-glycerolphosphate, 250 µl 1M Tris pH 7.4, 250 µl 10% deoxycholate, 50 µl 0.5M EDTA pH 7.5, 25 µl 20% SDS (sodium dodecyl sulfate), 50 µl sodium-ortha-vanadate, 7.5 µl PMSF (phenylmethylsulfanylfluoride), and

distilled water to a total volume of 5 ml). Cell lysates were scraped from wells using a micropoliceman and Ripa/lysis suspension was transferred to a 1.5 ml Eppendorf tube. Cell debris was removed by centrifugation at 21,890 x g for 10 minutes at 4°C. Supernatants containing isolated protein were transferred to 1.5 ml Eppendorf tubes.

2.8.2. Quantification of Protein in Cell Lysates

Protein within cell lysates was quantified by comparison with BSA standards. Five microliters of each sample and known amounts of BSA: 0 μg, 2.5 μg, 5.0 μg, 10 μg, and 20 μg, were loaded in duplicate into a flat bottom 96-well plate. 5 μl of lysis buffer was added to each well containing BSA. After addition of 300 μl/well BCA protein assay reagent (50:1, reagent A : reagent B) (Pierce, Rockford, IL), plates were incubated for 30 minutes at 37°C. Absorbance at 562 nm was measured using a Polorstar Optima Plate reader (BMG, Durham, NC) and protein concentration was assessed using FLUOstar OPTIMA version 1.30 software (BMG).

2.8.3. Electrophoresis of Cell Lysates

Cell lysates were separated by SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis). An appropriate volume of lysate to give 30 μg of protein was removed from each sample and denatured in 100°C dry heat for 3 minutes. Following centrifugation at 16,060 x g for 1 minute to further remove cellular debris, samples were mixed with 10 μl loading buffer and loaded onto 8.5 % SDS-PAGE gels. The loading buffer consisted of 5X sample buffer, 5% SDS, 10% glycerol, 0.5X stacking

gel buffer, β-mercapoethanol, and bromophenyl blue to color. To access protein product size, gels were simultaneously loaded with 10 µl BenchMark™ Pre-Stained Protein Ladder (Invitrogen).

The SDS acrylamide gel was divided into two components: stacking gel and 8.5% running gel. The stacking gel consisted of 1.5 ml acrylamide, 1.15 ml stacking buffer (Tris HCl buffer) pH 6.8, 0.05 ml 20% SDS, 7 ml H_2O, 5 µl TEMED, and 0.3 ml 2.8% APS (ammonium persulfate). Running gel consisted of 5.66 ml acrylamide, 5 ml running buffer (1.5M Tris HCL buffer) pH 8.9, 0.1 ml 20% SDS, 8.6 ml H_2O 20 µl TEMED, and 0.66 ml 2.8% APS. Cell lysates were electrophoresed in Mini-PROTEIN® Cell electrophoresis chambers (Bio-Rad) containing running buffer (1.5 M Tris HCl buffer) pH 8.9 at 15 mA constant current until sample separations were below 5.5 cm from gel bottom, then current was increased to 17 mA until separation was completed.

2.8.4. Transferring Protein Products

Nitrocellulose membranes were activated by soaking in 100% methanol for 10 seconds, in distilled water for 10 minutes, and in transfer buffer until use. Gels were removed from electrophoresis apparatus and placed in transfer buffer (20% methanol) for 10 minutes. The transfer cassette was assembled as follows: plastic base, brillo pad (pre-soaked in transfer buffer), two 3M filter papers (pre-soaked in transfer buffer), nitrocellulose membrane, gel(s), two 3M filter paper (pre-soaked in transfer buffer), brillo pad (pre-soaked in transfer buffer), and plastic top. The transfer cassette was placed in a

Bio-Rad Trans-blot cell (Bio-Rad), which itself was immersed in a circulating 6°C water bath. Transfer was performed at 100 V for 60 minutes.

2.8.5. Immunodetection of Proteins

Following transfer, sticky surfaces of membranes were blocked by 45 minute incubation in Blotto Solution (20 ml 1.0M Tris HCl buffer pH 7.6, 56ml 5M NaCl, 20 ml 10% Tween 20, 5% non-fat dry evaporated milk). Membranes were then incubated overnight at 4 °C while rocking with a predetermined concentration of primary Ab (Table 2.8) diluted in Blotto solution containing 1 µl per ml 20% sodium azide solution. To remove unbound primary antibodies, membranes were washed 5 times in a TBST bath for 5 minutes while rocking. TBST solution was the same as Blotto solution with the exemption of dry milk. Next, membranes were incubated with an appropriate secondary Ab labeled with HRP (Table 2.9) diluted to a predetermined concentration in Blotto Solution without sodium azide. Membranes were then washed with TBST, once for 5 minutes, then 4 times for 10 minutes. Finally, the location of bound Ab was revealed by incubating membranes in SuperSignal® West Pico Chemiluminescent Substrate (Pierce) (1:1 Peroxide solution: Enhancer solution) for 5 minutes while rocking.

2.8.6. Interpretation of Western Blot Results

Proteins immobilized onto membranes were detected using a traditional ECL Western blotting system where a HRP-conjugated secondary Ab in conjunction with a chemiluminescent substrate, luminal, generates a signal that can be captured on film.

Table 2.8. Primary antibodies used in the detection of proteins by Western blotting.

Antibody	Isotype	Conc.	Reference/Source
anti-P-Smad3 (anti-serum)	Rabbit IgG	1/2000	Murphy et al. 2004 [231]
anti-Smad3 (purified)	Rabbit IgG	1 μg/ml	Zymed Laboratories Inc. (Cat# 51-500)
anti-P-Smad2 (Ser 465/467)	Rabbit IgG	1/1000	Cell Signaling Technology (Cat# 3101)
anti-Smad2	Mouse IgG1	1/500	Transduction Laboratories (Cat# 610842)
anti-αTublin (Clone DM1A) (ascites fluid)	Mouse IgG1	1/5000	Sigma-Aldrich (T-9026)

Table 2.9. Secondary antibodies used in the detection of proteins by Western blotting.

Antibody	Concentration	Reference/Source
Goat anti-Rabbit HRP-Linked (H&L)	1/15,000	Cell Signaling Technology (Cat# 7074)
Goat anti-Mouse IgG-HRP	1/10,000	Santa Cruz Biotechnology (sc-2055)

After quick exposure of membranes to AGAF X-ray film in a dark room, X-rays were developed using a Konica SRX-101A developer. Protein expression was determined on the basis of presence or absence of appropriate band size.

2.8.7. Reprobing Membranes

Membranes to be reprobed were stripped of antibody by immersion in a stripping solution bath for 30 minutes at 55°C while rocking. Stripping solution contained 42.5 ml distilled water, 5 ml 20% SDS, 2.1 ml 1.5M Tris stripping buffer pH 6.8, and 405 µl β-mercaptoethanol. Once stripped, membranes were washed twice for 5 minutes in a TBST bath while rocking, and sticky surfaces were blocked by 1 hour incubation in Blotto Solution. Membranes were then incubated overnight with primary Ab (Table 2.8) at 4 °C as before (Section 2.8.5).

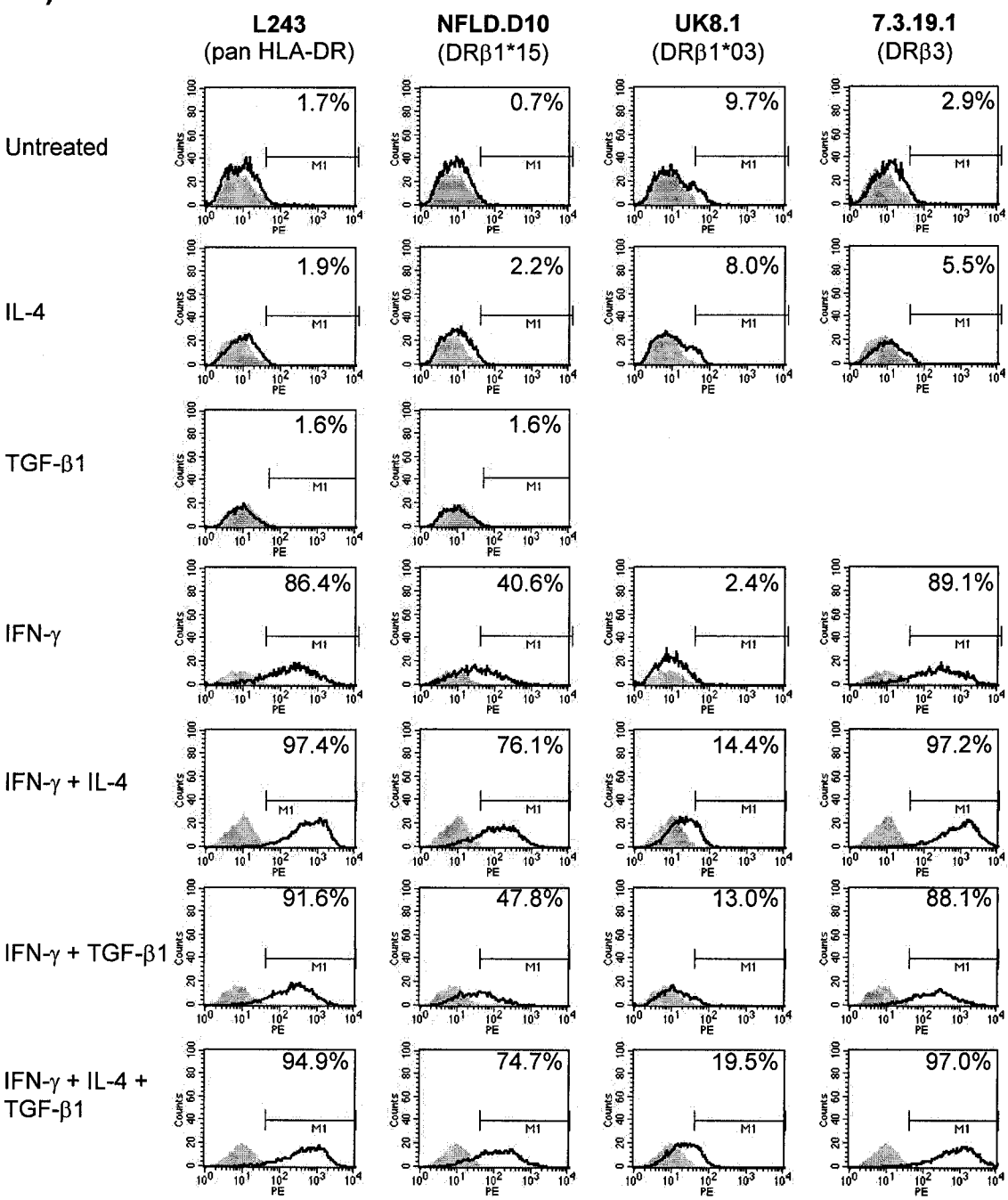
Chapter 3: Results

3.1. Preliminary Experiments to Assess the Effects of Different Cytokines on HLA-DR Expression.

The purpose of this set of experiments was to evaluate whether IL-4 and/or TGF-β1 individually or in combination with IFN-γ modulated HLA-DRβ allelic expression on an ER+ (MCF-7) and an ER- (BT-20) breast cancer cell line (BCCL). Cells were cultured and treated with cytokine combinations in standard E_2-containing media. Resulting HLA-DR expression was measured using antibodies L243 (pan HLA-DR), NFLD.D10 (DRβ1*15), UK8.1 (DRβ1*03, DRβ1*13), 7.3.19.1 (DRβ3), NFLD.D1 (pan DRβ1*04), 359-13F10 (pan DRβ1*04) and flow cytometry following 96 hour incubation with cytokines.

The results, depicted in Figures 3.1, revealed no constitutive HLA-DR (L243) or allelic expression (NFLD.D10, UK8.1, 7.3.19.1) on MCF-7. Expression was not modulated by treatment with IL-4 or TGF-β1, but IFN-γ strongly up-regulated HLA-DR, DRβ1*15, and DRβ3. DRβ1*03 was not up-regulated by IFN-γ alone, as indicated by poor UK8.1 binding (2.4% positive cells) (Figure 3.1A) and fluorescence intensity (Figure 3.1B). IL-4 augmented IFN-γ-induced overall and allelic HLA-DR expression, while TGF-β1 had no modulatory effect. IFN-γ + IL-4 and IFN-γ + TGF-β1 did slightly increase the percentage of cells bound by UK8.1, however the fluorescent intensity of these cells was negligible. The combination of all three cytokines induced similar generic and allelic expression as IFNγ + IL-4.

A)


L243
(pan HLA-DR)
NFLD.D10
(DRβ1*15)
UK8.1
(DRβ1*03)
7.3.19.1
(DRβ3)
Untreated
1.7%
0.7%
9.7%
2.9%
IL-4
1.9%
2.2%
8.0%
5.5%
TGF-β1
1.6%
1.6%
IFN-γ
86.4%
40.6%
2.4%
89.1%
IFN-γ + IL-4
97.4%
76.1%
14.4%
97.2%
IFN-γ + TGF-β1
91.6%
47.8%
13.0%
88.1%
IFN-γ + IL-4 + TGF-β1
94.9%
74.7%
19.5%
97.0%
Counts
PE
M1

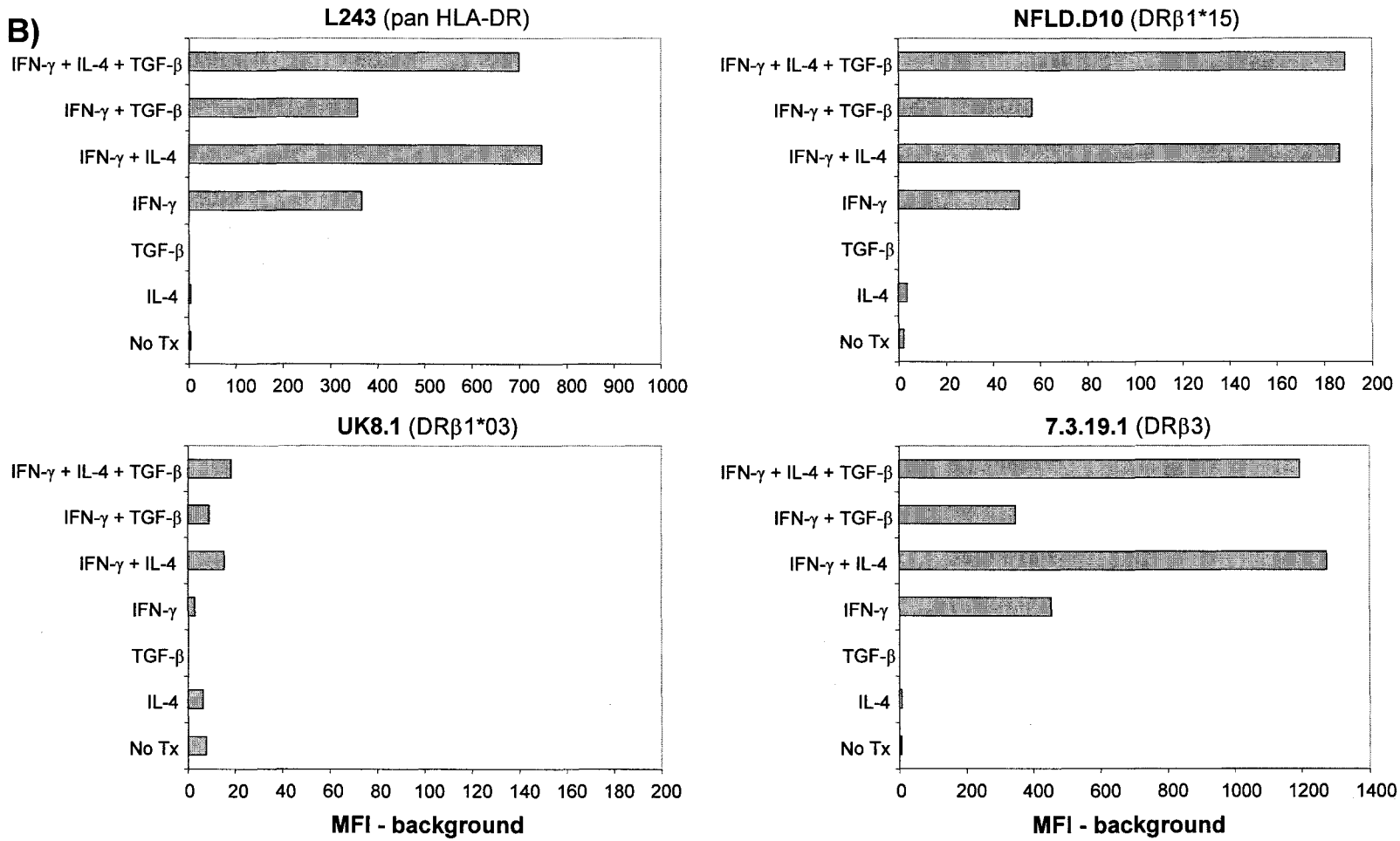
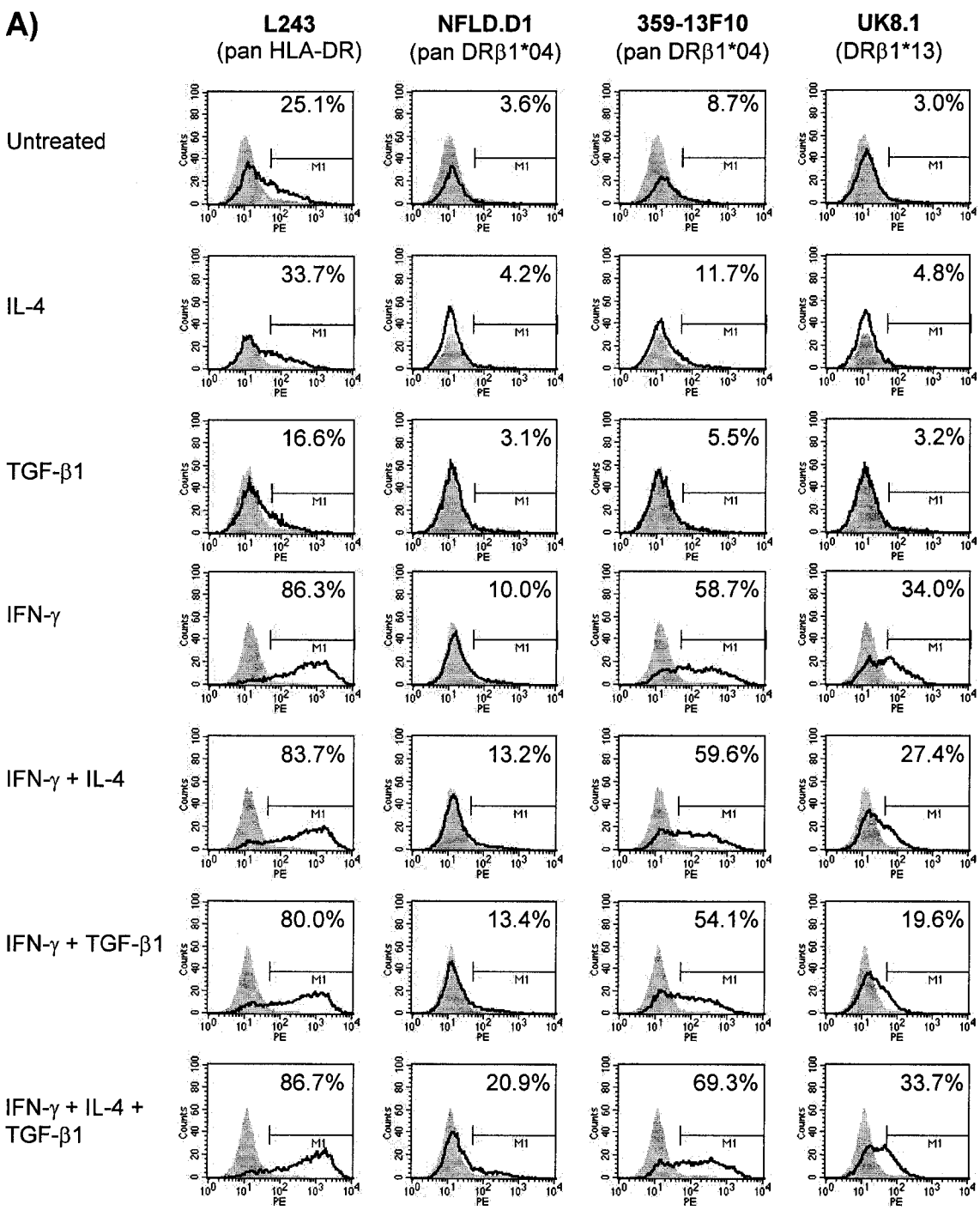


Figure 3.1. Preliminary data determining the effects of cytokine treatment on HLA-DR expression on MCF-7. Cells were cultured in standard E_2-containing media and HLA-DR expression was assessed using the antibodies: L243 (pan HLA-DR); NFLD.D10 (DRβ1*15); UK8.1 (DRβ1*03); 7.3.19.1 (DRβ3) and flow cytometry. Data are reported as: (A) histogram overlays where filled histograms = IgG isotype control; open histogram = test mAb; percentages indicate the percent of positive cells, or (B) $MFI_{Test} - MFI_{Background}$.

Overall, the results for MCF-7 suggested that modulation of allelic HLA-DR expression in response to individual and combined cytokine treatments displayed similar trends to that of generic HLA-DR. However, failure to up-regulate DRβ1*03 suggested this allele was poorly expressed, or alternatively, that allotypic expression on MCF-7 was selectively-up-regulated in response to cytokine treatments.

The results for BT-20 differed from MCF-7 as it displayed low constitutive HLA-DR (L243) and DRβ1*04 (359-13F10) expression. There was a notable difference in the amount of DRβ1*04 detected by NFLD.D1 and 359-13F10 despite equivalent recognition of DRβ1*04 on B cell line control SAVC (Table 2.4). As these antibodies bind to different domains of HLA-DRβ1*04 (Sheila Drover, personal communication), this suggested that the 359-13F10 epitope was more accessible than the NFLD.D1 epitope on BT-20 and was thus more representative of DRβ1*04 expression on BT-20.

Constitutive HLA-DR and DRβ1*04 expression was rather unaffected by IL-4, despite the slight increase in percentage of positive cells (Figure 3.2A). On the other hand, TGF-β suppressed this constitutive expression, as indicated by a small decrease in the percentage of positive cells (Figure 3.2A) and fluorescence intensity (Figure 3.2B). IFN-γ strongly up-regulated HLA-DR and DRβ1*04, but only moderately up-regulated DRβ1*13 (UK8.1). Addition of IL-4 or TGF-β1 decreased IFN-γ induced pan- and allelic HLA-DR expression. However, treatment with IFN-γ + IL-4 + TGF-β1 had no modulatory effect on generic HLA-DR and DRβ1*04 expression compared to observations with IFN-γ-treatment alone, but all three cytokines together slightly


A)
L243
(pan HLA-DR)
NFLD.D1
(pan DRβ1*04)
359-13F10
(pan DRβ1*04)
UK8.1
(DRβ1*13)
Untreated
25.1%
3.6%
8.7%
3.0%
IL-4
33.7%
4.2%
11.7%
4.8%
TGF-β1
16.6%
3.1%
5.5%
3.2%
IFN-γ
86.3%
10.0%
58.7%
34.0%
IFN-γ + IL-4
83.7%
13.2%
59.6%
27.4%
IFN-γ + TGF-β1
80.0%
13.4%
54.1%
19.6%
IFN-γ + IL-4 +
TGF-β1
86.7%
20.9%
69.3%
33.7%
M1
Counts
PE

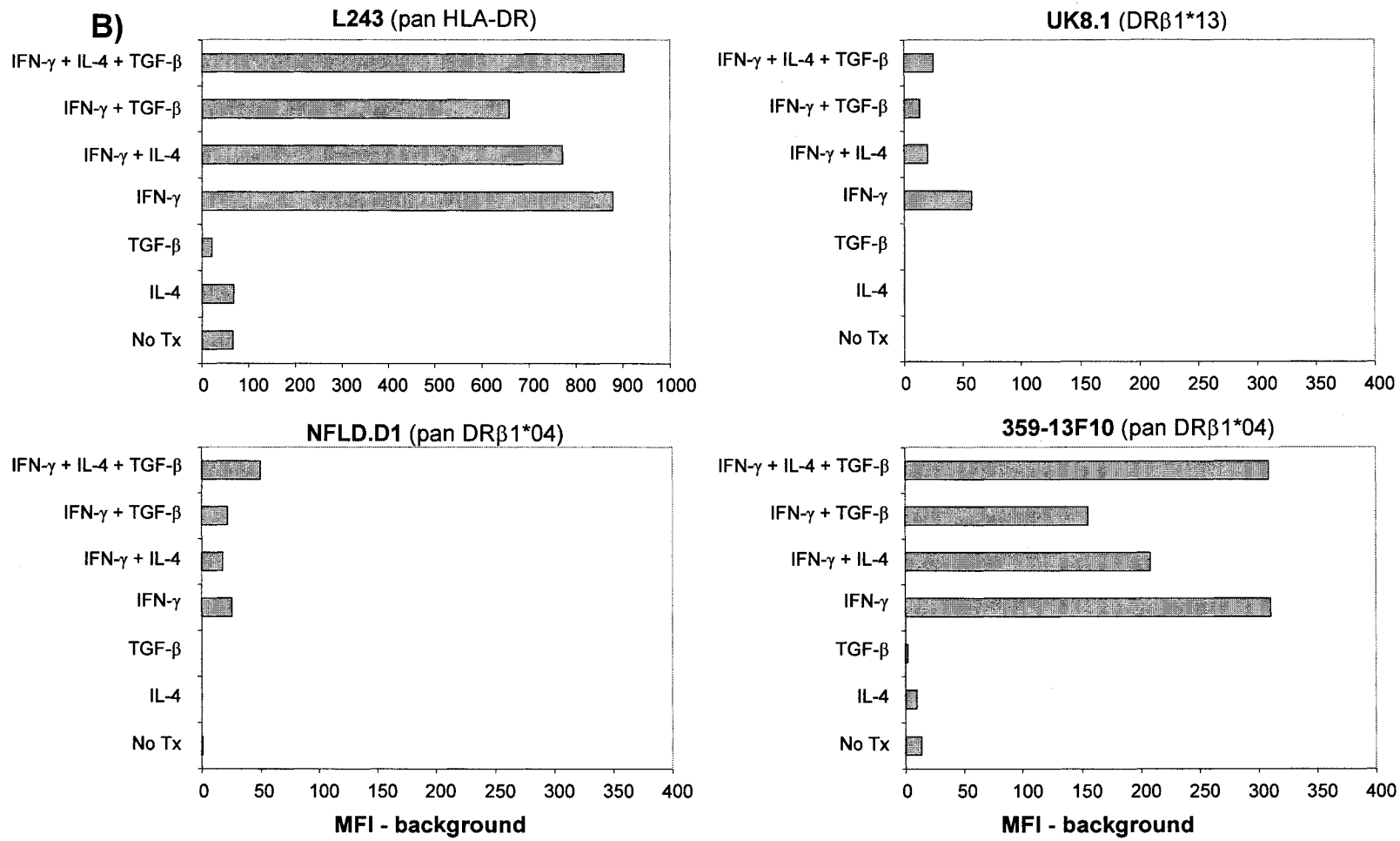


Figure 3.2. Preliminary data illustrating effects of cytokine combinations on HLA-DR allelic expression of BT-20. HLA-DR expression was assessed using the antibodies: L243 (panHLA-DR); NFLD.D1 (pan DRβ1*04); 359-13F10 (pan DRβ1*04); UK8.1 (DRβ1*13) and flow cytometry. Data are reported as: (A) histogram overlays where filled histograms = IgG isotype control; open histogram = test mAb; percentages indicate the percent of positive cells, or (B) $MFI_{Test} - MFI_{Background}$.

suppressed DRβ1*13 expression.

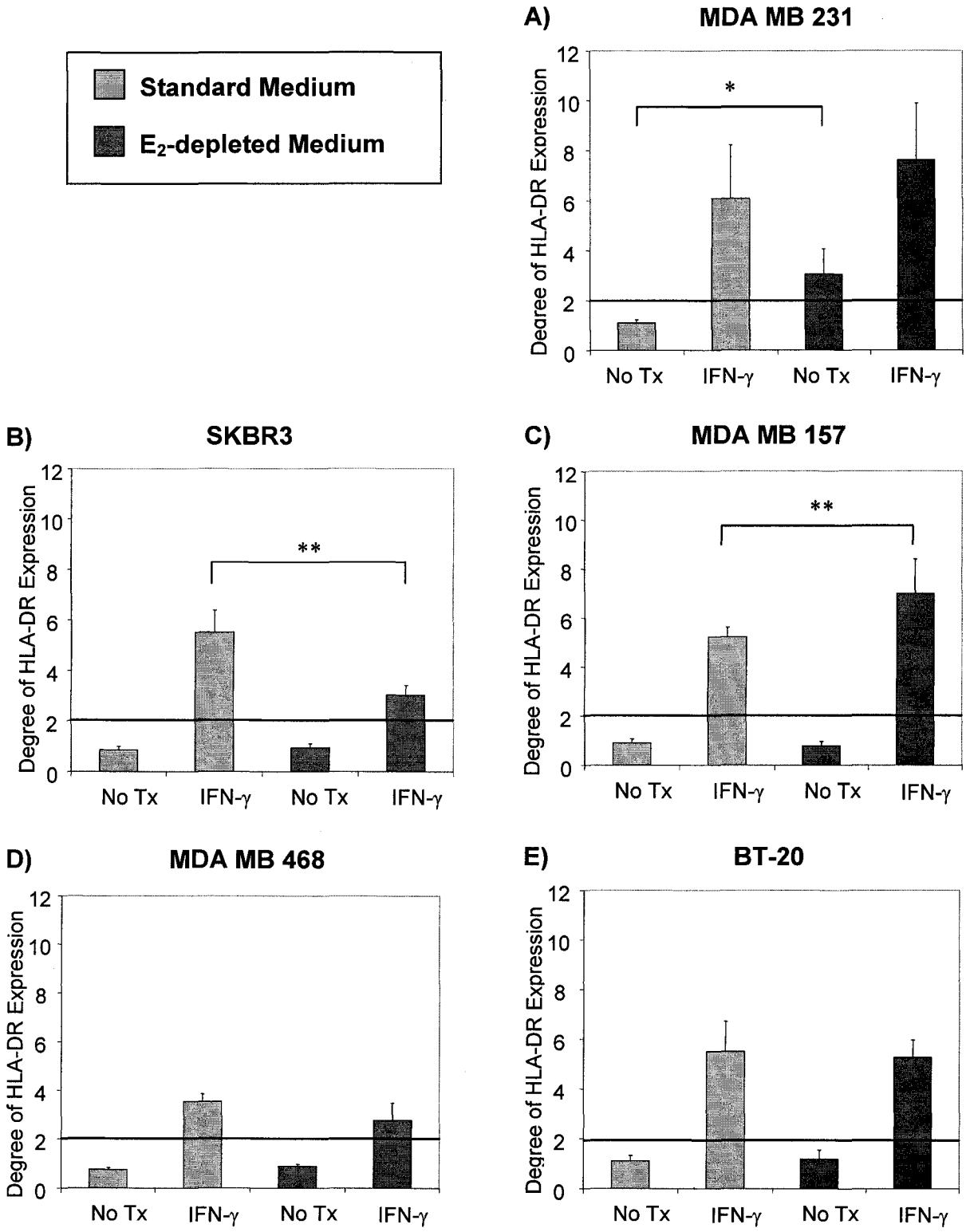
Taken together with the results for MCF-7, moderate up-regulation of DRβ1*13 on BT-20 in response to IFN-γ and selective suppressive of this allele by IFN-γ + IL-4 + TGF-β1 suggested that BCCL selectively modulated allelic expression in response to cytokine treatments. The results also suggested that ER+ and ER-BCCL may respond differently to individual and combined cytokine treatments, particularly as IFN-γ + TGF-β did not modulate HLA-DR expression on MCF-7 but suppressed expression on BT-20. Alternatively, the results may simply reflect differential responses to the various cytokine treatments by different HLA-DR alleles, which were not common to either cell line. Furthermore, other factors such as estrogen or estrogen-like components in medium and production of endogenous cytokines by BCCL may also influence HLA-DR expression. Therefore, we decided to compare both constitutive and IFN-γ induced HLA-DR expression on a panel of ER+ and ER- BCCL cultured in E_2-containing and E_2-depleted medium to determine the effects of exogenous estrogen and estrogen-like components on HLA-DR expression.

3.2. Establishing Optimal Culture Conditions to Investigate ERα and HLA-DR Expression on Breast Cancer Cell Lines

Media components may contain steroidal estrogens within FCS and non-steroidal estrogens which have hormonal activity. The popular pH indicator phenol red, present in a variety of culture medium, displays structural resemblance to some non-steroidal estrogens, allowing binding to ERα and stimulation of estrogenic activity, as indicated by induced proliferation and progesterone receptor expression [217, 232]. To determine whether such endogenous estrogens affect constitutive and IFN-γ-induced HLA-DR expression on BCCL, a panel of ER+ and ER- cells were cultured for 96 hours in both standard (E_2-containing) medium and E_2-depleted medium. HLA-DR expression was compared using L243 and CELISA.

As shown in Figure 3.3 (all panels), no BCCL cultured in standard medium displayed constitutive HLA-DR, but all except BT-474 up-regulated HLA-DR in response to IFN-γ. Following culture in E_2-depleted medium, only MDA MB 231 constitutively expressed HLA-DR (Figure 3.3A), while IFN-γ induced HLA-DR on all BCCL except BT-474. The reason why BT-474 does not up-regulate HLA-DR is presently unclear, but confirms previous findings in our laboratory [210]. BT-474 was shown to contain DRA and DRB mRNA following IFNγ treatment [210], therefore this problem does not appear to be at the transcriptional level and likely involves post-transcriptional modification.

Additionally, student two-tailed T-tests were used to analyze differences in constitutive and IFN-γ-induced HLA-DR expression between culture conditions.


Standard Medium
E2-depleted Medium
A)
MDA MB 231
Degree of HLA-DR Expression
*
No Tx
IFN-γ
No Tx
IFN-γ
B)
SKBR3
**
C)
MDA MB 157
**
D)
MDA MB 468
E)
BT-20

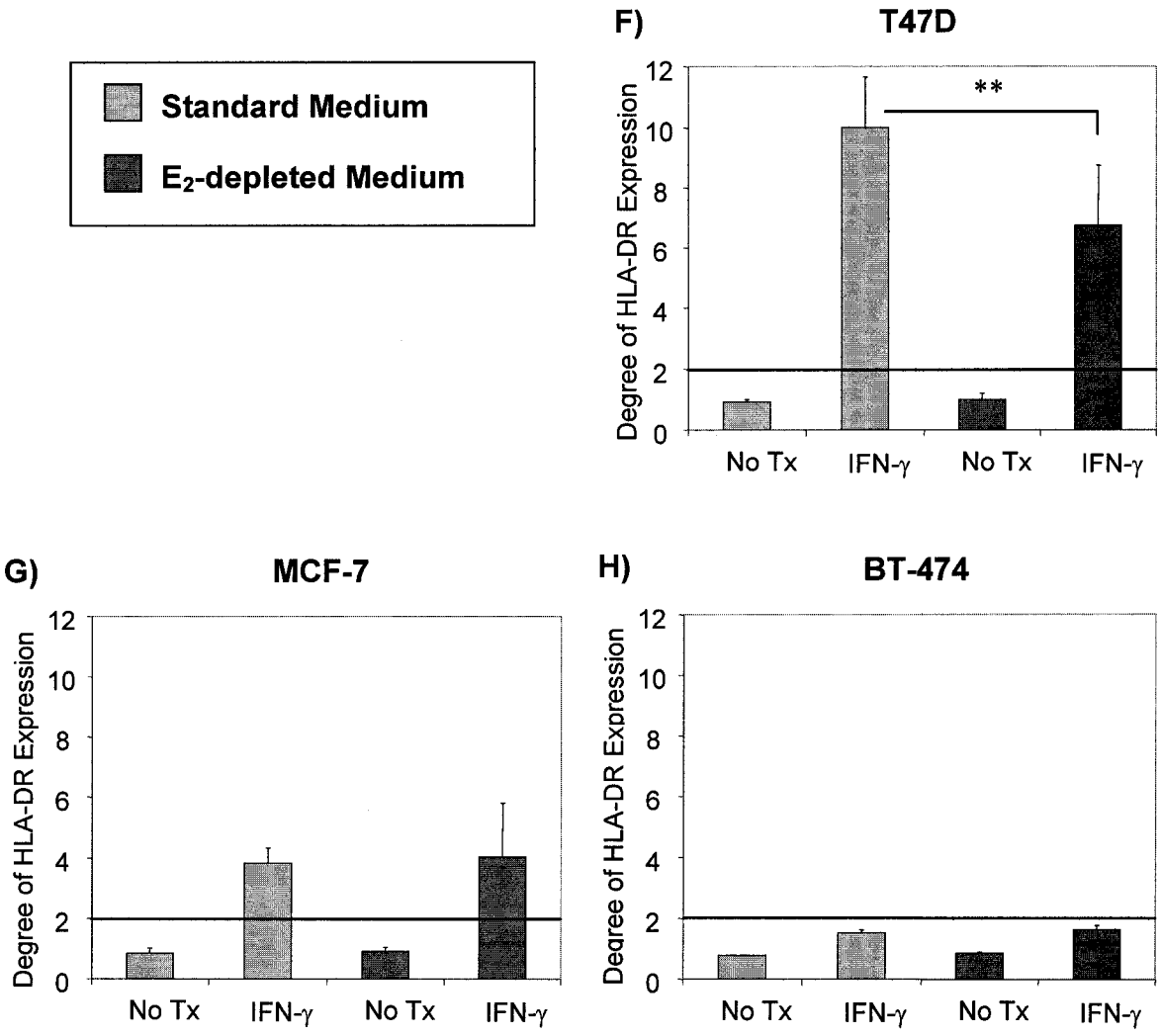


Figure 3.3. Effects of medium components on HLA-DR expression on breast cancer cell lines. Non-induced and IFN-γ-induced HLA-DR expression was assessed using the antibody L243 by CELISA after 96 hour culture in standard medium (light gray bars) or E_2-depleted medium (dark gray bars). Results are reported as the average and standard deviations of 2-3 experiments. Values greater than 2 are considered positive. $^{*}p < 0.05$, $^{**}p < 0.01$.

Constitutive HLA-DR expression on MDA MB 231 was higher in E_2-depleted medium than standard medium ($p = 0.017$) (Figure 3.3A). Differences in IFN-γ-induced expression were also found for SKBR3, MDA MB 157 and T47D. IFN-γ-induced HLA-DR expression was higher in standard medium on SKBR3 ($p < 0.01$) (Figures 3.3B) and T47D ($p < 0.01$) (Figure 3.3F), but higher in E_2-depleted media for MDA MB 157 ($p < 0.01$) (Figure 3.3C). No significant differences were observed for MDA MB 468, BT-20, MCF-7, or BT-474 (Figures 3.3D, 3.3E, 3.3G, and 3.3H, respectively).

Taken together, these results showed that medium components can affect constitutive and inducible generic HLA-DR expression on some BCCL. We have highlighted differences in terms of estrogen content as this suggested that exogenous estradiol treatment may affect HLA-DR expression in a cell line specific manner. That being said, we realize other factors such as insulin, progesterone, and testosterone may too differ between the two media types and also affect HLA-DR expression.

As the primary objective of this project was to assess the combined effects of 17β-estradiol and cytokines on allelic HLA-DR expression, we decided to perform all future experiments in E_2-depleted medium. By using E_2-depleted medium, we minimize the variables of phenol red and high endogenous estradiol, thus allowing better assessment of the effects exogenous estradiol treatment may have on HLA-DR expression.

3.3. Estrogen Receptor-alpha Status of BCCL

The effects of estradiol are primarily mediated through ERα and ERβ [233]. ERα status of each BCCL has been documented by the American Type Culture Collection (ATCC) and reported in various studies [216, 234, 235]. However, as cell lines may differ between laboratories and long term culture may change expression of various genes in cell lines, we decided to confirm the ERα status of each BCCL. Since expression of ERα also varies with amounts of estradiol components in medium [236, 237], we also compared the ERα status of cells cultured in standard medium to that in E2-depleted medium. To do this, we analyzed mRNA transcripts using an ERα exon 2 sequence specific primer and RT-PCR as described in Section 2.7.

As shown in Figure 3.4, ERα mRNA was constitutively transcribed in BT-474, T47D, MCF-7, BT-20 (weak), and MDA MB 157 (weak). No detectable ERα mRNA was present in MDA MB 231, MDA MB 468, or SKBR3. However, differences in ERα transcription were observed for cells grown in the different media. ERα transcripts in T47D and MCF-7 were more prominent in cells cultured in standard medium than E_2-depleted medium (Figure 3.4A). We also found weak amplification of ERα in MDA MB 157 cultured in both media (Figure 3.4B), which is in contrast to ATCC reports. As shown in Table 3.1 where results are also presented as a percentage of GAPDH expression, apart from MDA MB 157 our results agree with those published by the ATCC.

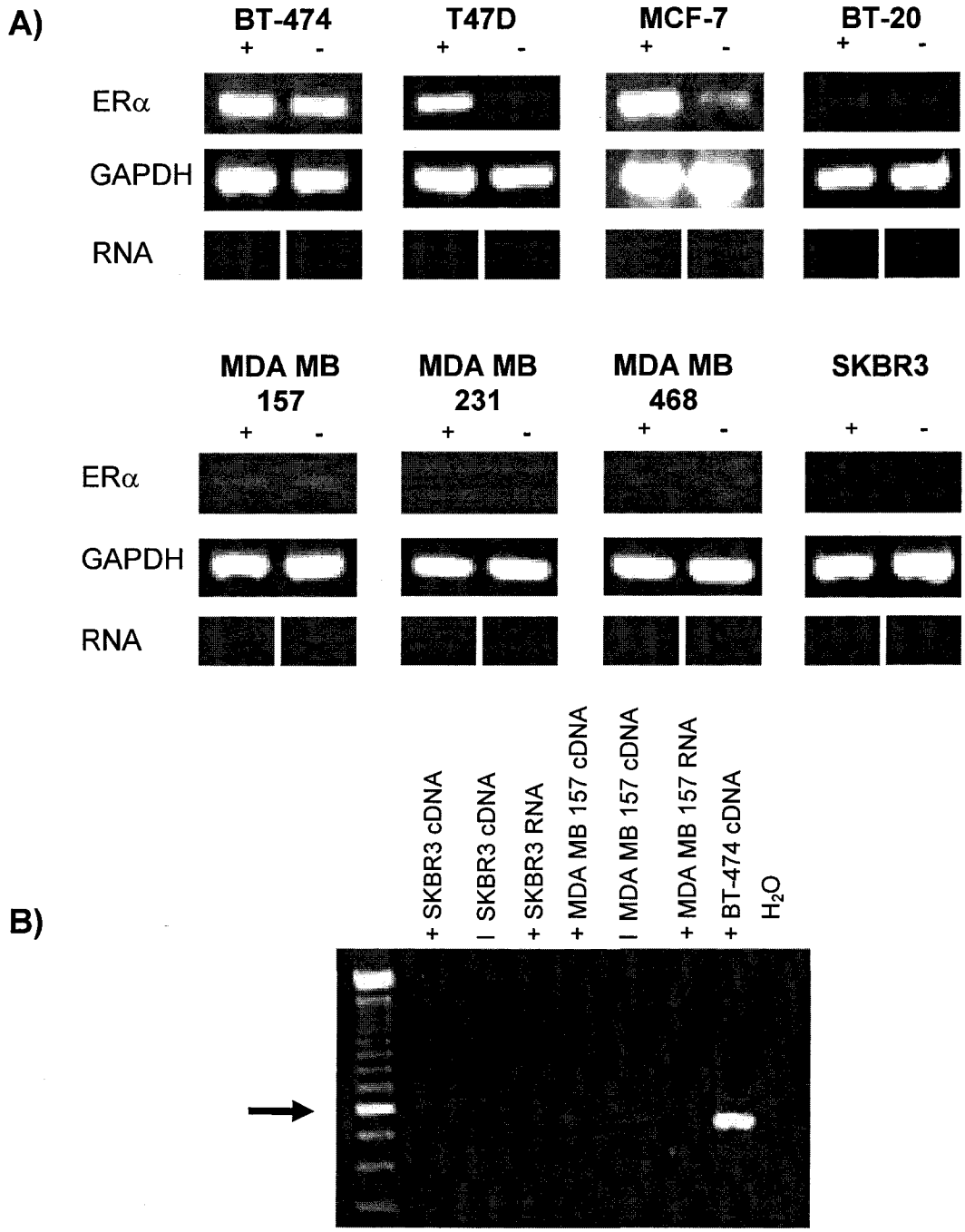


Figure 3.4. Representative gels depicting constitutive ERα expression in breast cancer cell lines cultured in standard medium (+) and estrogen-depleted medium (-). A) ERα transcription was analyzed by RT-PCR using sequence-specific primers. GAPDH was amplified as a control. RNA was subjected to PCR to ensure no DNA contamination. B) representative gel illustrating size of ERα transcripts in SKBR3, MDA MB 157 and BT-474 alongside a 100 bp ladder with an arrow indicating 600 bp.

Table 3.1. Estrogen receptor-alpha transcription in breast cancer cell lines cultured in standard (E_2-containing) medium or E_2-depleted medium assessed using sequence specific primers and RT-PCR.

Breast Cancer Cell Lines	**ERα Status (ATCC)**	**Constitutive mRNA**			
		Standard Medium		**E_2-depleted Medium**	
BT-474	+	+	(65.1 %)	+	(63.1 %)
T47D	+	+	(48.0 %)	+	(4.7 %)
MCF-7	+	+	(14.2 %)	+	(4.1 %)
BT-20	mRNA	+	(5.2 %)	+	(9.6 %)
MDA MB 157	-	+	(7.3 %)	+	(8.0 %)
MDA MB 231	-	-	(0.0 %)	-	(0.0 %)
MDA MB 468	-	-	(1.6 %)	-	(1.7 %)
SKBR3	-	-	(5.9 %)	-	(5.1 %)

( ) Values in brackets indicate expression normalized in relation to GAPDH as described in section 2.8.7; + indicates visible band, - indicates no visible band.

3.4. Determining the Optimal Concentration of 17β-estradiol for Modulating HLA-DR Expression

To determine the optimal concentration of 17β-estradiol, MCF-7 (ER+) and MDA MB 231 (ER-) were treated with 10^{-6} - 10^{-11} M estradiol in the presence / absence of 100 units/ml IFN-γ. Effects on HLA-DR expression were measured using the antibody L243 and CELISA. Since our estradiol was reconstituted in absolute ethanol and diluted 1/50 in PRF-IMDM medium, appropriate dilutions of reconstitution solvent were also assessed as controls for each estradiol concentration.

As shown in Figure 3.5, estradiol treatment did not induce HLA-DR expression on MCF-7 at any concentration tested. When co-cultured with IFN-γ (Figure 3.6), the effects of estradiol on HLA-DR expression displayed discordant results, particularly at 10^{-6} M, 10^{-7} M, 10^{-8} M, and 10^{-11} M. At such concentrations, effects due to estradiol could not be distinguished from effects due to ethanol. In fact, estradiol-mediated effects could only be consistently distinguished from the vehicle control at 10^{-9} M. At this molarity, estradiol did not modulate IFN-γ induction of HLA-DR while the vehicle control suppressed IFN-γ induction.

Analysis of estradiol treatment on MDA MB 231 also proved difficult. Again, the effects of estradiol could not be deciphered from the effects of ethanol on either constitutive (Figure 3.7) or IFN-γ-induced HLA-DR expression (Figure 3.8). Therefore, we tried other reconstitution solvents for estradiol in hope of finding one that had minimal effects on HLA-DR expression.

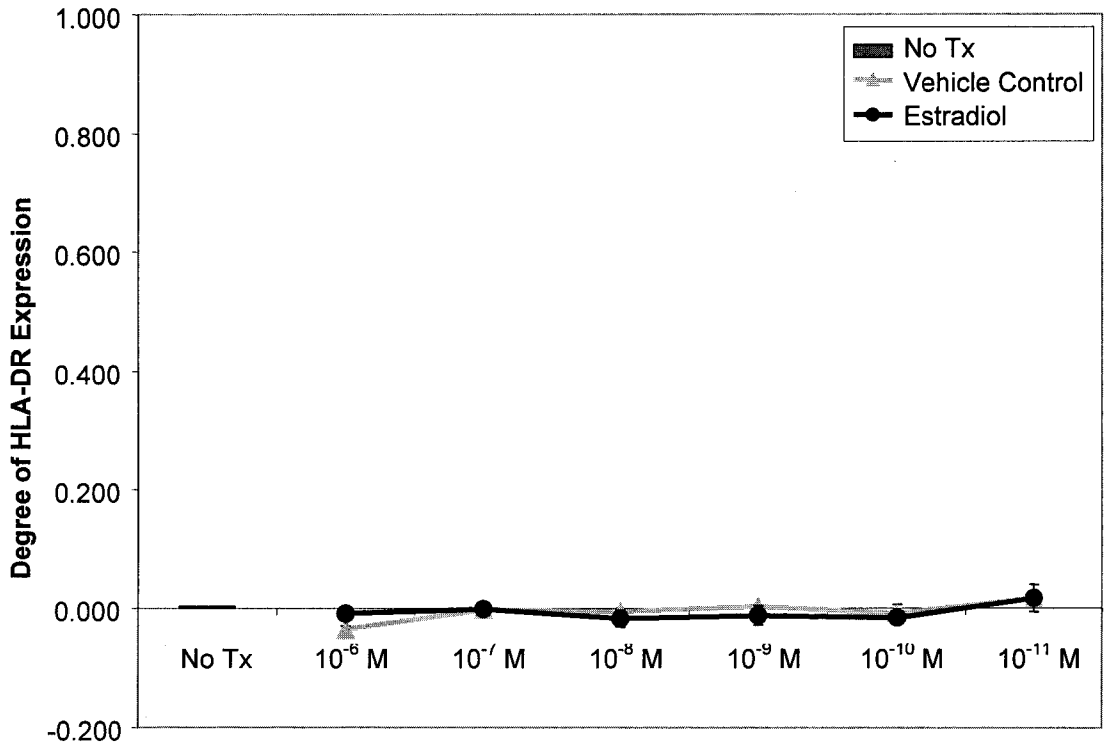


Figure 3.5. Effects of estradiol treatment on HLA-DR expression on MCF-7. HLA-DR expression was assessed using the antibody L243 and CELISA after 96 hour culture in E_2-depleted media in the presence of estradiol or vehicle control. Results are shown as $OD_{Test} - OD_{Background}$. Standard deviations were calculated from triplicates.

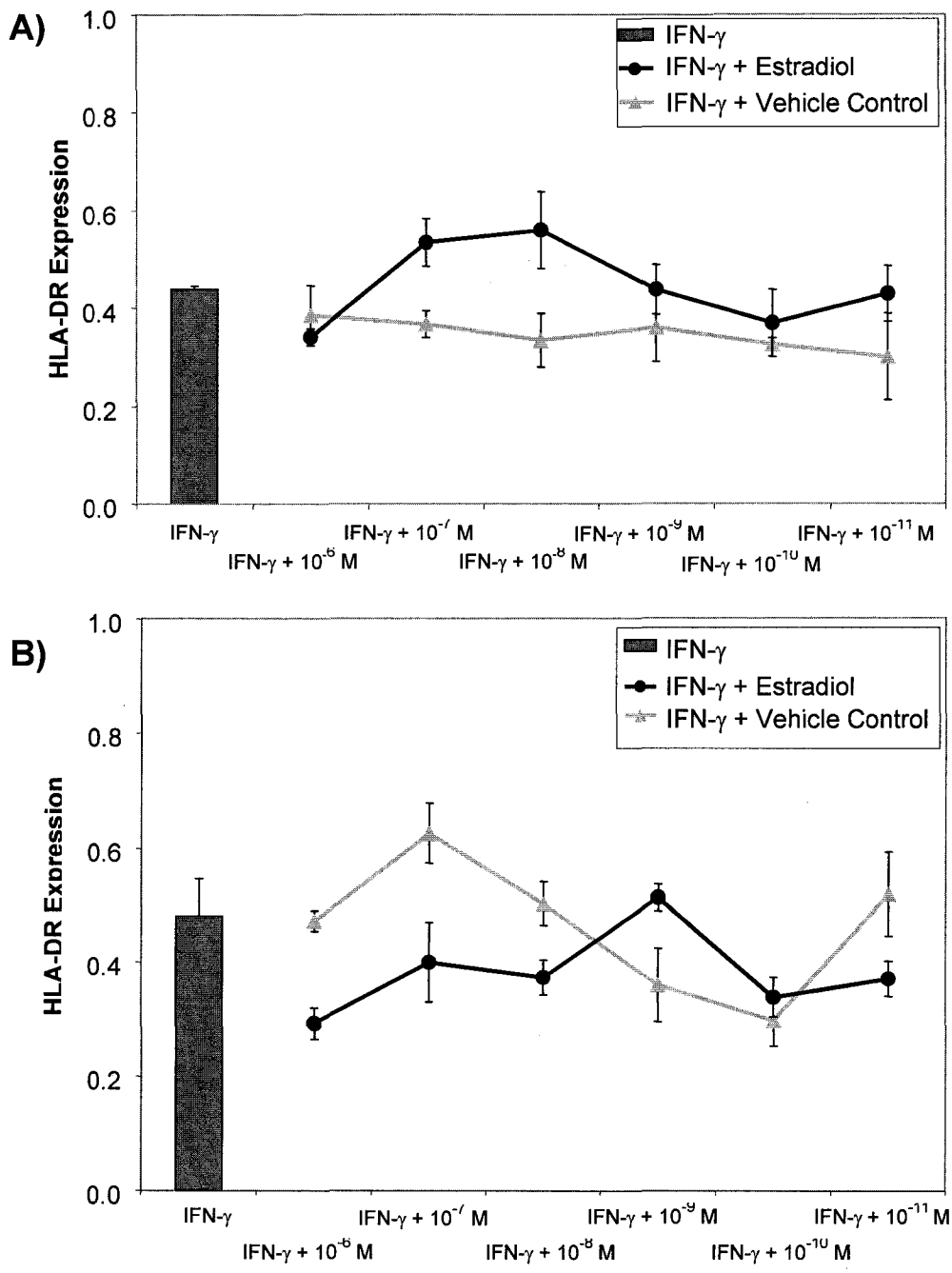


Figure 3.6. Effects of estradiol on IFN-γ-induced HLA-DR expression on MCF-7. HLA DR expression was assessed using the antibody L243 and CELISA after 96 hour culture in E_2-depleted media in the presence of 100 units/ml IFN-γ with estradiol or vehicle control. Results are shown as $OD_{Test} - OD_{Background}$. Standard deviations were calculated from triplicates. A) and B) depict replicate experiments.

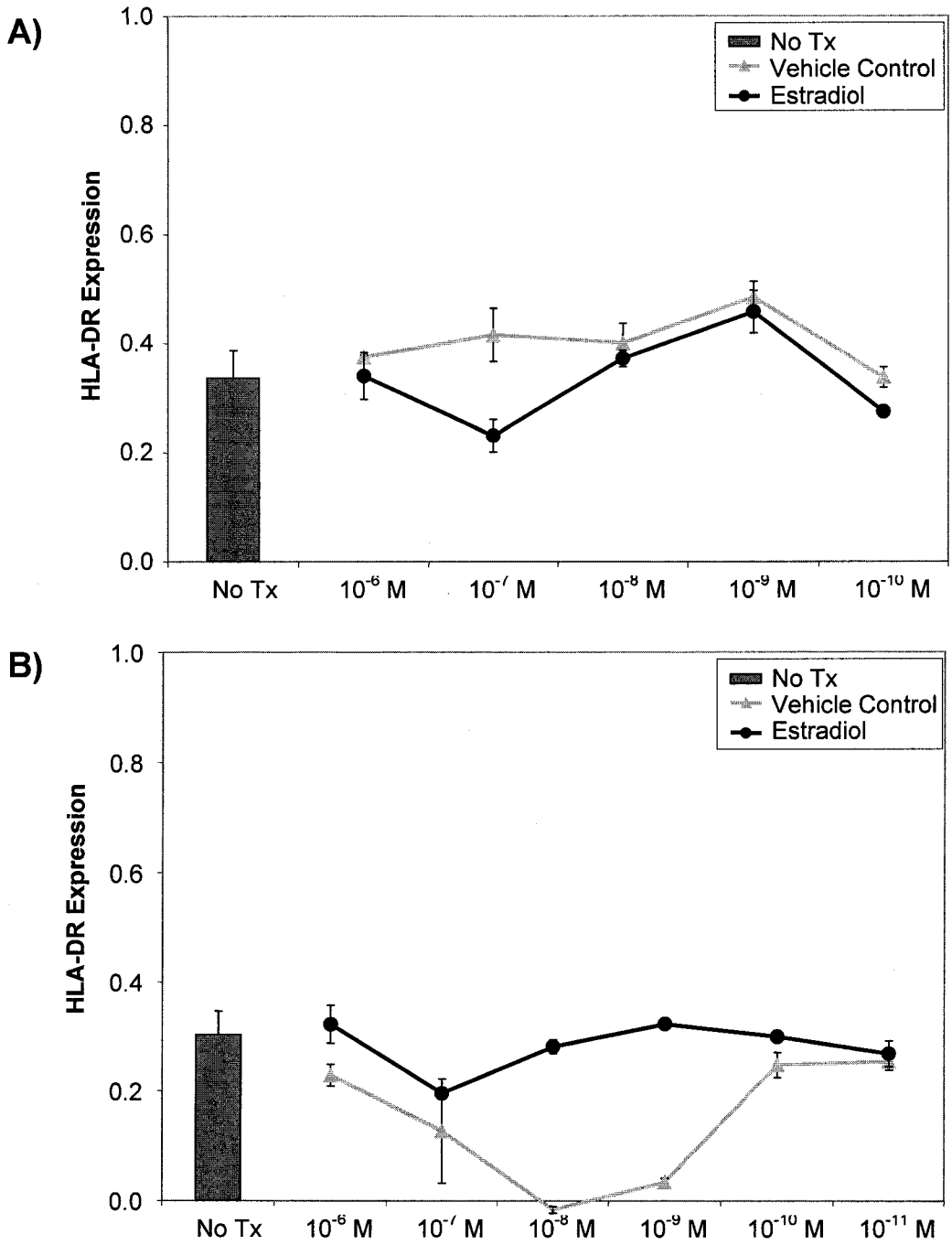


Figure 3.7. Effects of estradiol treatment on HLA-DR expression on MDA MB 231. HLA-DR expression was assessed using the antibody L243 by CELISA after 96 hour culture in E_2-depleted media in the presence / absence of estradiol or vehicle control. Results are shown as $OD_{Test} - OD_{Background}$. Standard deviations were calculated from triplicates. A) and B) depict replicate experiments.

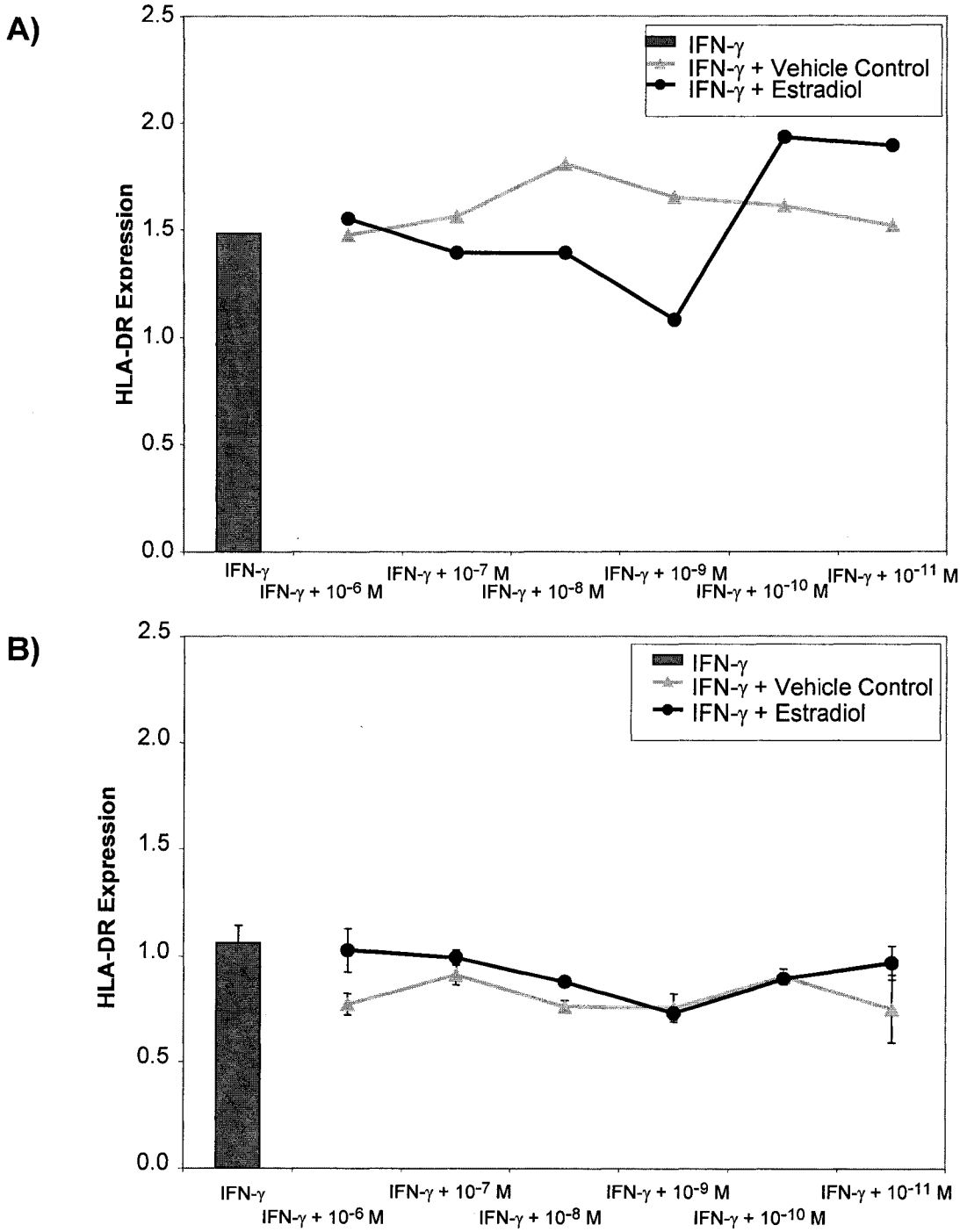


Figure 3.8. Effects of estradiol on IFN-γ-induced HLA-DR expression on MDA MB 231. HLA-DR expression was assessed using the antibody L243 by CELISA after 96 hour culture in E_2-depleted media in the presence of IFN-γ with estradiol or vehicle control. Results are shown as $OD_{Test} - OD_{Background}$. Standard deviations were calculated from triplicates. A) and B) depict replicate experiments.

3.5. Determining Optimal Reconstitution Solvent for Estradiol

As ethanol affected HLA-DR expression on both MCF-7 (ER+) and MDA MB 231 (ER-), an attempt was made to find another reconstitution solvent. According to the Merck Index [238], 17β-estradiol is insoluble in water, but soluble in alcohol, acetone, dioxane, and other organic solvents containing fixed alkali hydroxides. The manufacturer (Sigma) recommended we try dimethylsulfoxide (DMSO) in place of ethanol.

To test this, MCF-7 and MDA MB 231 were exposed to various dilutions of DMSO and ethanol, equivalent to that found in E_2 concentrations of 10^{-6} - 10^{-11} M, and effects on constitutive and IFN-γ-induced HLA-DR expression were compared using L243 and CELISA.

The results, depicted in Figures 3.09 and 3.10, revealed that both DMSO and ethanol did not induce HLA-DR expression on MCF-7 (Figure 3.9A), and had minimal effects on constitutive HLA-DR expression on MDA MB 231 (Figure 3.9B) at all molarities tested. When combined with IFN-γ treatment (Figure 3.10), DMSO and ethanol had minimal effects on IFN-γ-induced HLA-DR on MCF-7 (Figure 3.10A). However, DMSO suppressed IFN-γ induction of HLA-DR on MDA MB 231 more than ethanol at all molarities except 2.7 x 10^{-6} M, equivalent to DMSO present in 10^{-8} M estradiol (Figure 3.10B).

With DMSO now shown to suppress IFN-γ-induction of HLA-DR on MDA MB 231, other possible reconstitution solvents were tried, including 1-butanol, methanol, and acetone (results not shown). These solvents were also found to modulate both constitutive and IFN-γ-induced HLA-DR expression.

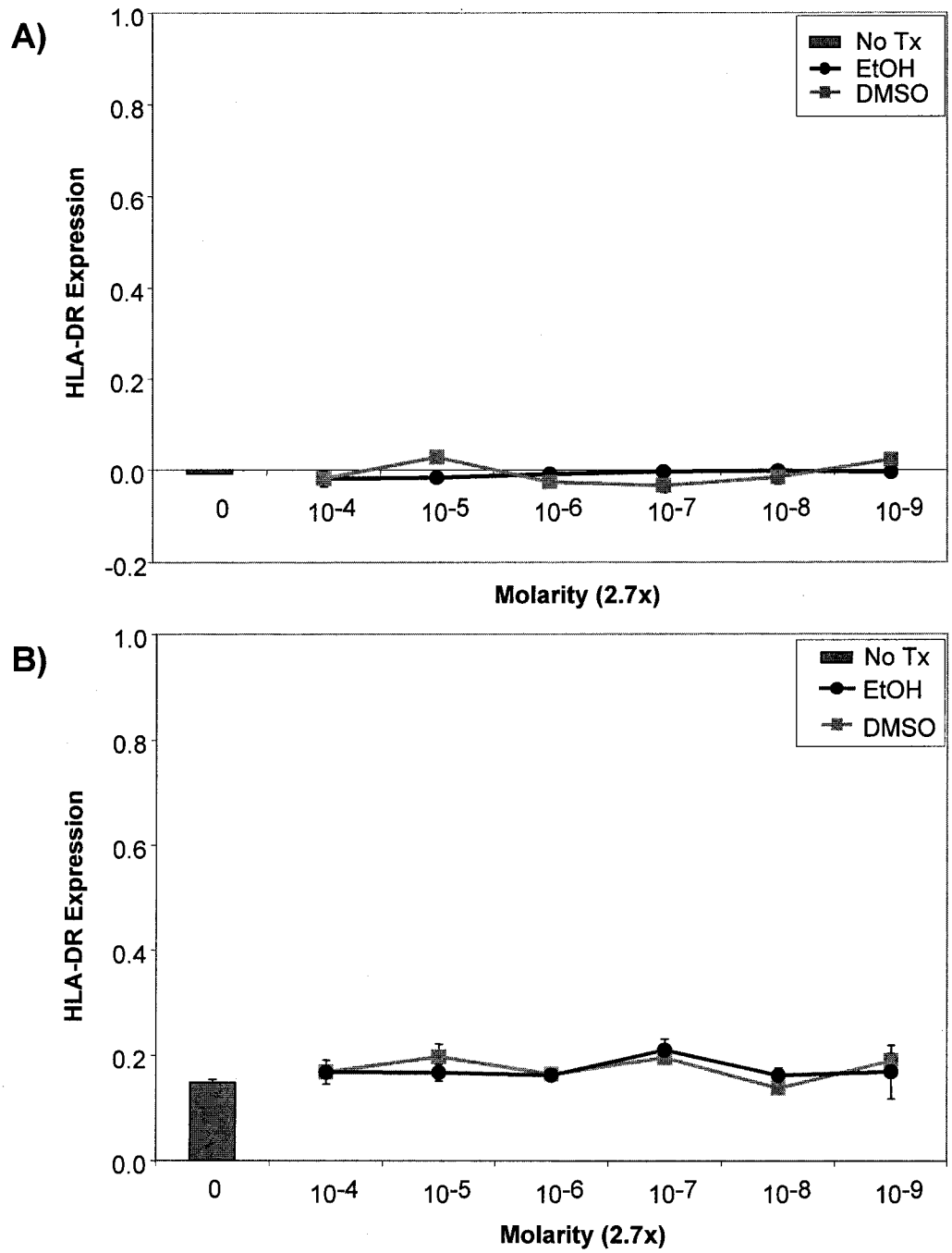


Figure 3.9. Effects of ethanol and DMSO on HLA-DR expression on MCF-7 (A) and MDA MB 231 (B). HLA-DR expression was measured using the antibody L243 and CELISA after 96 hour incubation in the presence / absence ethanol or DMSO. Results are presented as $OD_{Test} - OD_{Background}$. Standard deviations are from triplicates.

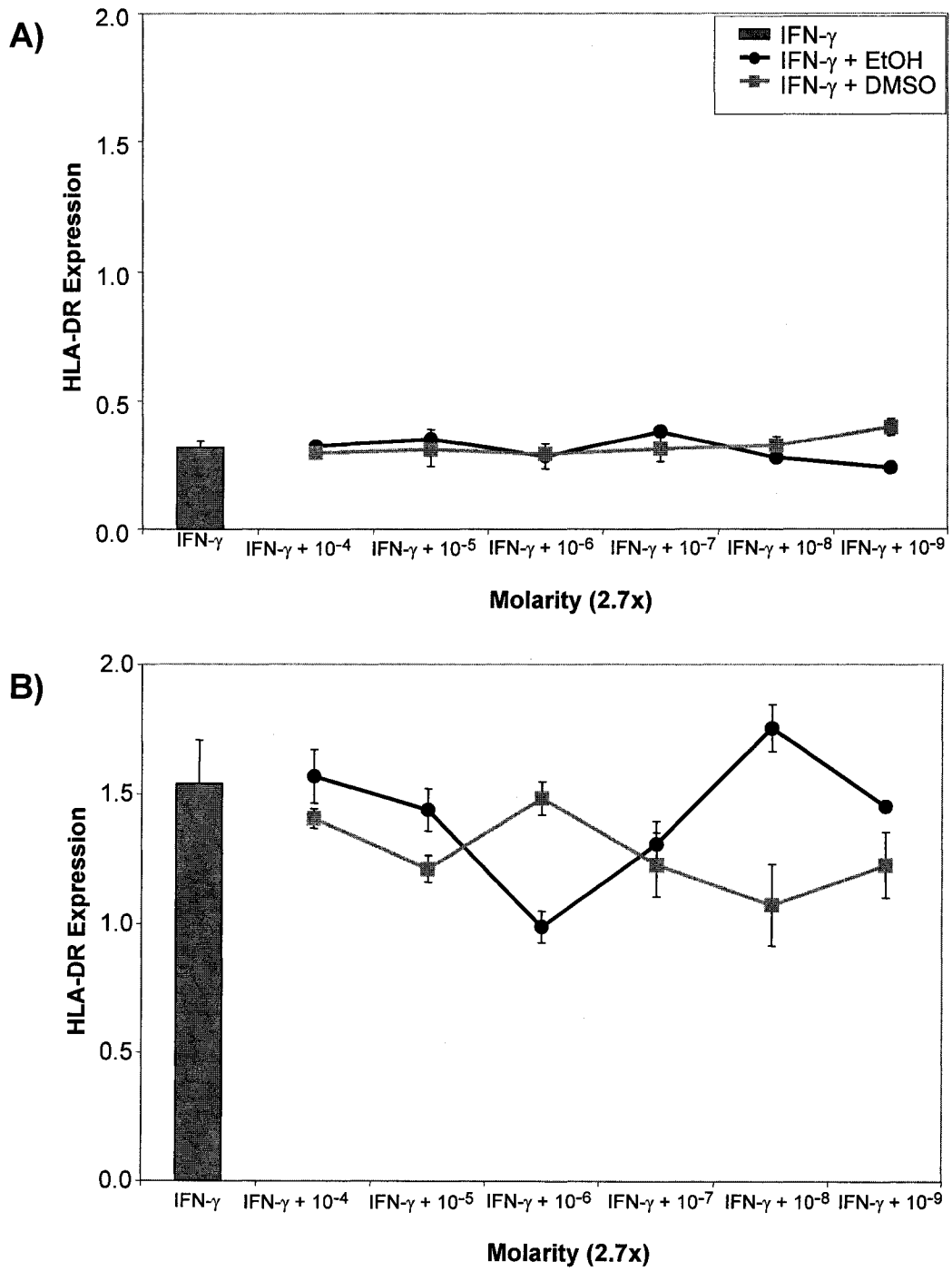


Figure 3.10. Effects of ethanol and DMSO on IFN-γ-induced HLA-DR expression on MCF-7 (A) and MDA MB 231 (B). HLA-DR expression was measured using the antibody L243 and CELISA after 96 hour incubation with IFN-γ in the presence of ethanol or DMSO. Results are presented as $OD_{Test} - OD_{Background}$. Standard deviations are calculated from triplicates.

Since we were unsuccessful in finding an appropriate reconstitution solvent, we continued with objective #2 of our project: investigating the effects of individual and combined cytokine treatments on HLA-DR expression on BCCL.

3.6. Constitutive Transcription of Cytokine Genes

Since a major objective of this study was to analyze the effects of exogenously added IFN-γ, IL-4, and TGF-β1 either individually or in combination on HLA-DR expression of BCCL, we questioned if the cells were making any of these cytokines endogenously. To address this, we analyzed for constitutive cytokine mRNA expression using sequence specific primers (Table 2.5) and RT-PCR. As before, mRNA analysis was performed on cells cultured in both standard and E_2-depleted media following 96 hour incubation.

Analysis of IFN-γ and IL-4 transcription, depicted in Figures 3.11 and 3.12, illustrated that no BCCL constitutively transcribed IFN-γ or IL-4 following culture in either media. Conversely, TGF-β1 transcripts were observed in all BCCL cultured in standard media, and all BCCL cultured in E_2-depleted media except SKBR3 (Figure 3.13). A weak band is visible in the gel photograph of SKBR3 cultured in standard media which may not be apparent in Figure 3.13.

Results of cytokine mRNA analysis, summarized in Table 3.2, show that differences in TGF-β1 amplification depended on media conditions. Normalizing TGF-β1 amplification to GAPDH indicated that transcripts were more intense in BT-474 and

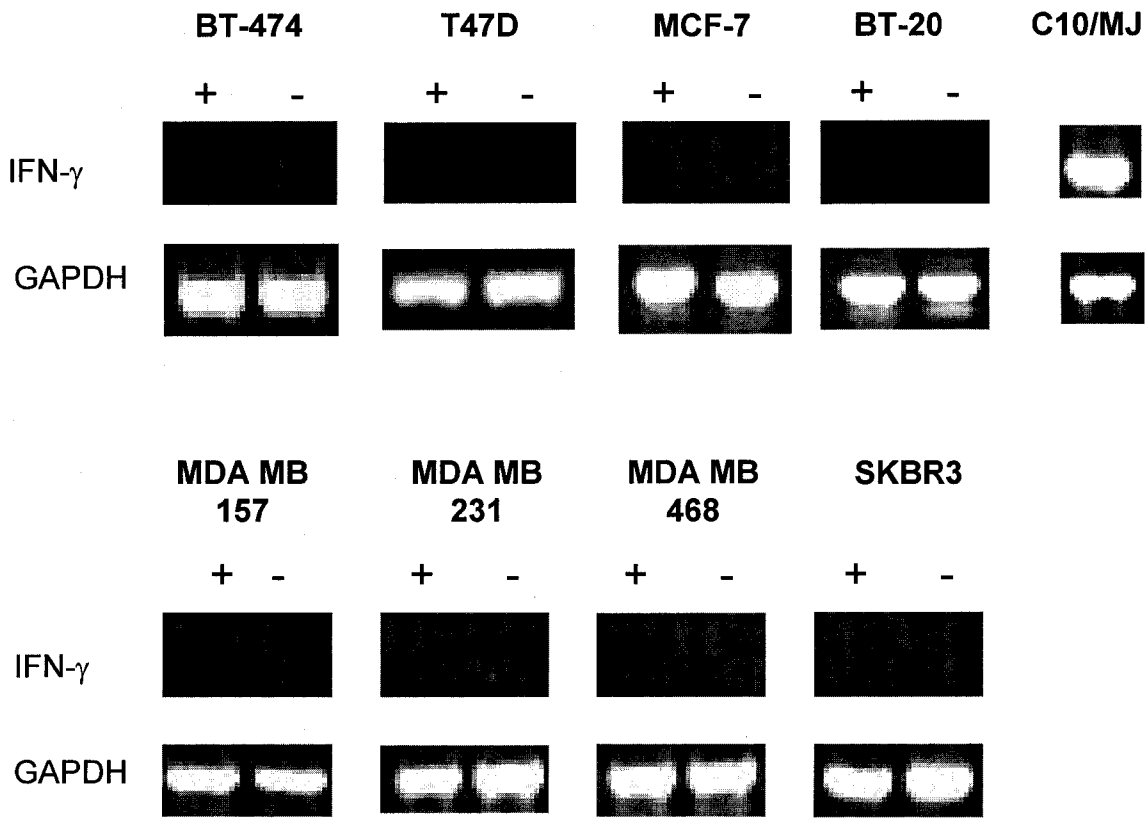


Figure 3.11. Representative gels depicting constitutive IFN-γ transcription in breast cancer cell lines cultured in standard medium (+) and estrogen-depleted medium (-). IFN-γ transcription was analyzed by RT-PCR using sequence-specific primers. C10/MJ cDNA was used as positive control for IFN-γ. GAPDH was amplified as a control.

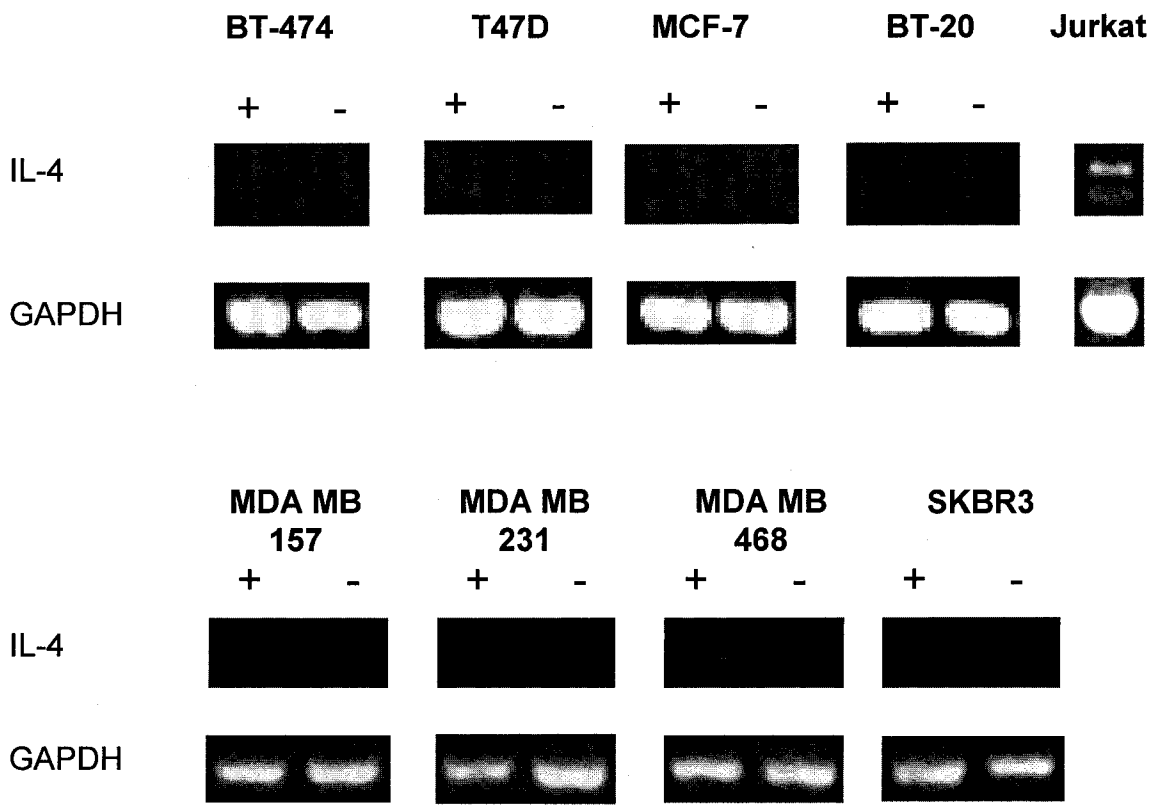


Figure 3.12. Representative gels depicting constitutive IL-4 transcription in breast cancer cell lines cultured in standard medium (+) and estrogen-depleted medium (-). IL-4 transcription was analyzed by RT-PCR using sequence-specific primers. Jurkat cDNA was used as positive control for IL-4. GAPDH was amplified as a control.

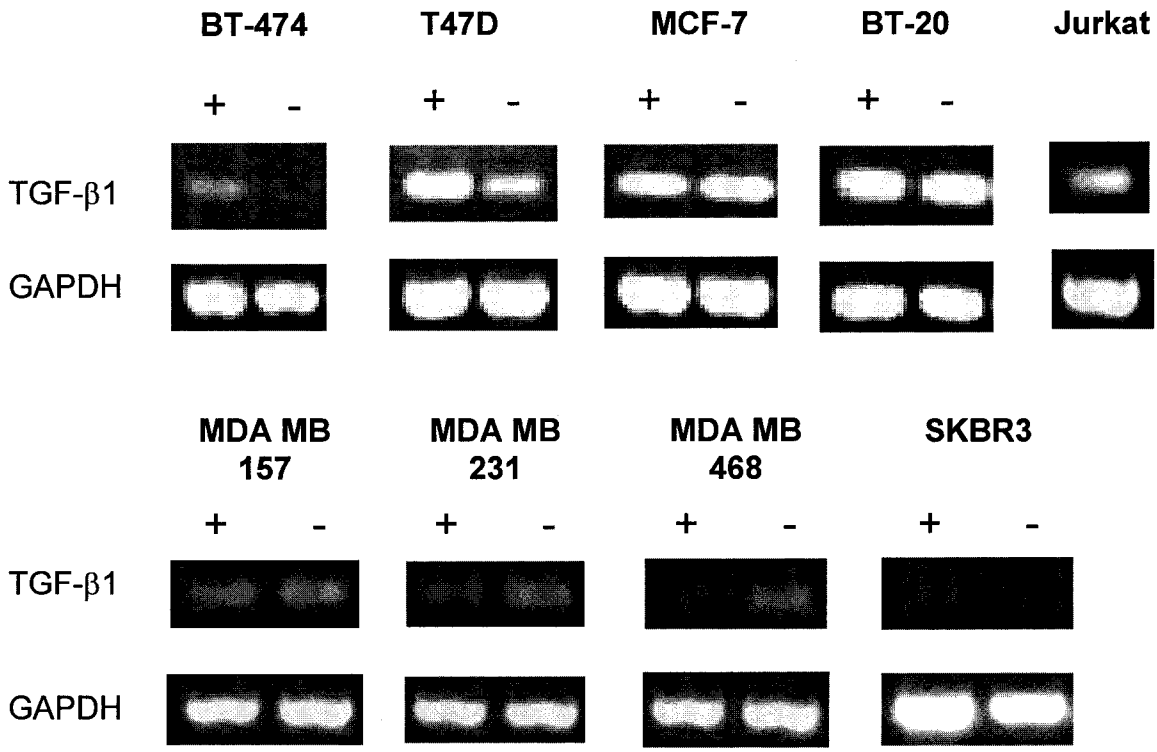


Figure 3.13. Representative gels depicting constitutive TGF-β transcription in breast cancer cell lines cultured in standard medium (+) and estrogen-depleted medium (-). TGFβ transcription was analyzed by RT-PCR using sequence-specific primers. Jurkat cDNA was used as positive control for TGFβ. GAPDH was amplified as a control.

Table 3.2. Cytokine transcription in breast cancer cell lines cultured in E_2-containing and E_2-depleted media as assessed using sequence specific primers and RT-PCR.

Breast Cancer Cell Lines	Media Type	Constitutive mRNA		
		IFN-γ	IL-4	TGF-β1
BT-474	E_2+	- (0.8%)	- (0.0%)	+ (25.1%)
	E_2-	- (3.3%)	- (0.0%)	+ (4.4%)
T47D	E_2+	- (0.0%)	- (0.0%)	+ (48.9%)
	E_2-	- (0.0%)	- (1.8%)	+ (30.5%)
MCF-7	E_2+	- (3.4%)	- (0.5%)	+ (53.4%)
	E_2-	- (2.4%)	- (1.2%)	+ (63.6%)
BT-20	E_2+	- (2.7%)	- (0.2%)	+ (62.9%)
	E_2-	- (6.5%)	- (1.3%)	+ (74.5%)
MDA MB 157	E_2+	- (1.1%)	- (0.4%)	+ (43.1%)
	E_2-	- (1.2%)	- (0.4%)	+ (49.5%)
MDA MB 231	E_2+	- (0.8%)	- (0.8%)	+ (24.1%)
	E_2-	- (3.1%)	- (0.5%)	+ (50.4%)
MDA MB 468	E_2+	- (2.1%)	- (0.7%)	+ (12.0%)
	E_2-	- (4.2%)	- (0.5%)	+ (40.7%)
SKBR3	E_2+	- (1.1%)	- (0.2%)	+ (2.4%)
	E_2-	- (2.2%)	- (0.6%)	- (0.8%)

( ) Values in brackets indicate expression normalized to GAPDH as described in section 2.8.7; + indicates visible band, - indicates no visible band.

T47D cultured in E_2-containing medium, while they were more intense in MDA MB 231, MDA MB 468, MCF-7 and BT-20 cells cultured in E_2-depleted medium. No difference in TGF-β1 amplification was found for MDA MB 157. As the above differences in intensity of TGF-β1 transcripts did not indicate trends in either ER+ or ER- BCCL, we report no correlation between differences in TGF-β1 amplification and ERα status.

3.7. Determining the Optimal Concentrations of Cytokines for Stimulating HLA-DR expression

Before performing preliminary experiments (Section 3.1), IFN-γ, IL-4, and TGF-β1 were titered on MDA MB 231 cultured in standard medium. MDA MB 231 was chosen as this cell line constitutively expresses HLA-DR, and IFN-γ strongly up-regulates HLA-DR expression [210]. Furthermore, reports indicate that MDA MB 231 contains high affinity IL-4 receptors [120], and is sensitive to TGF-β1 through retention of TβRII expression [239, 240]. Upon selection of optimal concentrations of these cytokines for stimulation of HLA-DR expression in standard medium, the same concentrations were used on breast cancer cell lines cultured in E_2-depleted medium.

3.7.1. IFN-γ

It is well documented that IFN-γ induces HLA-DR expression on most cell types including breast carcinoma [84, 130, 211-213, 241, 242]. Previous research in our laboratory determined that 500 units/ml IFN-γ was optimal for HLA Class II induction in synovial cells [211]. However, our laboratory also reported no difference in the up-regulation of cell surface HLA-DR on T47D upon treatment with 100 units/ml or 500 units/ml IFN-γ [210].

Therefore, to assess whether 100 units/ml IFN-γ was appropriate for induction of HLA-DR expression on other BCCL, MDA MB 231 and MCF-7 were exposed to 50 - 500 units/ml IFN-γ and resulting HLA-DR expression was measured using L243 and CELISA. Cells were treated for 96 hours as previous studies in our laboratory found this incubation period optimal for IFN-γ induction of HLA-DR on BCCL [210, 243].

As shown in Figure 3.14, HLA-DR induction on MCF-7 was virtually the same following 50 - 500 units/ml IFN-γ, while expression on MDA MB 231 appeared to occur in a dose dependent manner. Based on these results, 100 units/ml IFN-γ was selected to stimulate all BCCL as it provided appropriate up-regulation of HLA-DR on MCF-7 and MDA MB 231.

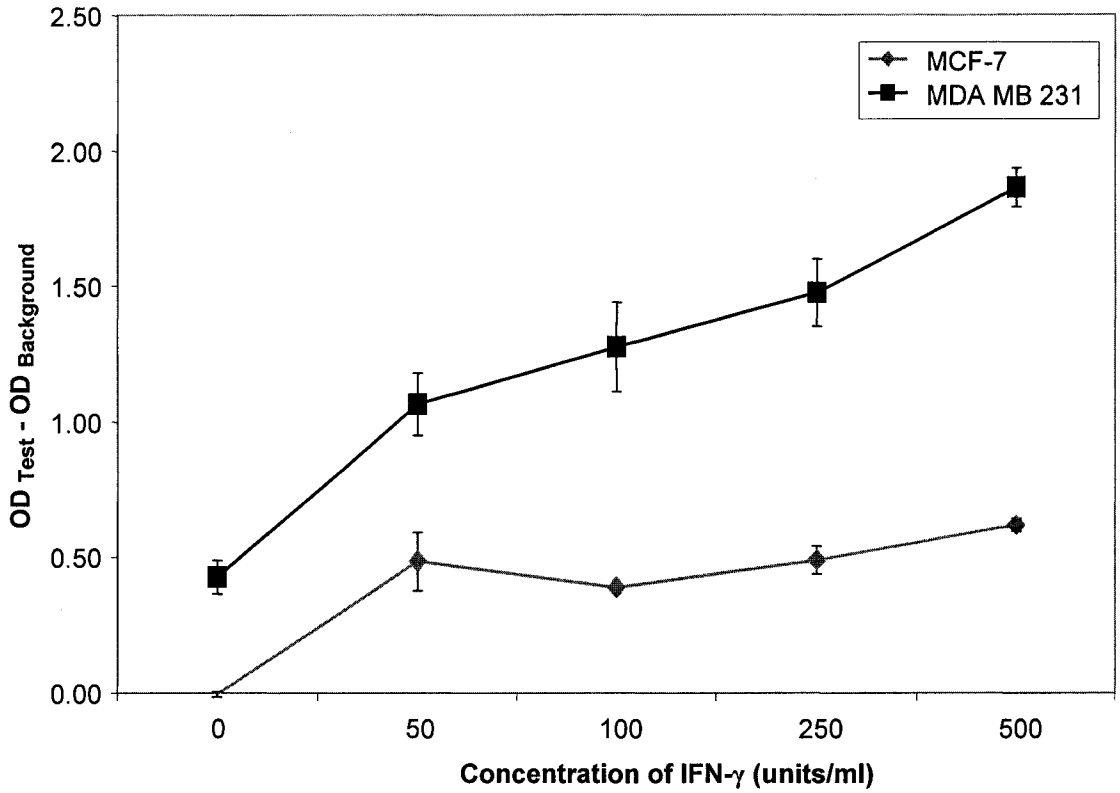


Figure 3.14. Effects of various concentrations of IFN-γ on HLA-DR expression on MDA MB 231 and MCF-7. HLA-DR expression was assessed using the antibody L243 and CELISA following 96 hour culture in standard medium. Results are shown as OD_{Test} - $OD_{Background}$. Standard deviations were calculated from triplicates.

3.7.2. IL-4

To determine the concentration of IL-4 for induction of HLA-DR in BCCL, MDA MB 231 was exposed to 50 - 500 units/ml IL-4 for 96 hours in the presence / absence of 100 units/ml IFN-γ. Resulting HLA-DR expression was measured using L243 and CELISA.

The results, depicted in Figure 3.15, showed that the greatest up-regulation of HLA-DR was found co-culturing 500 units/ml IL-4 with 100 units/ml IFN-γ. Therefore, the concentration of 500 units/ml was chosen for all IL-4 stimulation assays.

3.7.3. TGF-β1

To determine the optimal concentration of TGF-β1 for experimentation, MDA MB 231 was exposed to 1 - 10 ng/ml TGF-β1 for 96 hours in the presence or absence of 100 units/ml IFN-γ. Resulting HLA-DR expression was measured using L243 and CELISA.

As shown in Figure 3.16, TGF-β1 did not modulate constitutive HLA-DR expression at any concentration tested. Furthermore, when co-cultured with IFN-γ, no TGF-β1 concentration suppressed IFN-γ-induction of HLA-DR. Therefore, 10 ng/ml, which is commonly used in published studies [84, 86-88, 101], was considered a super-saturating dose of TGF-β1 and selected for subsequent experiments.

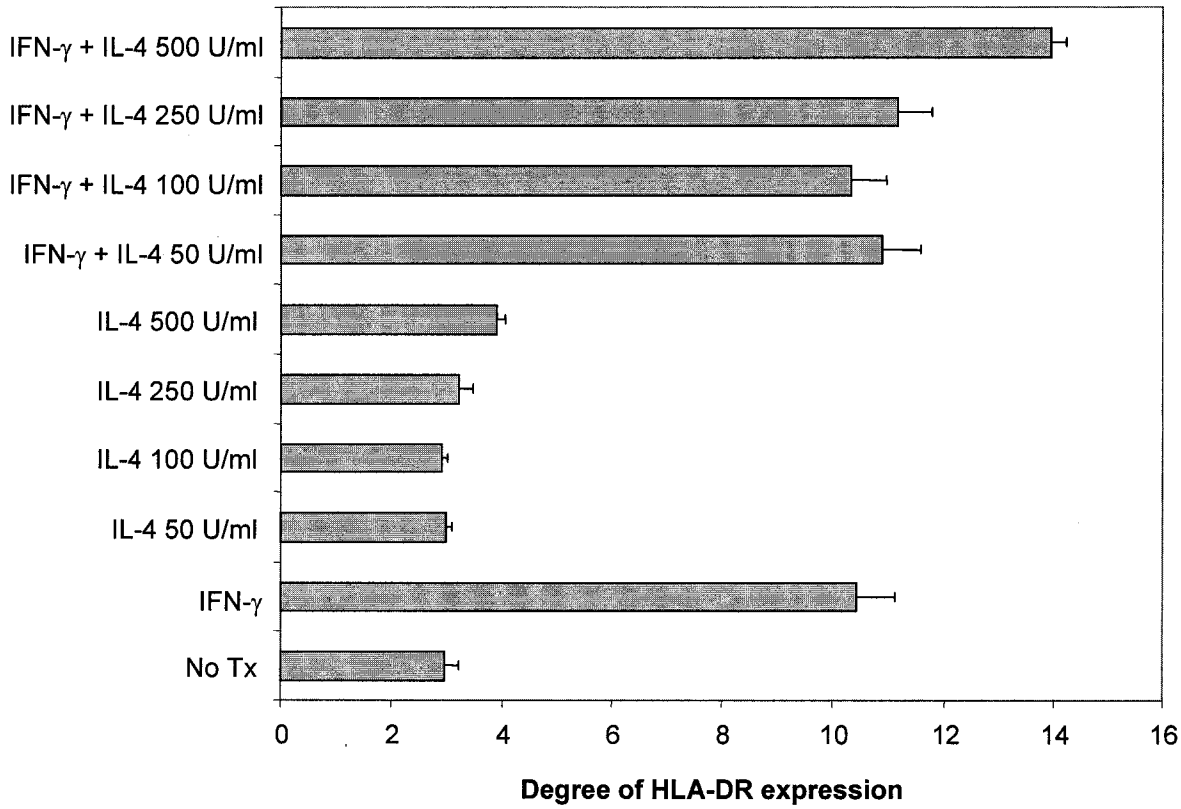


Figure 3.15. Effects of various concentrations of IL-4 on IFN-γ induction of cell surface HLA-DR on MDA MB 231. Cells were cultured in the presence or absence of IL-4 and/or 100 units/ml IFN-γ for 96 hours and assayed for HLA-DR expression using the antibody L243 and CELISA. Expression is shown as the ratio of OD_{Test} / $OD_{Background}$. Standard deviations were calculated from triplicates.

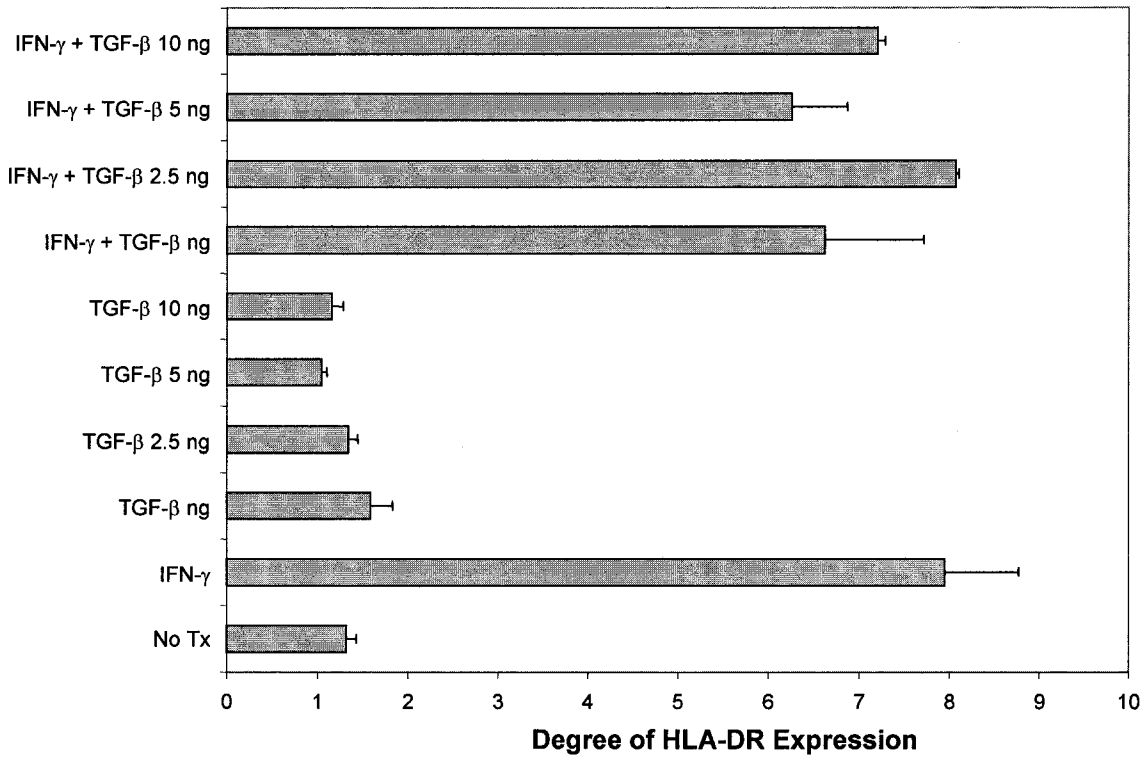


Figure 3.16. Effects of various concentrations of TGF-β1 on IFN-γ induction of HLA-DR on MDA MB 231. Cells were cultured in the presence or absence of TGF-β1 and/or IFN-γ for 96 hours and assayed for HLA-DR expression using the antibody L243 and CELISA. Expression is shown as the ratio of OD_{Test} / $OD_{Background}$. Standard deviations were calculated from triplicates.

3.8. Investigating Effects of Cytokine Combinations on HLA-DR Expression

Preliminary data (Section 3.1) conducted in standard media illustrated the effects of combined cytokine treatment on HLA-DR expression on MCF-7 (ER+) and BT-20 (ER-) BCCL. We decided to extend this study by examining BCCL cultured in E_2-depleted medium. We questioned if cells cultured in the absence of estrogen would also display cytokine-induced differences in HLA-DR expression in ER+ and ER- BCCL.

To test this, ER+ (MCF-7 and T47D) and ER- (BT-20, MDA MB 157, MDA MB 231, and SKBR3) BCCL were treated with IFN-γ and/or IL-4 and/or TGF-β1. Resulting effects on HLA-DR allelic expression were assessed using specific mAbs (Table 2.3) and both flow cytometry and CELISA. All assays were conducted following 96 hour culture in E_2-depleted media.

3.8.1. Estrogen Receptor-alpha Negative Breast Cancer Cell Lines

3.8.1.1 MDA MB 157

To evaluate the effects of cytokine co-culture on MDA MB 157, HLA-DR allelic expression was detected using the antibodies L243, pan HLA-DR; NFLD.D1, pan DRβ1*04; NFLD.D10, DRβ1*04 and DRβ1*15. CELISA analysis also incorporated NFLD.D7 for detection of DRβ1*15. Expression of DRβ4 and DRβ5 proteins could not be determined due to lack of specific mAbs.

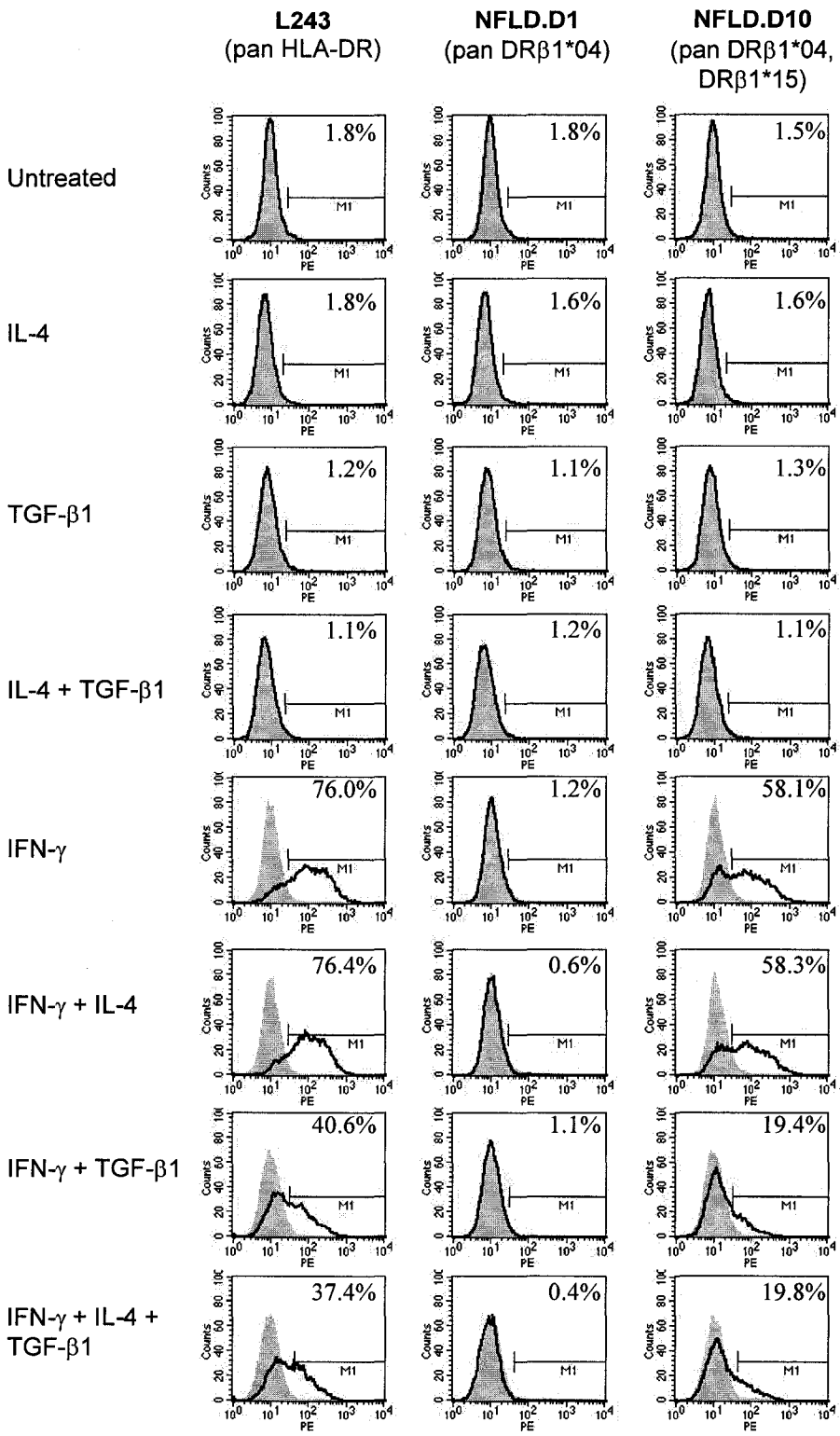
The results of flow cytometry, depicted in Figures 3.17A and 3.17B, showed that MDA MB 157 did not constitutively express generic HLA-DR (L243), and expression was unmodulated following treatment with IL-4, TGF-β1, or IL-4 + TGF-β1. Similar

results were also observed with NFLD.D1 (DRβ1*04) and NFLD.D10 (DRβ1*04, DRβ1*15). In contrast, IFN-γ strongly up-regulated generic HLA-DR (L243) and a -DR epitope recognized by NFLD.D10, as indicated by increased percentage of positive cells (Figure 3.17A) and fluorescence intensity (Figure 3.17B). However, NFLD.D1 binding was less than twice the background, suggesting that DRβ1*04 was not up-regulated on MDA MB 157. This is consistent with a previous study which showed poor DRβ1*04 expression on MDA MB 157 [210]. Thus, we assume that NFLD.D10 staining indicated DRβ1*15 expression.

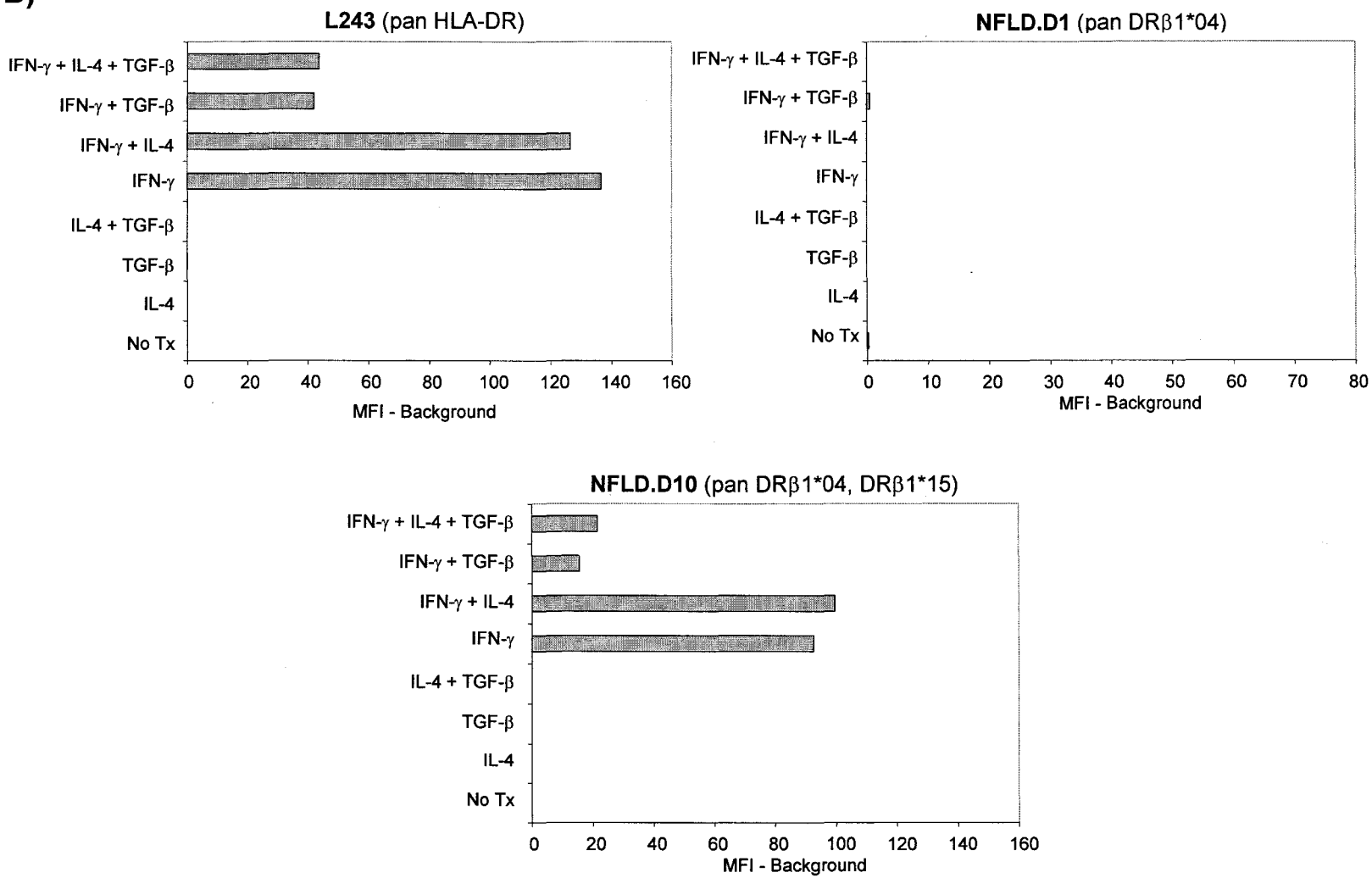
Addition of IL-4 to IFN-γ had no effect on the percentage of positive cells or the expression of generic HLA-DR and DRβ1*15. In contrast, addition of TGF-β inhibited IFN-γ induction of generic HLA-DR and DRβ1*15, as indicated by marked reductions in the percentage of positive cells and fluorescence intensity. Moreover, treatment of MDA MB 157 with the combination of IFN-γ + IL-4 + TGF-β1 also resulted in severe suppression of generic HLA-DR and DRβ1*15.

CELISA analysis (Figure 3.17C) confirmed flow cytometry results. Detection of DRβ1*04 was less than twice the background following IFN-γ treatment, confirming that DRβ1*04 was poorly expressed on MDA MB 157. Therefore, NFLD.D7 staining indicated DRβ1*15 expression. Again, generic HLA-DR and DRβ1*15 expression induced by IFN-γ or by IFN-γ + IL-4 were inhibited by addition of TGF-β1, as indicated by sizeable reductions in optical density. Thus, CELISA and flow cytometry data suggest that TGF-β1 inhibits IFN-γ-induced expression of HLA-DR on MDA MB 157. This

A)


L243
(pan HLA-DR)
NFLD.D1
(pan DRβ1*04)
NFLD.D10
(pan DRβ1*04, DRβ1*15)
Untreated
1.8%
1.8%
1.5%
IL-4
1.8%
1.6%
1.6%
TGF-β1
1.2%
1.1%
1.3%
IL-4 + TGF-β1
1.1%
1.2%
1.1%
IFN-γ
76.0%
1.2%
58.1%
IFN-γ + IL-4
76.4%
0.6%
58.3%
IFN-γ + TGF-β1
40.6%
1.1%
19.4%
IFN-γ + IL-4 + TGF-β1
37.4%
0.4%
19.8%
M1
Counts
PE

B)


L243 (pan HLA-DR)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 20 40 60 80 100 120 140 160
MFI - Background
NFLD.D1 (pan DRβ1*04)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 10 20 30 40 50 60 70 80
MFI - Background
NFLD.D10 (pan DRβ1*04, DRβ1*15)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 20 40 60 80 100 120 140 160
MFI - Background

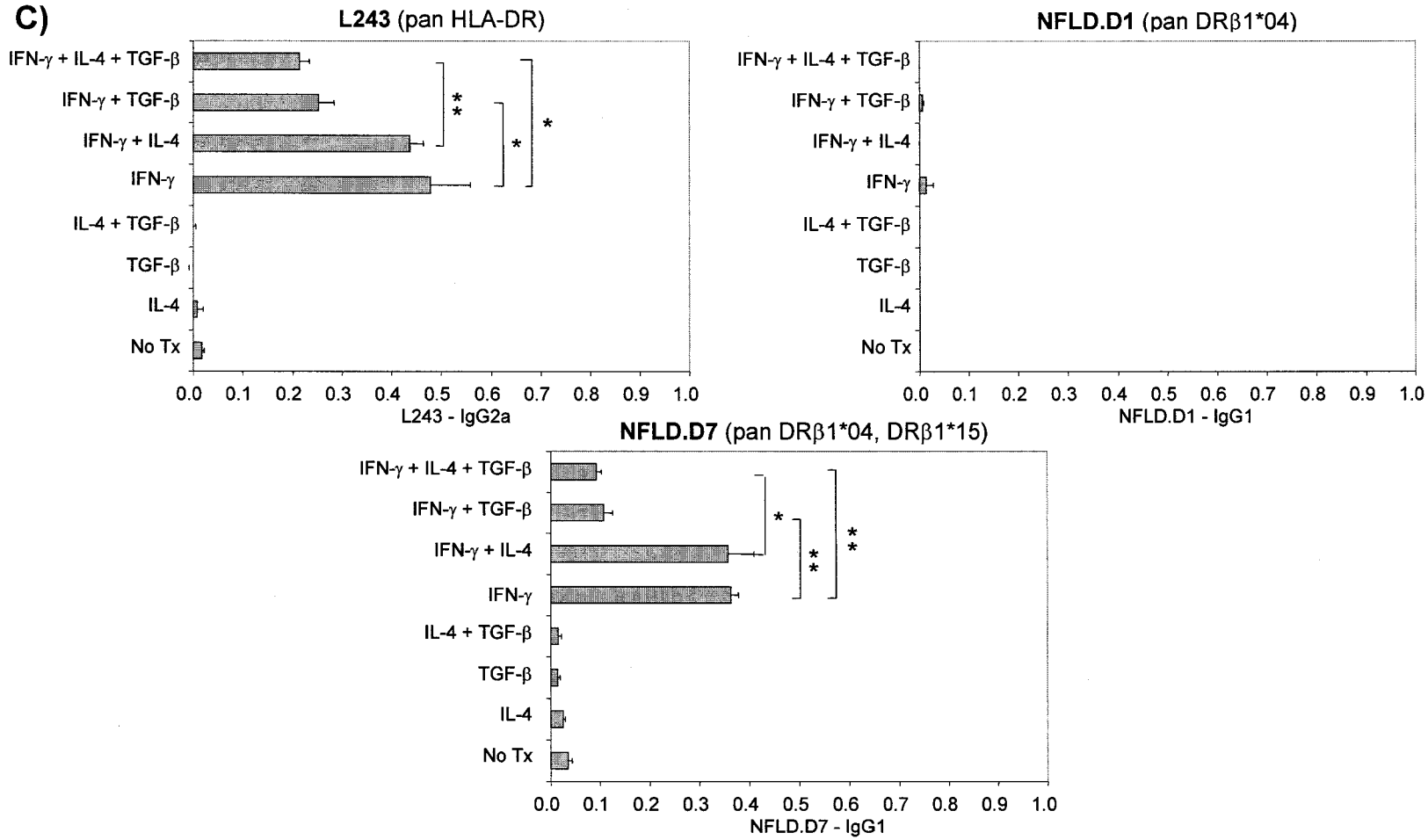


Figure 3.17. Effects of cytokines on HLA-DR allelic expression on MDA MB 157. HLA-DR expression was measured using the antibodies L243 (pan HLA-DR); NFLD.D1 (pan HLA-DRβ1*04); NFLD.D7 (pan DRβ1*04, DRβ1*15); NFLD.D10 (pan DRβ1*04, DRβ1*15). Results are reported as: (A) histogram overlays where filled histograms = IgG isotype control, open histogram = Test mAb and percents indicate the percentage of positive cells; (B) $MFI_{Test} - MFI_{Backgorund}$ as measured by flow cytometry; and (C) $OD_{Test} - OD_{Background}$ as measured by CELISA. $^{*}p < 0.05$. $^{**}p < 0.01$.

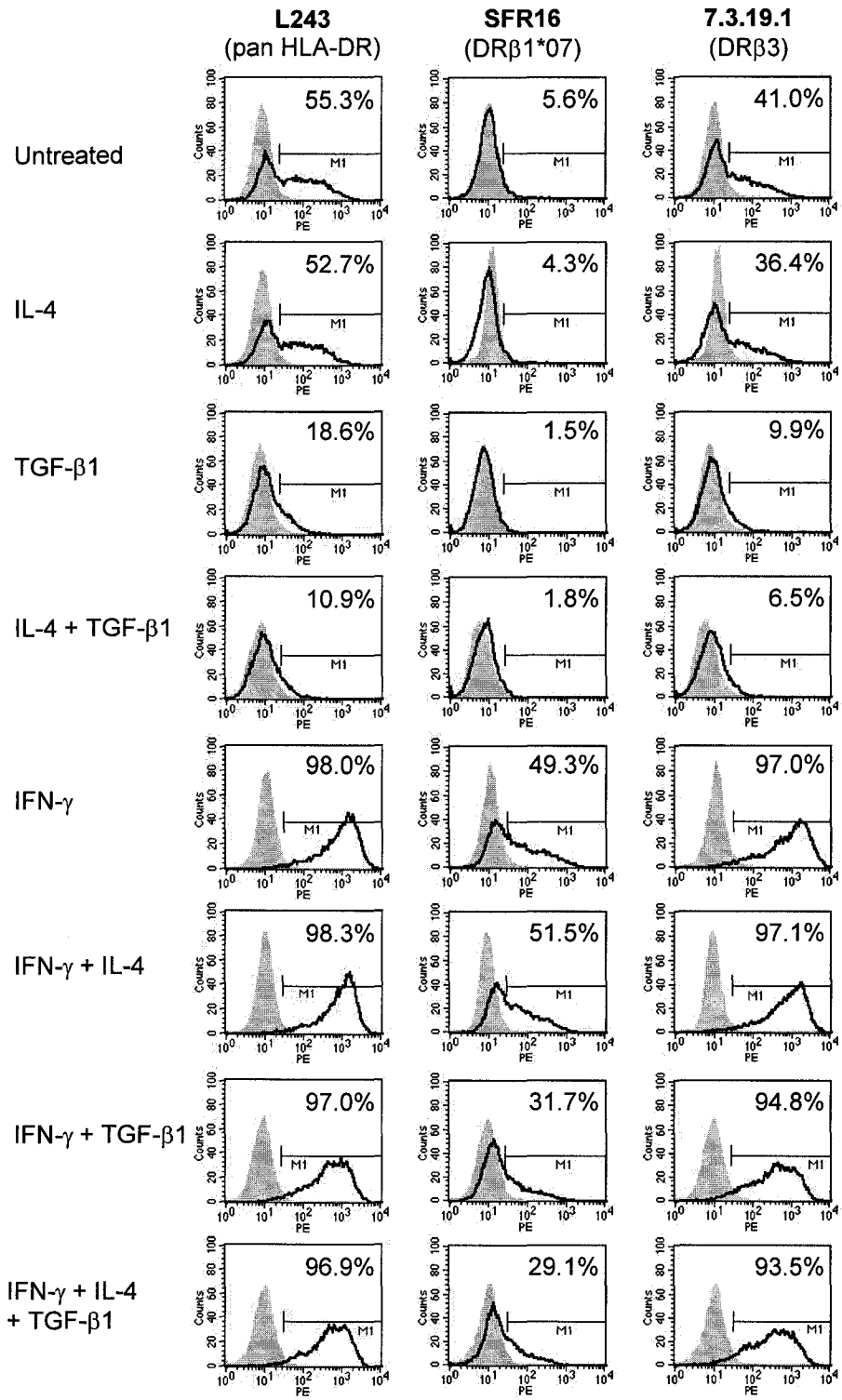
finding also correlates with previous studies involving astrocytes, monocytes, microglial cells, fibrosarcoma cells, and synovial cells [84, 87, 88]. Aside from lack of DRβ1*04 expression, allelic expression displayed similar trends as generic HLA-DR in terms of cytokine-mediated modulation.

3.8.1.2. MDA MB 231

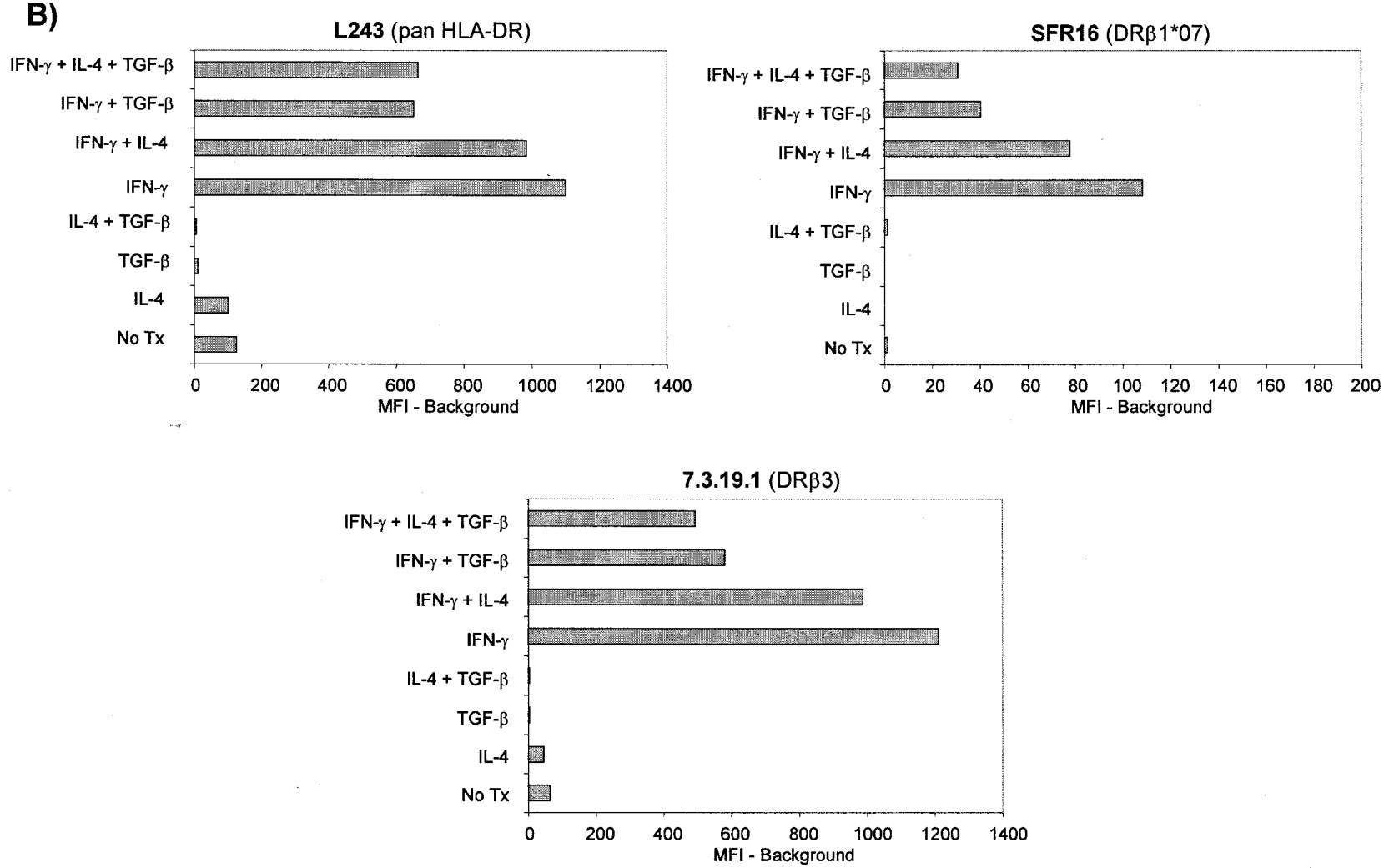
The effects of cytokine combinations on allelic expression of MBA MB 231 were detected using the antibodies L243, pan HLA-DR; SFR16, HLA-DRβ1*07; and 7.3.19.1, HLA-DRβ3. HLA-DRβ4 expression could not be determined as no specific mAbs were available.

Flow cytometry analysis, Figures 3.18A and 3.18B, showed strong constitutive expression of generic HLA-DR (L243) and DRβ3 (7.3.19.1) on MDA MB 231, while DRβ1*07 (SFR16) expression was negligible. Expression of generic HLA-DR, DRβ1*07, and DRβ3 was not altered by IL-4, but was inhibited by TGF-β1 and the combination of IL-4 and TGF-β1, as indicated by the percentage of positive cells (Figure 3.18A), and fluorescence intensity of L243 and 7.3.19.1 (Figure 3.18B). In contrast, IFN-γ strongly up-regulated the percentage of positive cells and expression of generic HLA-DR and DRβ3, while the percentage of DRβ1*07 positive cells was strongly up-regulated and DRβ1*07 expression was poorly up-regulated.

A)


L243
(pan HLA-DR)
SFR16
(DRβ1*07)
7.3.19.1
(DRβ3)
Untreated
55.3%
5.6%
41.0%
IL-4
52.7%
4.3%
36.4%
TGF-β1
18.6%
1.5%
9.9%
IL-4 + TGF-β1
10.9%
1.8%
6.5%
IFN-γ
98.0%
49.3%
97.0%
IFN-γ + IL-4
98.3%
51.5%
97.1%
IFN-γ + TGF-β1
97.0%
31.7%
94.8%
IFN-γ + IL-4
+ TGF-β1
96.9%
29.1%
93.5%
Counts
PE
M1

B)


L243 (pan HLA-DR)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 200 400 600 800 1000 1200 1400
MFI - Background
SFR16 (DRβ1*07)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 20 40 60 80 100 120 140 160 180 200
MFI - Background
7.3.19.1 (DRβ3)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 200 400 600 800 1000 1200 1400
MFI - Background

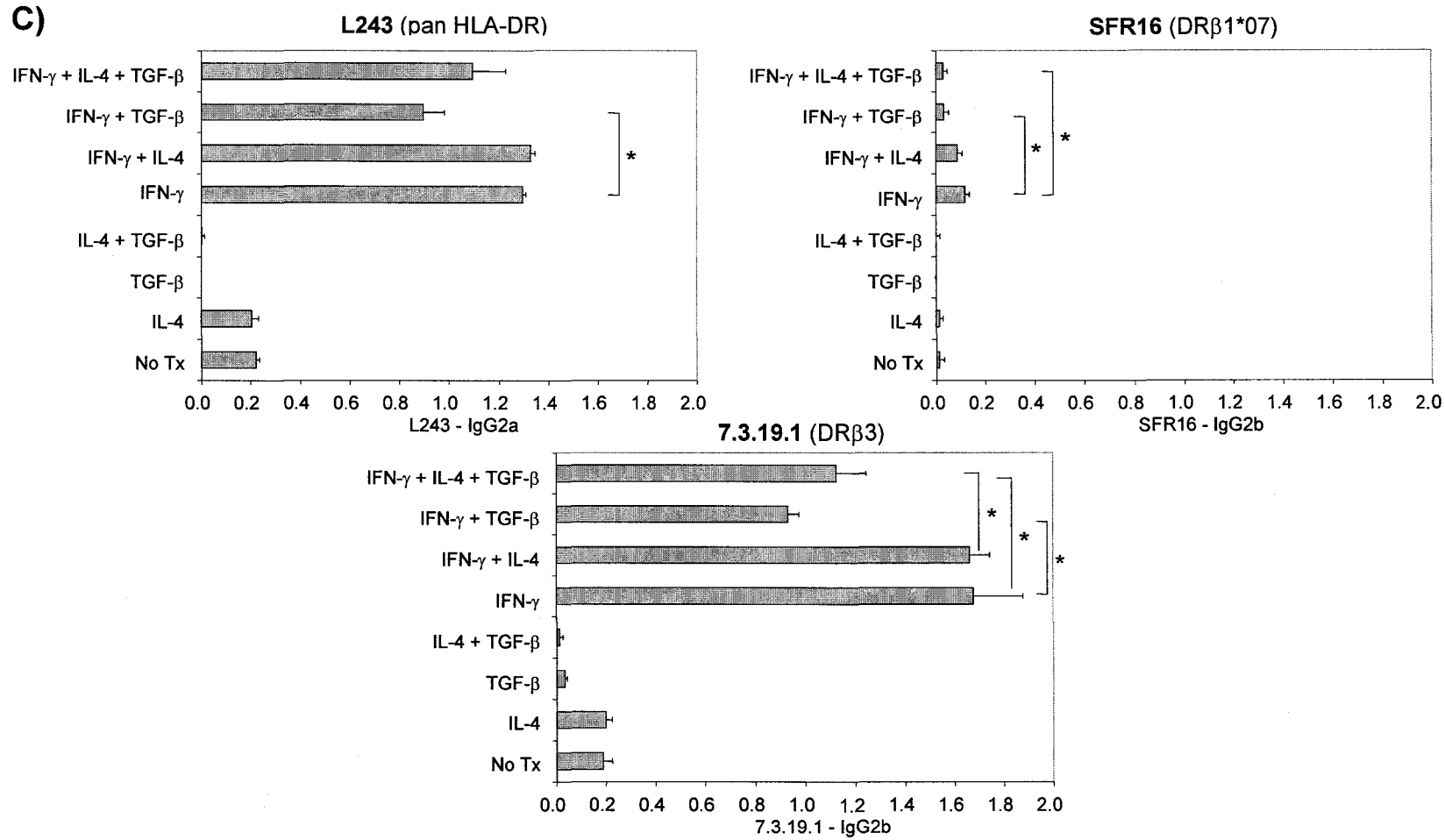


Figure 3.18. Effects of cytokines on HLA-DR allelic expression on MDA MB 231. Cells were cultured in E_2-depleted media and HLA-DR expression was measured using the antibodies L243 (panHLA-DR); SFR16 (DRβ1*07); 7.3.19.1 (DRβ3). Results are reported as: (A) histogram overlays where filled histograms = IgG isotype control, open histogram = Test mAb and percents indicate the percentage of positive cells; (B) $MFI_{Test} - MFI_{Backgorund}$ as measured by flow cytometry; and (C) $OD_{Test} - OD_{Background}$ as measured by CELISA. $^{*}p < 0.05$.

Addition of IL-4 to IFN-γ had no effect on the percentage of cells stained with L243, SFR16, and 7.3.19.1, but reduced fluorescence intensity of generic HLA-DR, DRβ1*07, and DRβ3. IFN-γ + TGF-β1 and IFN-γ + IL-4 + TGF-β1 specifically reduced the percentage of cells stained with SFR16, but both treatments caused considerable reduction in expression of generic HLA-DR, DRβ1*07, and DRβ3.

CELISA analysis (Figure 3.18C) confirmed flow cytometry findings, except, IL-4 did not suppress IFN-γ induction of generic HLA-DR, DRβ1*07, or DRβ3. As previously indicated, TGF-β1 did remarkably inhibit IFN-γ-induced generic HLA-DR, DRβ1*07 and DRβ3. Furthermore, IFN-γ + IL-4 + TGF-β1 inhibited, while IFNγ + IL-4 did not modulate, IFNγ-induced DRβ1*07 and DRβ3.

All together, the results for MDA MB 231 suggest that BCCL can display differential expression and induction of HLA-DR alleles, as DRβ1*07 was not constitutively expressed and was poorly up-regulated by cytokine treatments. In addition, the results for MDA MB 231 are similar to those of MDA MB 157 and suggest that TGF-β1 inhibits IFN-γ induced HLA-DR expression on ER- breast cancer cell lines.

3.8.1.3. SKBR3

SKBR3 has a similar HLA-DR phenotype (DRβ1*07, DRβ1*13, DRβ3, DRβ4) to MDA MB 231, and both are ER-. Given this, we wondered if SKBR3 would display similar DR expression as MDA MB 231 following cytokine treatments. To answer this, we used the same antibodies: L243, pan HLA-DR; SFR16, HLA-DR1*07; and 7.3.19.1,

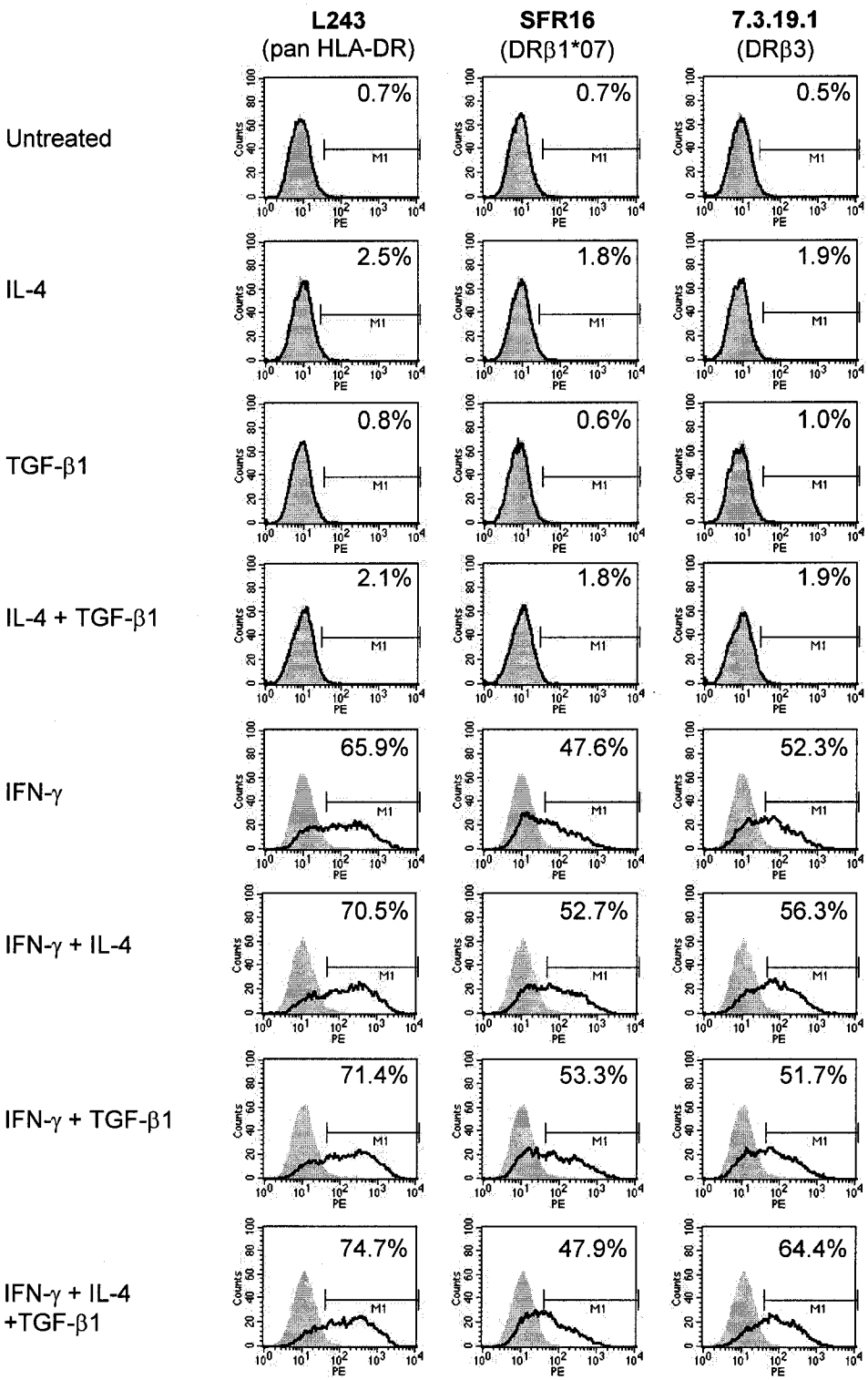
HLA-DRβ3. As previously stated, HLA-DRβ4 expression could not be examined as no specific mAb was available.

The results of flow cytometry analysis, Figures 3.19A and 3.19B, revealed that SKBR3 did not constitutively express generic HLA-DR (L243), DRβ1*07 (SFR16) or DRβ3 (7.3.19.1), and expression was unmodulated by treatment with IL-4, TGF-β1, or IL-4 + TGF-β1. However, IFN-γ strongly up-regulated generic HLA-DR, DRβ1*07, and DRβ3, as indicated by increased percentage of positive cells (Figure 3.19A) and fluorescence intensity (Figure 3.19B).

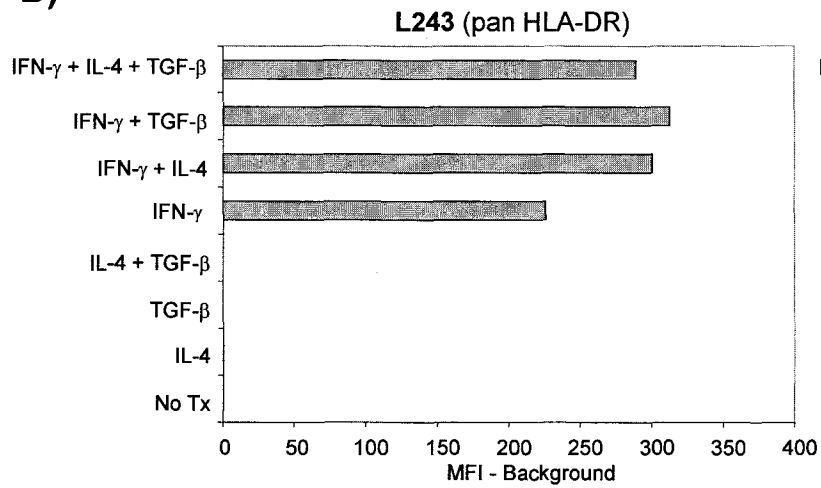
Addition of IL-4 augmented IFN-γ induction of generic HLA-DR, DRβ1*07 and DRβ3, as indicated by increased percentage of positive cells, and more specifically, by increased fluorescence intensity. Similarly, addition of TGF-β1 to IFN-γ increased the percentage of cells labeled with L243, SFR16, and 7.3.19.1, and increased fluorescence intensity. This suggested that either IL-4 or TGF-β1 could act synergistically with IFN-γ to induce HLA-DR expression on SKBR3. However, IFN-γ + IL-4 + TGF-β1 resulted in the highest percentage of generic HLA-DR and DRβ3 positive cells and increased the expression of generic HLA-DR and DRβ3, but did not modulate the percentage or expression of DRβ1*07, indicating selective up-regulation of HLA-DR alleles.

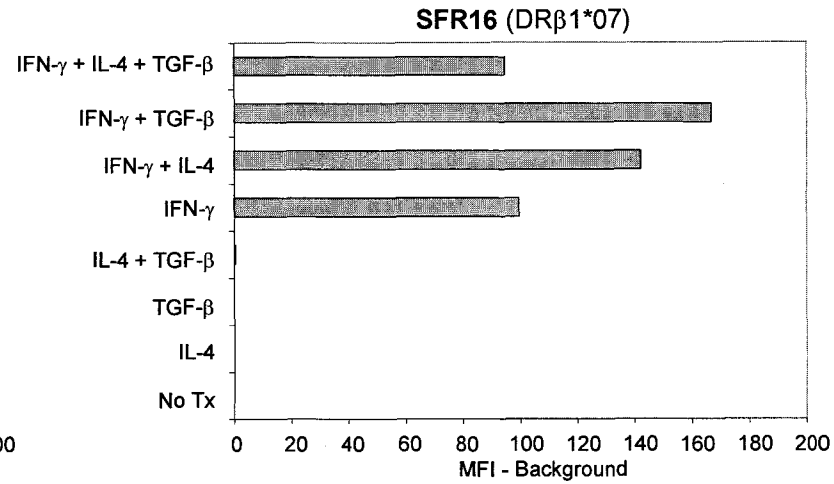
CELISA analysis, depicted in Figure 3.19C, verified most flow cytometry findings. However, DRβ1*07 was poorly up-regulated, indicating a discrepancy with flow cytometry data that will be addressed in Chapter 4. Addition of IL-4 slightly increased IFN-γ induction of generic HLA-DR, but did not affect DRβ1*07 or DRβ3.

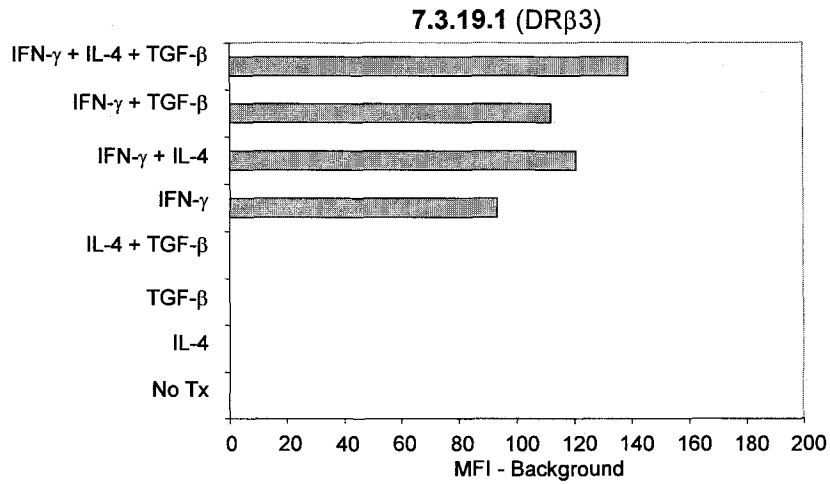
A)


L243
(pan HLA-DR)
SFR16
(DRβ1*07)
7.3.19.1
(DRβ3)
Untreated
0.7%
0.7%
0.5%
IL-4
2.5%
1.8%
1.9%
TGF-β1
0.8%
0.6%
1.0%
IL-4 + TGF-β1
2.1%
1.8%
1.9%
IFN-γ
65.9%
47.6%
52.3%
IFN-γ + IL-4
70.5%
52.7%
56.3%
IFN-γ + TGF-β1
71.4%
53.3%
51.7%
IFN-γ + IL-4
+TGF-β1
74.7%
47.9%
64.4%
M1
Counts
PE

B)


L243 (pan HLA-DR)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 50 100 150 200 250 300 350 400
MFI - Background


SFR16 (DRβ1*07)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 20 40 60 80 100 120 140 160 180 200
MFI - Background


7.3.19.1 (DRβ3)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 20 40 60 80 100 120 140 160 180 200
MFI - Background

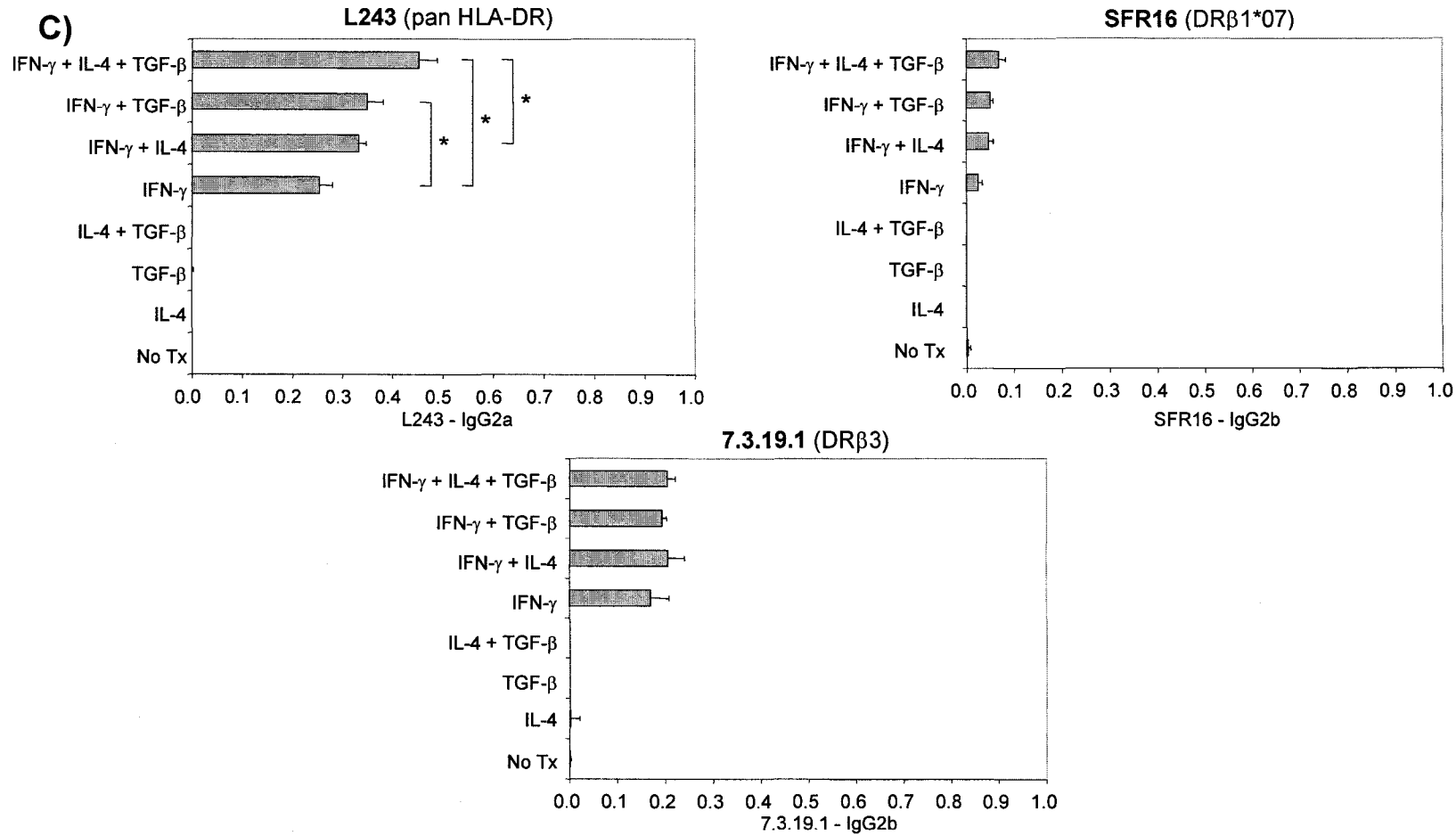


Figure 3.19. Effects of cytokines on HLA-DR allelic expression on SKBR3. HLA-DR expression was measured using the antibodies L243 (pan HLA-DR); SFR16 (DRβ1*07); 7.3.19.1 (DRβ3). Results are reported as: (A) histogram overlays where filled histograms = IgG isotype control, open histogram = Test mAb and percents indicate the percentage of positive cells; (B) $MFI_{Test} - MFI_{Backgorund}$ as measured by flow cytometry; and (C) $OD_{Test} - OD_{Background}$ as measured by CELISA. $^{*}p < 0.05$.

IFNγ + TGF-β1 up-regulated generic HLA-DR, while expression of DRβ1*07 and DRβ3 was relatively unmodulated, suggesting that DRβ1*13 or DRβ4 was up-regulated. Moreover, the highest expression of generic HLA-DR was found following IFN-γ + IL-4 + TGF-β1, suggesting that TGF-β was not inhibitory to SKBR3.

Taken together, the results of SKBR3 are in contrast to the results of MDA MB 157 and MDA MB 231 as we showed that TGF-β does not inhibit IFN-γ induction of generic or allelic HLA-DR on SKBR3. Moreover, as the BCCL examined thus far have all been ER-, these current results suggest that TGF-β is not inhibitory to all BCCL. Furthermore, as SKBR3 has similar HLA-DR phenotype to MDA MB 231, we now suggest that the effects mediated by cytokine combinations on HLA-DR expression may be cell line specific.

3.8.1.4. BT-20

To further examine the effects of cytokine combinations on ER- BCCL, allelic expression on BT-20 was detected using the antibodies L243, pan HLA-DR; NFLD.D1, pan DRβ1*04; 7.3.19.1, DRβ3. CELISA analysis also incorporated TAL8.1 for detection of HLA-DRβ1*13. As stated previously, we were unable to detect DRβ1*04 with 359-16F10 due to shortage of this antibody.

The results of flow cytometry, depicted in Figures 3.20A and 3.20B, revealed a small amount of constitutive generic HLA-DR (L243), which was unaffected by IL-4, but was decreased by TGF-β treatment. Since constitutive DRβ1*04 (NFLD.D1) and DRβ3 (7.3.19.1) was not observed, we assumed that HLA-DRβ1*13 and/or HLA-DRβ4 were

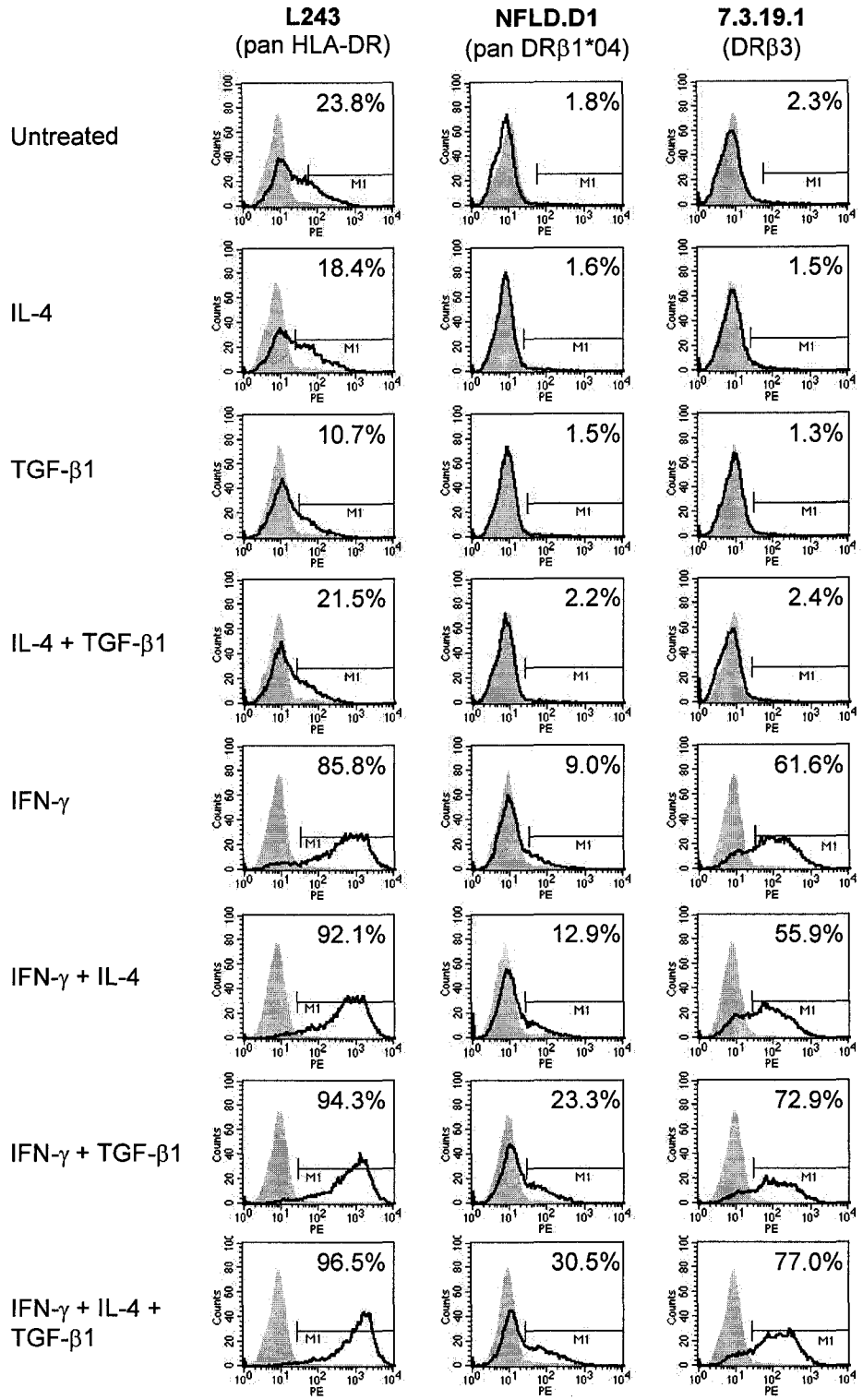
expressed. However, DRβ1*13 and DRβ4 proteins were not ascertained by flow cytometry due to shortage or lack of specific mAbs. Alternatively, it is possible that low levels of HLA-DR allelic products were indictable using allele specific mAbs, but were detectable using L243 since this Ab recognizes all DR types collectively.

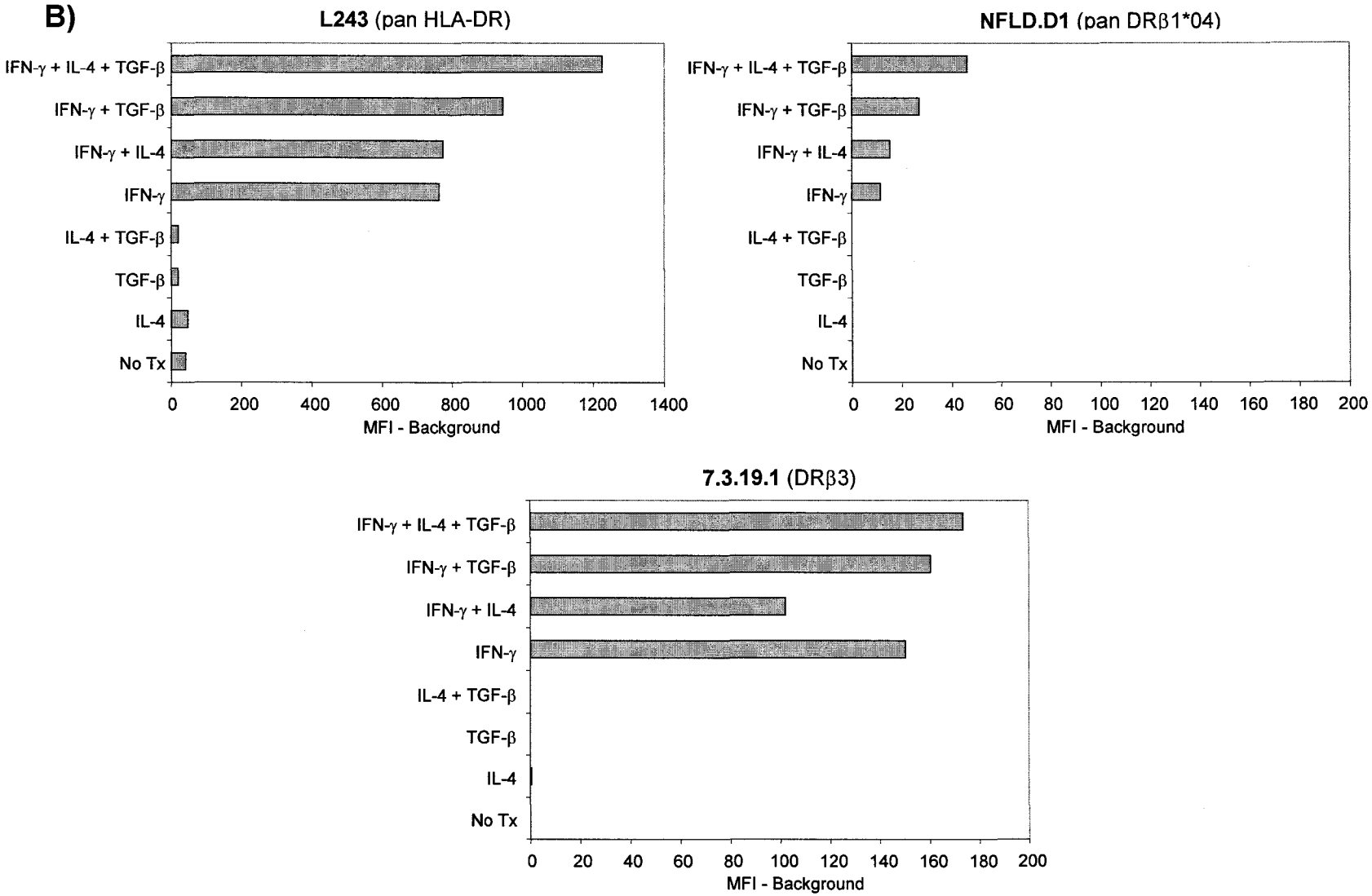
Neither IL-4, TGF-β1, nor IL-4 + TGF-β1 modulated expression of DRβ1*04 or DRβ3. In contrast, IFN-γ strongly up-regulated generic HLA-DR and DRβ3, but only slightly increased DRβ1*04, as indicated by increased percentage of positive cells (Figure 3.20A) and fluorescence intensity (Figure 3.20B). Addition of IL-4 to IFNγ marginally increased the percent of positive cells stained with L243 and NFLD.D1, but did not affect fluorescence intensity, while it reduced IFN-γ induction of DRβ3.

In contrast, IFNγ + TGF-β1 increased the percentage of cells expressing generic HLA-DR, DRβ1*04, and DRβ3, but only substantially increased expression of generic HLA-DR when fluorescent intensity was analyzed. IFN-γ + IL-4 + TGF-β1 treatment resulted in the highest percentage and expression of generic HLA-DR, DRβ1*04, and DRβ3, suggesting that these three cytokines could act synergistically to modulate HLA-DR on BT-20.

CELISA analysis (Figure 3.20C) confirmed flow cytometry results for the most part. However, expression of constitutive generic HLA-DR was negligible, and although IL-4 slightly increased generic HLA-DR expression, this detection was less than twice the background and was also considered negligible. Furthermore, IFN-γ, IFN-γ + IL-4, IFN-γ + TGF-β1, and IFN-γ + IL-4 + TGF-β1 did not up-regulate DRβ1*04 as

A


L243
(pan HLA-DR)
NFLD.D1
(pan DRβ1*04)
7.3.19.1
(DRβ3)
Untreated
23.8%
1.8%
2.3%
IL-4
18.4%
1.6%
1.5%
TGF-β1
10.7%
1.5%
1.3%
IL-4 + TGF-β1
21.5%
2.2%
2.4%
IFN-γ
85.8%
9.0%
61.6%
IFN-γ + IL-4
92.1%
12.9%
55.9%
IFN-γ + TGF-β1
94.3%
23.3%
72.9%
IFN-γ + IL-4 + TGF-β1
96.5%
30.5%
77.0%
M1
Counts
PE


B)
L243 (pan HLA-DR)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 200 400 600 800 1000 1200 1400
MFI - Background
NFLD.D1 (pan DRβ1*04)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 20 40 60 80 100 120 140 160 180 200
MFI - Background
7.3.19.1 (DRβ3)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0 20 40 60 80 100 120 140 160 180 200
MFI - Background

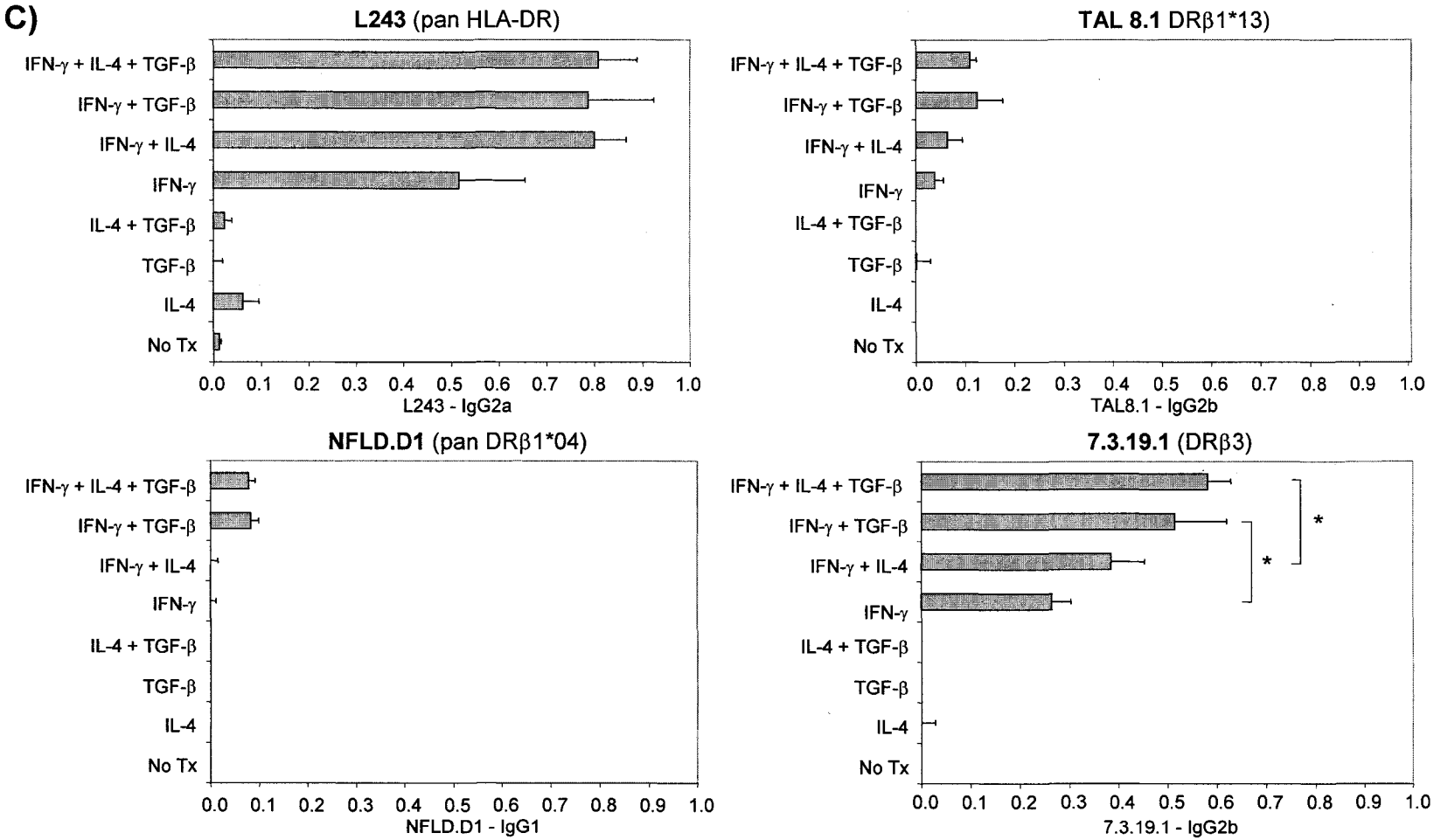


Figure 3.20. Effects of cytokines on HLA-DR allelic expression on BT-20. HLA-DR expression was assessed using antibodies: L243 (pan HLA-DR); TAL8.1 (DRβ1*13); NFLD.D1 (pan DRβ1*04); 7.3.19.1 (DRβ3). Results are reported as: (A) histogram overlays where filled histograms = IgG isotype control, open histogram = Test mAb, and percents indicate the percentage of positive cells; (B) $MFI_{Test} - MFI_{Backgorund}$ as measured by flow cytometry; and (C) $OD_{Test} - OD_{Background}$ as measured by CELISA. $^{*}p < 0.05$. $^{**}p < 0.01$.

expression was less than twice the background. Other discrepancies included IFNγ + IL-4, IFNγ + TGF-β1 and IFNγ + IL-4 + TGF-β1 inducing similar amounts of generic HLA-DR expression, suggesting that either IL-4 or TGF-β1 could act synergistically with IFN-γ. All three cytokines together also induced the highest expression of DRβ3, but did not further augment generic HLA-DR or DRβ1*04 as previously indicated by flow cytometry. The discrepancies identified here will be addressed in Chapter 4.

CELISA analysis also revealed that DRβ1*13 (TAL 8.1) was not constitutively expressed on BT-20, suggesting that constitutive HLA-DR (L243) expression was HLA-DRβ4. DRβ1*13 expression was unaffected by IL-4, TGF-β1, or IL-4 + TGF-β1. Expression also remained less than twice the background following IFN-γ and IFN-γ + IL-4. Addition of TGF-β1 to IFN-γ induced positive levels of expression, and IFN-γ + IL-4 + TGF-β1 did not indicate any synergistic activity of all three cytokines on DRβ1*13 expression.

Taken together, flow and CELISA results for BT-20 suggest that DRβ1*04 was poorly up-regulated by IFN-γ and combined cytokine treatments. Our results also suggest that TGF-β1 inhibits constitutive HLA-DR expression on BT-20, but in combination with IFN-γ, TGF-β1 augments IFN-γ induction of generic and allelic HLA-DR. This suggests that TGF-β1 alone acts differently than when combined with IFN-γ to modulate HLA-DR expression on BT-20. Furthermore, as we showed that IFN-γ + TGF-β1 induced HLA-DR expression on BT-20 and SKBR3, but suppressed expression on MDA MB 157 and MDA MB 231, we suggest the effects mediated by these cytokines are cell line specific.

3.8.1.5. Summary of the Differential Effects Mediated by Cytokines on HLA-DR Expression on ER-negative Breast Cancer Cell Lines

A summary of the differential effects mediated by individual and combined cytokine treatments on ER- BCCL is presented in Table 3.3. Only MDA MB 231 and BT-20 constitutively expressed HLA-DR, and in each case, expression was down-regulated by TGF-β1. IL-4 + TGF-β1 also reduced constitutive HLA-DR on MDA MB 231, but not BT-20. In contrast, IL-4 did not modulate generic or allelic HLA-DR expression on any ER- breast cancer cell line.

All ER- cell lines were responsive to IFN-γ and up-regulated HLA-DR to varying degrees. This induction may depend on the allotypes expressed as DRβ1*04 was not up-regulated on MDA MB 157, and BT-20 poorly up-regulated DRβ1*04 and DRβ1*13.

IFN-γ + IL-4 had inconsistent effects on MDA MB 231, SKBR3, and BT-20. However, flow cytometry indicated that IL-4 had antagonistic effects on IFN-γ induced HLA-DR on MDA MB 231, but augmentative effects on SKBR3 which carries the same allotypes as MDA MB 231. TGF-β1 also had antagonistic effects on IFN-γ induced HLA-DR on MDA MB 231 and MDA MB 157, while it had the opposite effect on SKBR3 and BT-20.

All three cytokines together followed similar expression patterns as IFN-γ + TGF-β for each cell line. Thus, it appears that the modulatory effects of IFN-γ + IL-4, IFN-γ + TGF-β1, and IFN-γ + IL-4 + TGF-β do not occur in an allotype-specific manner in ER- breast cancer cell lines.

Table 3.3. Summary of the differential effects [a] on HLA-DR allelic products mediated by cytokine treatments on ER- breast cancer cell lines.

	Cell Line	HLA-DR allele	Constitutive expression	Effects of Cytokine Treatment on HLA-DR Expression				Effects on HLA-DR Expression in Relation to IFNγ		
				IL-4	TGF-β1	IL-4 + TGF-β1	IFN-γ	IFN-γ + IL-4	IFN-γ + TGF-β1	IFN-γ + IL-4 + TGF-β1
ER-	MDA MB 157	HLA-DR	-	-	-	-	↑	-	↓	↓
		DRβ1*04	-	-	-	-	-	-	-	-
		DRβ1*15	-	-	-	-	↑	-	↓	↓
	MDA MB 231	HLA-DR	+	-	↓	↓	↑	↓ (-)	↓	↓
		DRβ1*07	-	-	-	-	$\uparrow_w$	↓ (-)	↓	↓
		DRβ3	+	-	↓	↓	↑	↓ (-)	↓	↓
	BT-20	HLA-DR	+ (-)	-	↓ (-)	-	↑	- (↑)	↑	↑
		DRβ1*04	-	-	-	-	$\uparrow_w$ (-)	-	↑ (-)	↑ (-)
		DRβ1*13	-	-	-	-	-	-	↑	↑
		DRβ3	-	-	-	-	$\uparrow_w$	↓ (↑)	↑	↑
	SKBR3	HLA-DR	-	-	-	-	↑	↑	↑	↑
		DRβ1*07	-	-	-	-	↑ (-)	↑ (-)	↑ (-)	-
		DRβ3	-	-	-	-	↑	↑	↑ (-)	↑ (-)

[a] Expression was considered positive if values were twice the background

+ indicates detectable expression; - indicates no effect; ↑ indicates increased expression; $\uparrow_w$ = weak increased expression;

↓ indicates decreased expression;

( ) indicates discrepant CELISA results.

3.8.2. Estrogen Receptor-alpha Positive Breast Cancer Cell Lines

3.8.2.1. MCF-7

The effects of cytokine combinations on allelic expression of MCF-7 was detected using the antibodies L243, pan HLA-DR; NFLD.D10, HLA-DRβ1*15; and NFLD.M1, HLA-DRβ3. CELISA analysis also incorporated TAL8.1 for detection of HLA-DRβ1*03. Expression of HLA-DRβ5 could not be evaluated due to lack of specific mAbs.

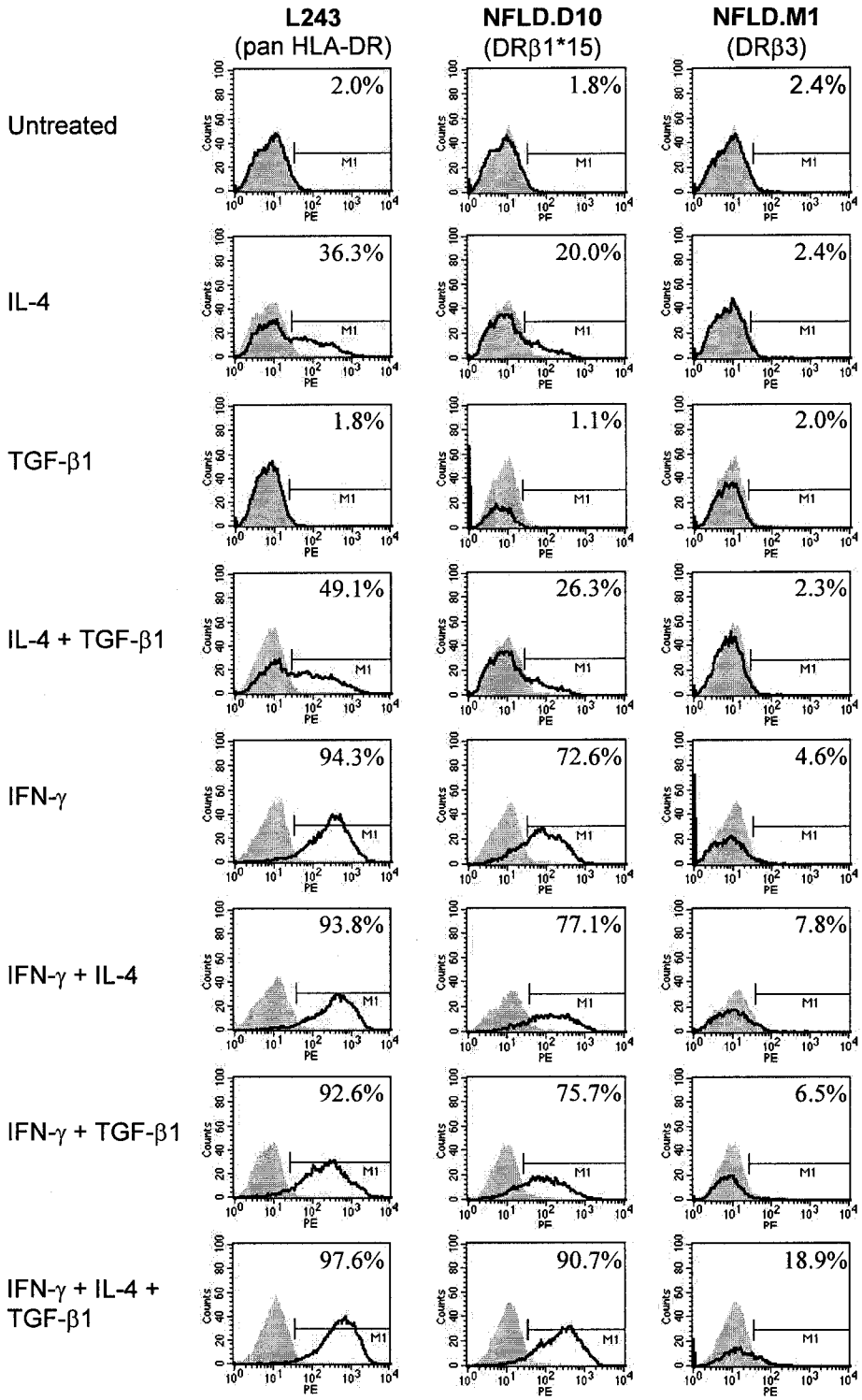
Flow cytometry analysis did not reveal any constitutive generic HLA-DR (L243), DRβ1*15 (NFLD.D10), or DRβ3 (NFLD.M1) expression on MCF-7. Generic HLA-DR and DRβ1*15 were induced by IL-4 and IL-4 + TGF-β1, but not by TGF-β1, as indicated by increased percentage of positive cells (Figure 3.21A) and fluorescence intensity (Figure 3.21B). However, neither IL-4, nor TGF-β1, nor IL-4 + TGF-β1 modulated DRβ3 expression. IFN-γ strongly up-regulated expression of generic HLA-DR and DRβ1*15, but not DRβ3, suggesting that DRβ3 was poorly expressed by MCF-7.

The combination of IL-4 and IFN-γ increased the fluorescence intensity of cells stained with L243 and NFLD.D10, but had marginal effects on the percentage of positive cells. In contrast, IFN-γ + TGF-β1 had no effect on HLA-DR or DRβ1*15. IFN-γ + IL-4 and IFN-γ + TGF-β both increased the percentage of cells stained with NFLD.M1, but in both cases, expression was less than twice the background and was therefore considered negligible. The highest percentage and expression of HLA-DR, DRβ1*15, and DRβ3 resulted following IFN-γ + IL-4 + TGF-β1 treatment.

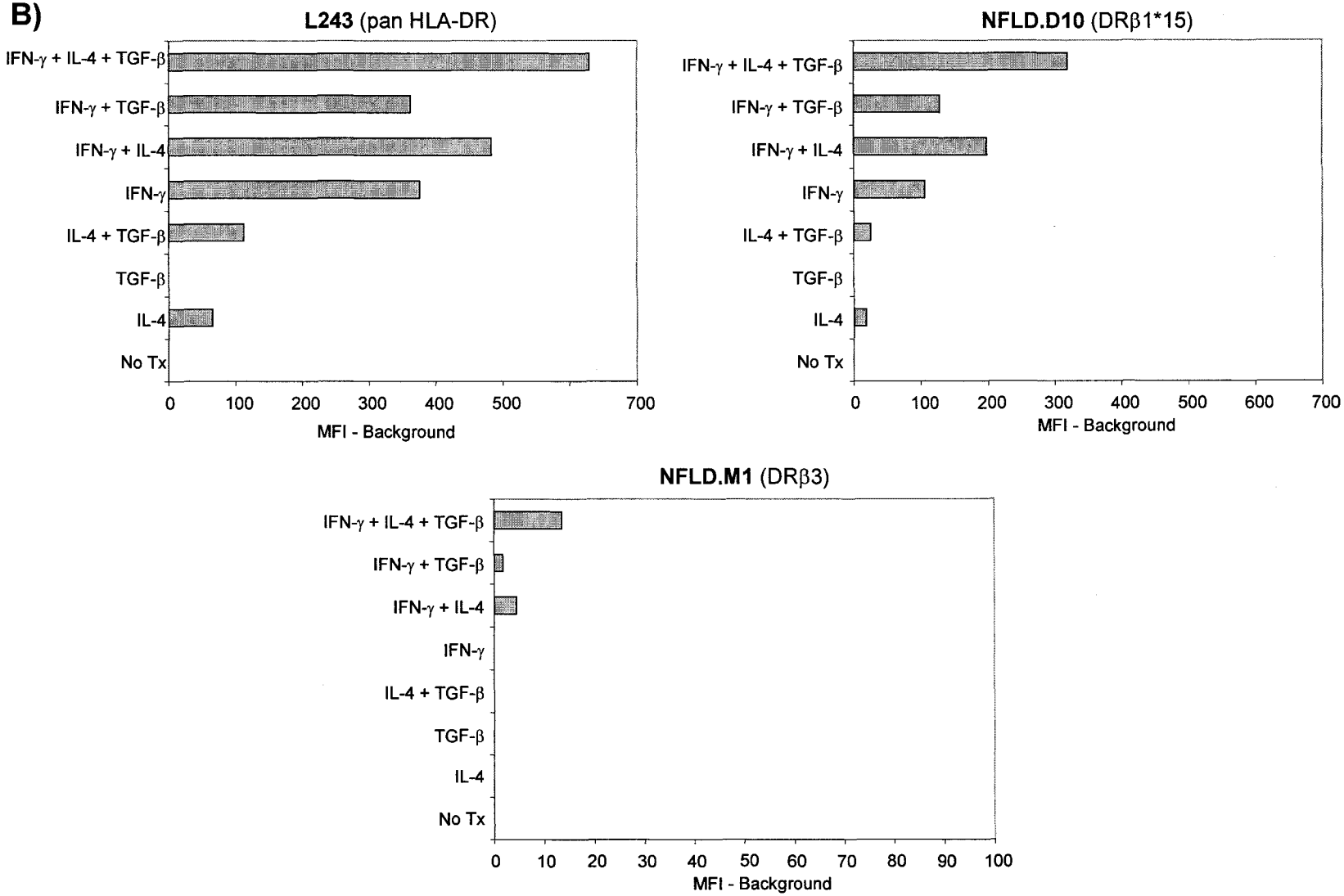
The results of CELISA analysis, depicted in Figure 3.21C, revealed some discrepancies with flow cytometry results, which will be addressed in Chapter 4. Neither IL-4 nor IL-4 + TGF-β1 induced generic HLA-DR (L243), DRβ1*15 (NFLD.D10), or DRβ1*03 (TAL 8.1). Furthermore, addition of TGF-β to IFN-γ had no effect on general HLA-DR expression, while its effects on DRβ1*15, DRβ1*03, and DRβ3 were negligible. Similarly, addition of IL-4 did not augment IFNγ-induced generic HLA-DR or DRβ1*03, and the slight up-regulation of DRβ1*15 was negligible.

Taken together, flow and CELISA results establish that TGF-β does not inhibit IFN-γ-induced HLA-DR on MCF-7. The results also indicate that TGF-β1 may act synergistically with IFN-γ + IL-4 to up-regulate, rather than suppress, generic and allelic HLA-DR expression on ER+ BCCL. Differential up-regulation of HLA-DR allotypes was displayed by MCF-7 as both DRβ1*03 and DRβ3 were not up-regulated by IFN-γ.

A)


L243
(pan HLA-DR)
NFLD.D10
(DRβ1*15)
NFLD.M1
(DRβ3)
Untreated
2.0%
1.8%
2.4%
IL-4
36.3%
20.0%
2.4%
TGF-β1
1.8%
1.1%
2.0%
IL-4 + TGF-β1
49.1%
26.3%
2.3%
IFN-γ
94.3%
72.6%
4.6%
IFN-γ + IL-4
93.8%
77.1%
7.8%
IFN-γ + TGF-β1
92.6%
75.7%
6.5%
IFN-γ + IL-4 +
TGF-β1
97.6%
90.7%
18.9%
Counts
PE
M1

B)


L243 (pan HLA-DR)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0
100
200
300
400
500
700
MFI - Background
NFLD.D10 (DRβ1*15)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0
100
200
300
400
500
600
700
MFI - Background
NFLD.M1 (DRβ3)
IFN-γ + IL-4 + TGF-β
IFN-γ + TGF-β
IFN-γ + IL-4
IFN-γ
IL-4 + TGF-β
TGF-β
IL-4
No Tx
0
10
20
30
40
50
60
70
80
90
100
MFI - Background

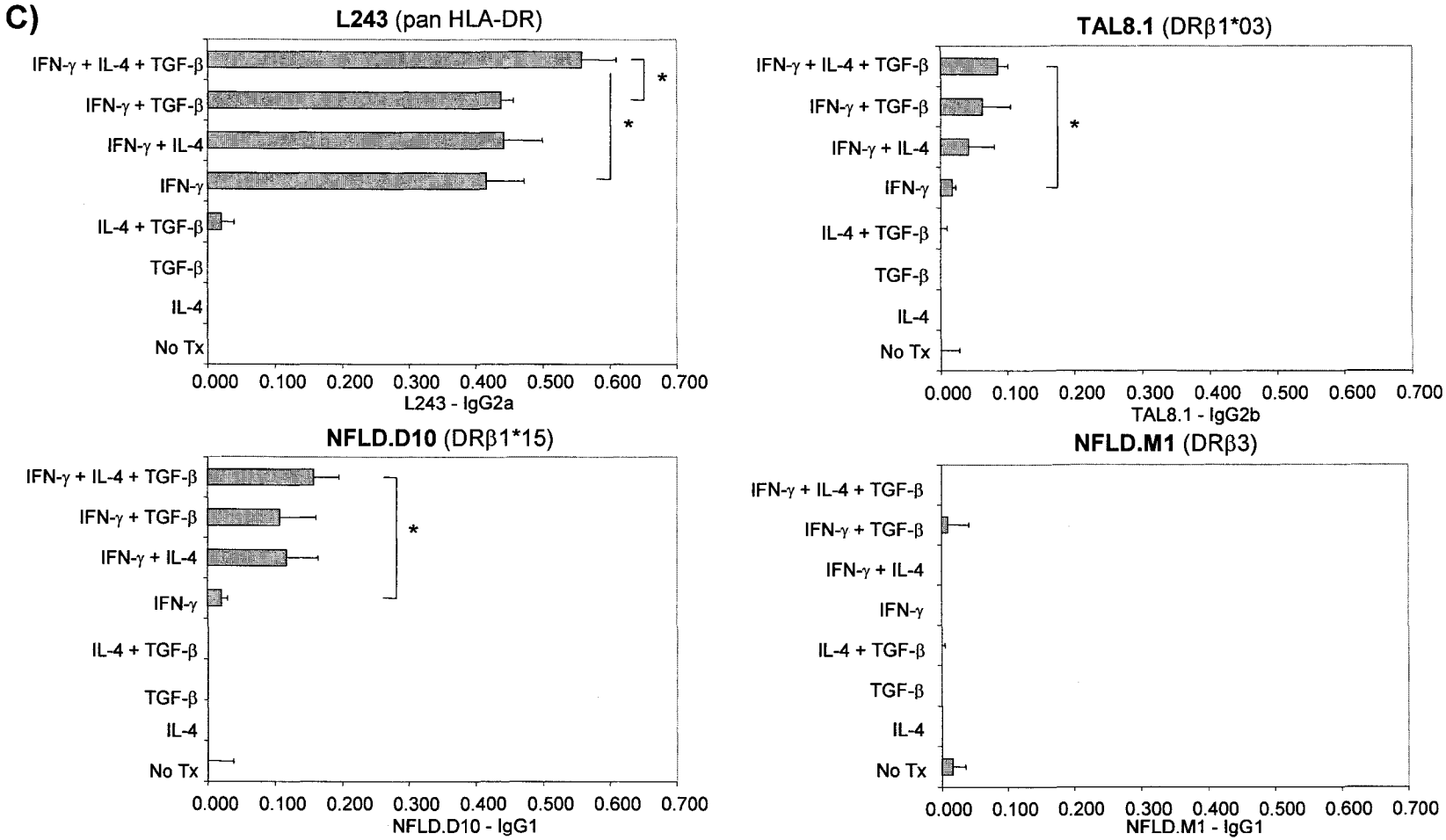


Figure 3.21. Effects of cytokines on HLA-DR allelic expression on MCF-7. HLA-DR expression was assessed using Abs: L243 (pan HLA-DR); TAL8.1 (DRβ1*03); NFLD.D10 (DRβ1*15); NFLD.M1 (DRβ3). Results are reported as: (A) histogram overlays, filled histograms = IgG isotype control, open histogram = Test mAb, and % indicate the percentage of positive cells; (B) $MFI_{Test} - MFI_{Backgorund}$ as measured by flow cytometry; and (C) $OD_{Test} - OD_{Background}$ as measured by CELISA. $^{*}p < 0.05$.

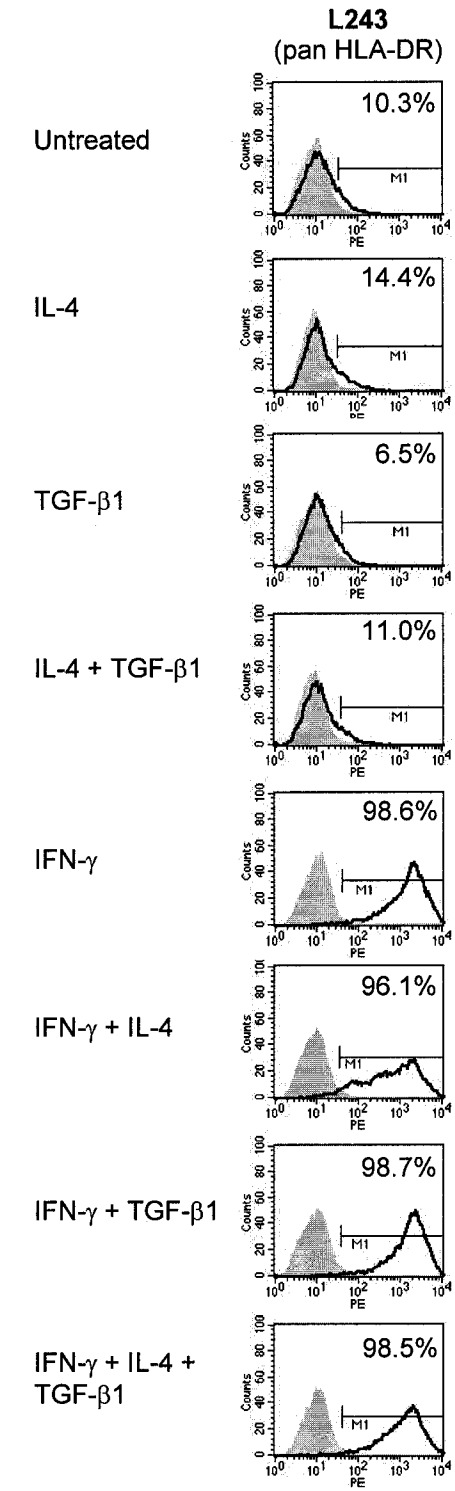
3.8.2.2. T47D

To further evaluate the effects of cytokine co-culture on ER+ BCCL, experiments were performed on T47D. Since T47D only carries HLA-DRβ1*0102 [210] cytokine effects were detected using L243.

Negligible amounts of constitutive HLA-DR were detected on T47D by flow cytometry (Figures 3.22A and 3.22B) and CELISA (Figure 3.22C). Expression was unmodulated by IL-4, TGF-β1, or IL-4 + TGF-β1, but was strongly induced by IFN-γ, as indicated by increased percentage of positive cells (Figure 3.22A), fluorescence intensity (Figure 3.22B), and optical density (Figure 3.22C). Although the percentage of positive cells was relatively unaffected by addition of either IL-4 or TGF-β1 to IFN-γ treatment, IL-4 inhibited IFN-γ-induced HLA-DR fluorescence intensity and optical density, while TGF-β1 had no effect. Treatment with IFN-γ + IL-4 + TGF-β1 also resulted in inhibition of IFN-γ induced HLA-DR expression.

As both T47D and MCF-7 are ER+ BCCL, these results confirm TGF-β1 does not inhibit IFN-γ-induced HLA-DR on ER+ BCCL. Furthermore, since TGF-β1 did not augment IFN-γ + IL-4 induction of generic and allelic HLA-DR on T47D, as previously observed on MCF-7, and these two cell lines have no common HLA-DR alleles, these results suggest that the effects mediated by cytokine combinations on HLA-DR expression are cell line specific.

A)


L243
(pan HLA-DR)
Untreated
10.3%
IL-4
14.4%
TGF-β1
6.5%
IL-4 + TGF-β1
11.0%
IFN-γ
98.6%
IFN-γ + IL-4
96.1%
IFN-γ + TGF-β1
98.7%
IFN-γ + IL-4 + TGF-β1
98.5%
M1
Counts
PE

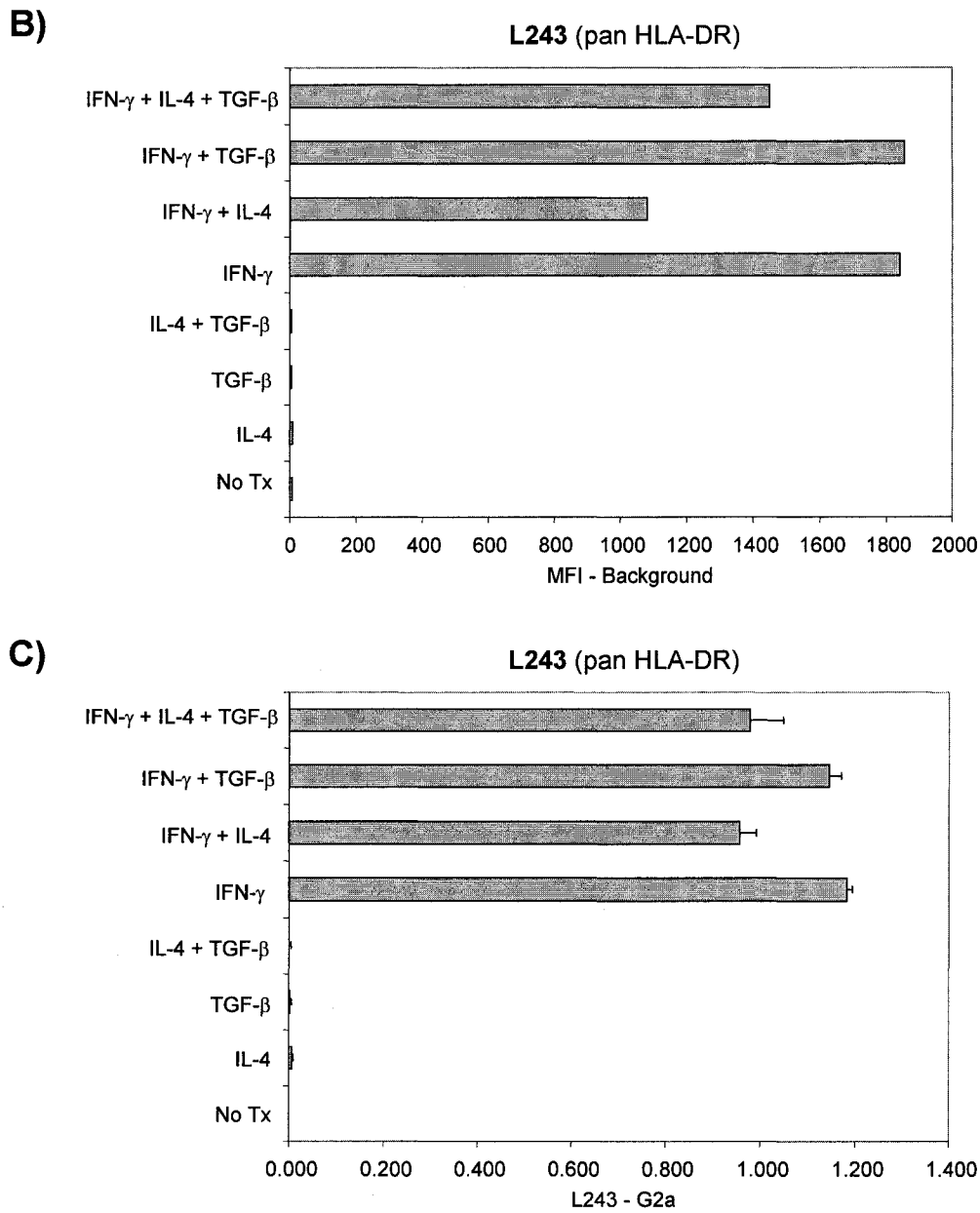


Figure 3.22. Effects of cytokines on HLA-DR expression on T47D. HLA-DR expression was measured using the antibody L243 (pan HLA-DR). Results are reported as: (A) histogram overlays where filled histograms = IgG isotype control, open histogram = Test mAb, and percents indicate the percentage of positive cells; (B) $MFI_{Test} - MFI_{Backgorund}$ as measured by flow cytometry; and (C) $OD_{Test} - OD_{Background}$ as measured by CELISA.

3.8.2.3. Summary of the Differential Effects Mediated by Cytokines on HLA-DR Expression on ER-positive breast cancer cell lines.

A summary of the differential effects mediated by individual and cytokine combinations on HLA-DR expression for ER+ breast cancer cell lines is presented in Table 3.4. Neither MCF-7 nor T47D constitutively expressed HLA-DR, and in both cases expression was unaffected by TGF-β1. Results for IL-4 and IL-4 + TGF-β1 were inconsistent on MCF-7, but flow cytometry suggested that IL-4 up-regulated generic HLA-DR and DRβ1*15. In contrast, T47D was unaffected by either treatment involving IL-4.

Both cell lines were responsive to IFN-γ to varying degrees, but DRβ1*03 and DRβ3 were not up-regulated on MCF-7, suggesting that these proteins were poorly expressed.

IL-4 augmented IFN-γ induced generic HLA-DR and DRβ1*15 on MCF-7, but antagonized HLA-DR on T47D. This suggested that the modulatory effects mediated by cytokines were cell line specific. However, TGF-β1 did not modulate expression on either cell line, indicated that TGF-β1 was not inhibitory to ER+ breast cancer cell lines.

Intriguingly, weak positive expression of DRβ1*03 and DRβ3 was only detected on MCF-7 following IFN-γ + IL-4 + TGF-β1, suggesting that allotypic expression may depend on the surrounding cytokine milieu. This treatment also up-regulated generic HLA-DR and DRβ1*15 on MCF-7, while it down-regulated HLA-DR on T47D, again indicated that modulatory effects were cell line specific.

Table 3.4. Summary of the differential effects [a] on HLA-DR allelic products mediated by cytokine treatments on ER+ breast cancer cell lines.

	Cell Line	HLA-DR allele	Constitutive expression	Effects of Cytokine Treatment on HLA-DR Expression				Effects on HLA-DR Expression in Relation to IFNγ		
				IL-4	TGF-β1	IL-4 + TGF-β1	IFN-γ	IFN-γ + IL-4	IFN-γ + TGF-β1	IFN-γ + IL-4 + TGF-β1
ER+	MCF-7	HLA-DR	-	↑ (-)	-	↑ (-)	↑	↑ (-)	-	↑
		DRβ1*03	-	-	-	-	-	-	-	↑
		DRβ1*15	-	↑ (-)	-	↑ (-)	↑ (-)	↑	-	↑
		DRβ3	-	-	-	-	-	-	-	↑ (-)
	T47D	HLA-DR	-	-	-	-	↑	↓	-	↓

[a] Expression was considered positive if values were twice the background.
+ indicates detectable expression; - indicates no effect; ↑ indicates increased expression; ↓ indicates decreased expression
( ) indicates discrepant CELISA results.

3.9. Determining BCCL TGF-β1 Sensitivity

Western blots were performed to determine BCCL responsiveness to TGF-β1 stimulation, as indicated by phosphorylation of Smad2 and Smad3 proteins. As described in Section 1.4.3, Smad2/3 are key proteins involved in TGF-β intracellular signaling that become activated upon phosphorylation, forming a complex with Smad4 which translocates to the nucleus and effects the transcription of TGF-β sensitive genes. The presence of phosphorylated-Smad2/3 (P-Smad2, P-Smad3) indicates active internal TGF-β signaling and functional TGF-β receptors on the cell surface. Thus, the absence of P-Smad2/3 suggests the absence of one or more of the TGF-β receptors (TGFβ-RI, TGFβ-RII) on the cell surface, or alternatively, that the receptors are present but nonfunctional.

As shown in Figure 3.23, anti-P-Smad3 analysis indicated two bands in BT-20, MDA MB 157, MDA MB 231, MDA MD 468, and SKBR3. This work was performed in collaboration with Dr. Jules Dore using an Ab derived from rabbit anti-serum. P-Smad3 is the lower of these two bands, with the upper band believed to be cross-reaction with total Smad3 or evidence of a contaminating Ab (Jules Dore , personal communication).

P-Smad3 was found in all ER- BCCL, illustrating that ER- BCCL were sensitive to TGF-β1 treatment. Weak P-Smad3 expression was noted in SKBR3 compared to other ER- cell lines. Among the ER+ BCCL, P-Smad3 was detected in MCF-7, but not BT-474 or T47D. Weak bands were visible in BT-474 and T47D, however, as these cell lines lacked Smad3 expression, this detection was believed to be artifactual or due to contaminating Ab in the rabbit anti-serum. Immunoblotting with anti-Smad3 confirmed there were equivalent amounts of Smad3 in treated and untreated cells. These results are

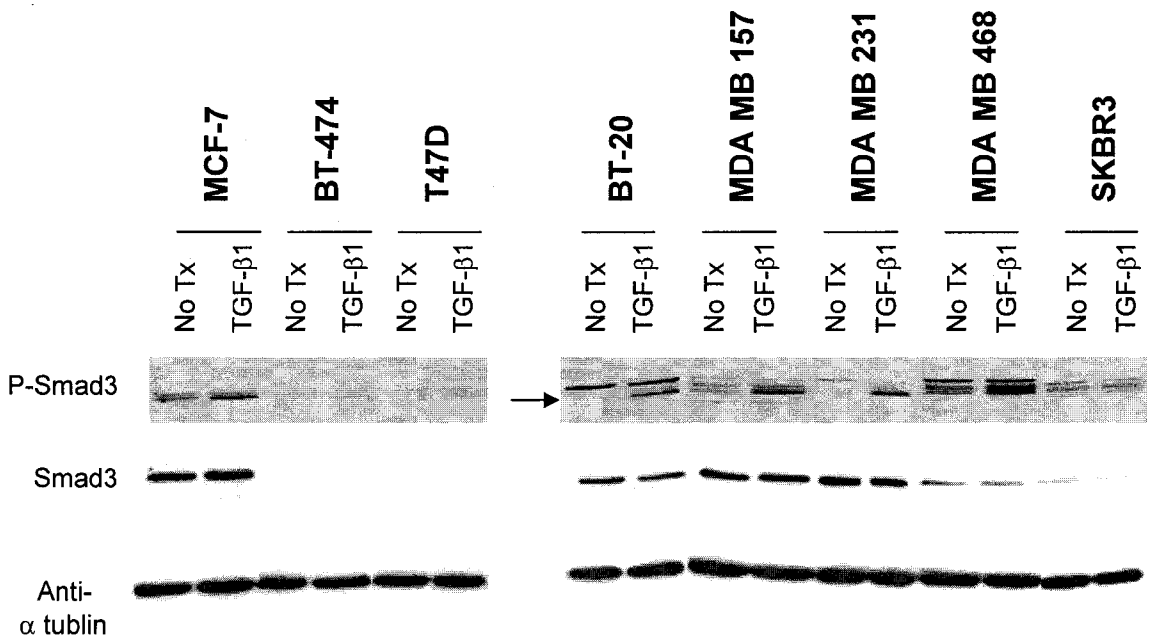


Figure 3.23. TGF-β1-mediated phosphorylation of Smad3 in breast cancer cell lines. Cells were stimulated with 5 ng/ml TGF-β1 for 1 hour and Smad3 phosphorylation was determined by Western blotting. Total Smad3 and anti-α tubulin were detected as controls. The arrow indicates the location of P-Smad3. Results are representative examples of duplicate experiments.

consistent with a loss of TGF-β sensitivity in most ER+ BCCL, as indicated in several previous studies [240, 244-248].

Notably, MCF-7, MDA MB 157, MDA MB 468, and SKBR3 (weak) were found to express constitutive P-Smad3. Furthermore, TGF-β1 treatment increased the amount of P-Smad3 in MCF-7, MDA MB 157, MDA MB 468, but not SKBR3.

In contrast to P-Smad3, expression of P-Smad2 appeared more cell-line specific. P-Smad2 was detected in MDA MB 157, MDA MB 231, MDA MB 468 and BT-474, but was not detected in MCF-7, T47D, BT-20, or SKBR3. Furthermore, of cell lines displaying P-Smad2, none appeared to do so constitutively. Immunoblotting with anti-Smad2 confirmed there were equivalent amounts of Smad2 in treated and untreated cells. These results suggest that only selective BCCL are capable of mediating P-Smad2-dependent signaling in response to TGF-β1 stimulation.

Taken together, analysis of Smad 2 and Smad 3 phosphorylation in BCCL suggest that only T47D may lack or have a nonfunctional TGF-β receptor on the cell surface as neither P-Smad2 nor P-Smad3 was detected in this cell line. All other BCCL displayed P-Smad2 and/or P-Smad3, or increased levels of P-Smad3 in response to TGF-β1 stimulation (Table 3.5). However, no P-Smad2 or increase in constitutive P-Smad3 was detected in SKBR3, suggesting that TGF-β1 augmentation of IFN-γ-induced HLA-DR expression in SKBR3 may be mediated through a Smad-independent pathway.

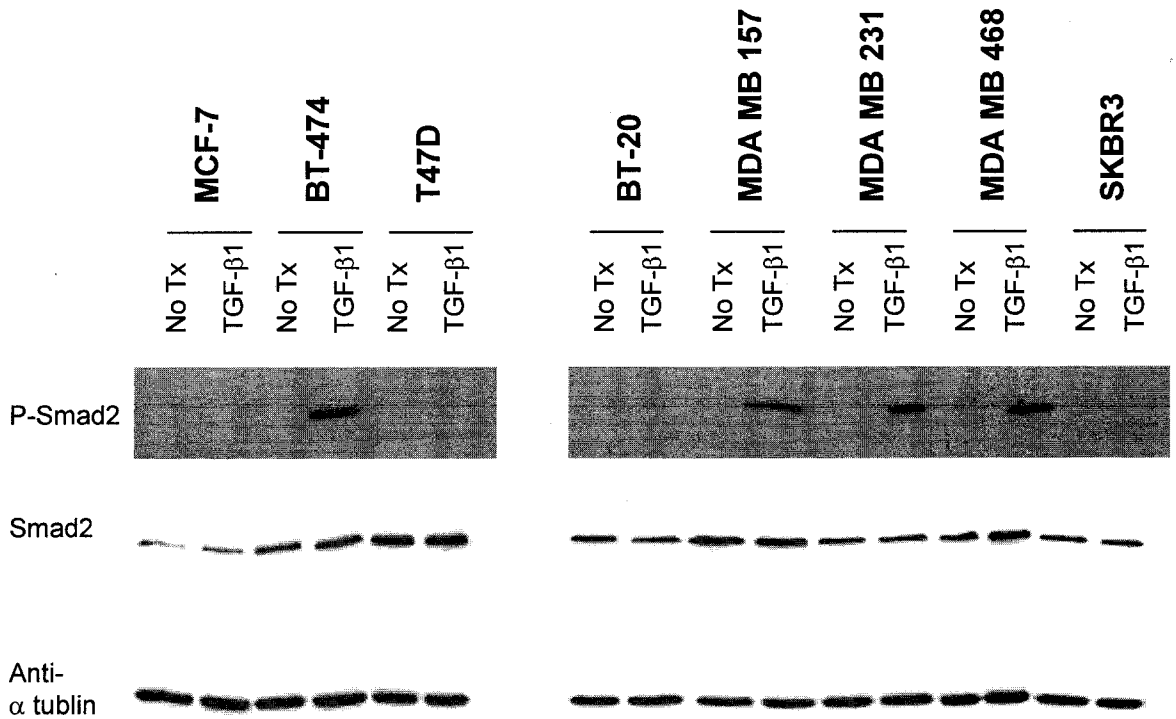


Figure 3.24. TGF-β1-mediated phosphorylation of Smad2 in breast cancer cell lines. Cells were stimulated with 5 ng/ml TGF-β1 for 1 hour and Smad2 phosphorylation was determined by Western blotting. Total Smad2 and anti-α tubulin were detected as controls. Results are representative examples of duplicate experiments.

Table 3.5. Detection of Smad proteins in breast cancer cell lines through Western blotting.

Breast Cancer Cell Line	Smad 3		P-Smad3		Smad2		P-Smad2	
	No Tx	TGF-β	No Tx	TGF-β	No Tx	TGF-β	No Tx	TGF-β
MCF-7	+	+	+	+	$+_w$	$+_w$	-	-
BT-474	-	-	-	-	+	+	-	+
T47D	-	-	-	-	+	+	-	-
BT-20	+	+	-	+	+	+	-	-
MDA MB 157	+	+	$+_w$	+	+	+	-	+
MDA MB 231	+	+	-	+	+	+	-	+
MDA MB 468	$+_w$	$+_w$	+	+	+	+	-	+
SKBR3	$+_w$	$+_w$	$+_w$	$+_w$	+	+	-	-

+ = detectable expression; $+_w$ = weak detectable expression; - = no detectable expression.

Chapter 4: Discussion

Ongoing research in our laboratory is focused on the mechanisms and significance of HLA-class II expression on breast cancer cells. A previous study by A.E. Edgecombe [210] clearly showed that a minority of breast cancer cell lines have constitutive class II expression, while the majority is inducible by treatment with IFN-γ. Her work also revealed differential expression of HLA-class II (DR, DQ and DP) and co-chaperones. Recently, our laboratory showed that DR-positive tumor cells in breast carcinoma tissues differentially express their DR alleles [249]. Subsequently, it was found that expression is influenced by IFN-γ, IL-4 and TGF-β and is clinically associated with ERα and age of diagnosis (Sharon Oldford, personal communication). This implies that HLA-DR expression by breast cancer cells is modulated by estrogen and the cytokine milieu. Based on the background of these findings, the work described in this thesis was aimed at determining how these interacting factors affect HLA-DR expression in ER+ and ER- cell lines.

4.1. Summary of Key Findings

- Constitutive and IFN-γ-induced HLA-DR expression on ER+ and ER- BCCL was affected by culturing the cells in E_2-containing and E_2-depleted medium, suggesting that estrogen had modulatory effects on DR expression. The individual effects of estradiol could not be ascertained as reconstitution solvents (ethanol, DMSO, 1-butanol, and methanol) suppressed HLA-DR expression on breast cancer cell lines.

- TGF-β1 mRNA in ER+ and ER- BCCL was affected by culturing the cells in E_2-containing and E_2-depleted medium, suggesting transcription was modulated by estradiol concentrations.
- Two of 8 breast cancer cell lines constitutively expressed HLA-DR, which was inhibited by TGF-β1 and correlated with differential expression of constitutive P-Smad3 and P-Smad2.
- IFN-γ up-regulated generic HLA-DR molecules on all cell lines to varying degrees. Aside from DRβ1*04, which was poorly up-regulated on BT-20 and MDA MB 157, all other allotypes were up-regulated. Overall, the degree of IFN-γ induction did not depend on the allotypes expressed by particular breast carcinoma cells.
- IL-4 alone and combined with IFN-γ, selectively modulated HLA-DR expression in a cell line-specific manner.
- TGF-β1 mediated differential effects on HLA-DR expression on breast cancer cell lines. In contrast to studies indicating TGF-β suppression of CIITA, TGF-β1 augmented DR expression on SKBR3 and BT-20. Analysis of P-Smad2 and P-Smad3 indicated these differential effects may be explained by differential expression of Smad signaling components, and suggested IFN-γ + TGF-β1 synergy may occur in a Smad-independent manner. TGF-β1 did not modulate DR on ER+ cell lines.

- The effects of IL-4 + TGF-β on constitutive and IFN-γ-induced HLA-DR expression suggest that the quantity and function of IL-4 and TGF-β receptors on the cell surface direct the effects of combined cytokine treatment.
- Our study indicates that breast cancer cell lines mediate cell-line specific effects. Thus, studies employing cell lines need to be carefully evaluated before the results of *in vitro* studies can be translated to *in vivo* situations.

4.2. Study Concerns

4.2.1. Cell Lines

Breast cancer cell lines used in this study were kindly provided by Dr. Shou-Ching Tang and Dr. Alan Pater (Section 2.1). The identity of each of these cell lines was previously confirmed by A.E. Edgecombe via comparison of short tandem repeat sequences published by the ATCC [210]. Whether these breast cancer cell lines are representative of the tumors from which they originated, and whether their diversity reflects inter-tumoral heterogeneity remains matters of debate. Some argue that prolonged *in vitro* culture of breast cancer cells in artificial conditions modify the properties of cell lines making them genetically different from the initial population. Furthermore, inter-laboratory differences are known to occur for commonly used cell lines, conflicting data comparison. For example, MCF-7 cell stocks from different laboratories have been shown to differ in sensitivity to 17β-estradiol, associated with variations in ER protein and mRNA levels [250].

4.2.2. Experimentation Concerns

Cytokine-mediated effects on HLA-DR expression on breast cancer cell lines were analyzed by both flow cytometry and CELISA. However, sometimes discrepancies were evident between these two experimental assays, causing limitations that will be discussed in Section 4.5.1.

4.3. Estrogen Modulation of HLA-DR expression on Breast Carcinoma Cell Lines

4.3.1. Media Composition Affects HLA-DR Expression on Breast Carcinoma Cell Lines

As previously reviewed in Section 1.5.1, numerous studies have indicated that 17β-estradiol has immunomodulatory effects on MHC class II expression on a variety of cell types. To analyze the effects of estradiol on HLA-DR expression on breast cancer cell lines, we began by comparing HLA-DR expression on cells cultured in standard medium and E_2-depleted medium which differed particularly in the content of steroidal and nonsteroidal estrogens.

Collectively these results indicated that media composition, and particularly estrogen in culture media, differentially affected constitutive and IFN-γ inducible HLA-DR expression on breast cancer cell lines. Since media composition did not modulate HLA-DR expression on MDA MB 468, BT-20, MCF-7, or BT-474, our results did not indicate unanimous trends for ER- or ER+ breast carcinoma cell lines. Thus, we suggest that the effects mediated by media composition and estrogen on HLA-DR expression are cell-line specific. Moreover, as IFN-γ induction of HLA-DR on SKBR3 (ER-) was higher in E_2-containing media, our results suggested that estrogen could affect HLA-DR

expression on both ER+ and ER- cells lines. This posed the question of how estrogen-mediated modulation may occur in ER- cell lines, and suggested that ER- breast cancer cells are not totally insensitive to estrogen. Through *in vitro* studies, others have also indicated that some ER- breast cancer cell lines respond to estrogens and antiestrogens, suggesting that these compounds act through an alternative mechanism than ERα [179].

Although we have concentrated on effects mediated potentially by differences in steroidal and non-steroidal estrogen content, it is possible that other factors may be implicated. For example, the amounts of creatine, glucose, insulin, thyroxine, and phosphorous differ between normal FCS and charcoal dextran-treated FCS according to the composition information provided by the manufactures (Invitrogen and Hyclone, respectively). The effects of such factors on HLA-DR expression on breast carcinoma cell lines is presently unknown, and thus may warrant further investigation.

Notably, our results are contradictory to previous studies reporting constitutive HLA-DR expression on BT-20, MDA MB 231, T47D, SKBR3, and MCF-7 cultured in standard medium [210, 251]. These discrepancies are not surprising, given that differences in cell line passage number may affect the ability to express different molecules, and cultured cells may acquire mutations over time. Batches of FCS may also vary in hormone and chemical content, altering medium composition.

In agreement with other studies [210, 212, 213, 252], we found strong HLA-DR up-regulation on MDA MB 231, SKBR3, MDA MB 157, BT-20, and T47D in response to IFN-γ.

4.3.2. Differential Expression of ERα mRNA in Breast Carcinoma Cell Lines

ERα transcription was examined in all 8 breast cancer cell lines by RT-PCR using primers specific to a 550-base-pair wild-type fragment from exon 2 of human ERα cDNA [226]. Consistent with documentation provided by the ATCC, we found ERα mRNA expression in BT-474, T47D, and MCF-7, but not in MDA MB 231, MDA MB 468 and SKBR3 (Figure 3.4A). In addition, we found ERα mRNA in BT-20, which is classified as ER- by ligand binding analysis, but was previously shown to express a variant ERα transcript that contains an exon 5 deletion [236, 253]. Sequence analysis of this BT-20 variant ER transcript has revealed a frame-shift alteration resulting in a stop codon at the 5' end of exon 6, which translates into expression of a truncated form of the receptor lacking the majority of the hormone-binding domain [254]. Since our ERα primers were specific for exon 2, this variant would be amplified by RT-PCR.

We also found ERα mRNA in MDA MB 157 which is in contrast to ATCC reports. We are confident that this discrepancy is not the result of a mislabeled cell line as the identity of each breast cancer cell line was confirmed by comparison with short tandem repeat sequences published by the ATCC [210].

The ERα transcript observed in MDA MB 157 was of similar size as wild-type ERα found in BT-474 (Figure 3.4B), but may also indicate an ERα variant. Variants include exon-deleted, exon-duplicated, or truncated ER mRNA transcripts that encode a variety of incomplete ER-like proteins missing some functional domains of the wild-type receptor [255, 256]. Variant ERα mRNAs have been reported in breast cancer cell lines

other than BT-20, including T47D and MCF-7 [253, 257-259]. A range of ER variant mRNAs also occur in clinical breast cancer and are believed associated with altered responsiveness to estrogens and/or antiestrogens [256, 260-262] and play a role in tumorgenesis [263, 264]. However, since transcription does not necessarily correlate with protein translation, future work to determine whether MDA MB 157 expresses ERα protein should include Western blot analysis and comparison of molecular weight of this protein with wild-type ERα.

An alternative explanation for ERα amplification in MDA MB 157 is cross-reaction of ERα primers with ERβ. Our primers were checked using the *Amplify* program for negative cross-reactivity with the wild-type ERβ sequence, but 5 ERβ isoforms are known to occur. In fact, four of these altered isoforms have been cloned and characterized from MDA MB 157 [265]. Since the human ERβ is highly homologous to human ERα, particularly in the amino-terminal domain (96% amino acid homology) where our primers bind [175], it is possible that one of these isoforms was amplified by our primers. A recent BLAST search also confirmed that our primers may recognize mRNA of estrogen-receptor related variant protein (Accession # M69296), estrogen-related receptor beta (Accession # NM_004452), and estrogen-related receptor beta2 (Accession # AF094517).

4.3.3. Media Composition Affects ERα mRNA in Breast Carcinoma Cell Lines

We found that ERα transcript amplification was much higher for T47D and MCF-7 cultured in standard medium than E_2-depleted medium, suggesting that estradiol levels influenced ERα transcription. These results may be explained by genetic instability provoked by cell culture conditions, such as was described in T47D-5 (T47D subline) which developed estradiol insensitivity when maintained for almost continuous exponential growth [266]. On the other hand, differences in ERα mRNA may involve factors that control expression of ERα.

The expression of ERα mRNA is differentially regulated by estrogen in estrogen-responsive breast cancer cell lines. Estrogen treatment has been shown to decrease ERα mRNA levels in MCF-7 cells [267, 268], and increase ERα mRNA levels in T47D cells [267, 269]. Studies examining short- and long-term culture of T47D and MCF-7 cells in phenol red-free and estrogen-depleted medium also report considerable changes in morphology, growth rate, receptor content, and response to hormones and antihormones [270, 271]. These studies indicated that ER content steadily increased in MCF-7 cultured in low or estrogen-depleted medium [270, 271], whereas ER content and ER mRNA decreased in T47D in response to short-term and long-term estrogen deprivation [218, 272]. Thus, our findings are in agreement with those reported for T47D, but are in contrast to those for MCF-7.

Differential regulation by estrogen may also be due to different promoter usage. Cloning and sequencing of human ERα DNA has identified three promoters (A, B, C) which are coordinately regulated by estrogen [236]. It has been shown that all three

promoters are used by both MCF-7 and T47D for expression of ERα [236]. However, estrogen down-regulated all three promoters in MCF-7, but up-regulated all promoters in T47D [236]. This demonstrated that estrogen regulation varies in a cell-specific manner but not in a promoter-specific manner, and excluded the possibility that a single promoter controlled the expression of ER mRNA levels in breast cancer cells. Furthermore, Donaghue et al. [236] reported that BT-20 cells expressed low levels of ERα transcripts which were derived solely from promoter A, suggesting that low levels of ERα expression in BT-20 could be due to differential promoter usage, the number of promoters used, or lower activity of all promoters. Future research could examine whether similar differential promoter usage or differential promoter activity occurs in MCF-7 and T47D cultured in E_2-depleted medium.

A variety of signaling pathways may also influence ERα expression. Hyperactivation of MAPK signaling pathways, as a result of overexpression or activation of epithelial growth factor receptor (EGF) and Her-2/neu [273, 274], correlates with down-regulation of ERα on breast carcinoma cells [275-277]. As T47D is Her-2/neu$^+$ [278] and MCF-7 is Her-2/neu$^-$ [216, 276], this could potentially explain the reduction of ERα in T47D but not in MCF-7. However, our results suggest that Her-2/neu expression does not always correlate with down-regulation of ERα in breast cancer cell lines, as BT-474, which overexpresses Her-2/neu [216, 276], did not display reduced ERα amplification when cultured in E_2-depleted medium. Consistent with our findings, reports indicate that ERα is down-regulated in MCF-7 cells cultured under estrogen-depleted conditions due to hyperactivation of ERK1/2 [279, 280]. This inhibition may also be

attributable to nuclear factor-kappa B (NFκB) signaling, as inhibition of NFκB partially restored ERα activity and expression in MCF-7 [281]. These breast cancer cell lines may be capable of activating MAPK signaling in an autocrine manner, as Martinez-Carpio et al. [282] demonstrated that MDA MB 231 secretes and possesses EGF receptors. Several reports also indicate that MAPK can directly phosphorylate ERα on serine residue 118 [283, 284]. Thus, if phosphorylation of this residue leads to ubiquination and degradation of ERα, then hyperactivation of MAPK as a result of culture in estrogen-depleted medium could lead to reduced ERα mRNA. Future research could examine whether medium composition modifies MAPK or NFκB signaling in MCF-7 and T47D.

4.3.4. Effects of 17β-estradiol Treatment on HLA-DR Expression on Breast Cancer Cell Lines

A primary objective in this thesis was to examine the effects of 17β-estradiol on constitutive and IFN-γ-induced HLA-DR expression on breast cancer cell lines. However, despite efforts to find alternative reconstitution solvents for estradiol, we were unable to determine the effects of estradiol on HLA-DR expression on MCF-7 or MDA MB 231 due to modulation of HLA-DR by ethanol, DMSO, 1-butanol, and methanol vehicle controls. These results thus emphasize the importance of including appropriate controls when examining the effects of estradiol on HLA-DR expression.

Ethanol is known to affect MHC Class II expression. Studies on mice have indicated that ethanol decreased MHC Class II expression on B cells, and suppressed MHC Class II molecule-mediated T cell responses, but not MHC Class I molecule-

mediated T cell responses, in a dose-response manner [285-287]. Ethanol also appears to mediate other effects on breast carcinoma cells, with results depending on ERα status. Reports indicate that culture of ER+ breast cancer cell lines in ethanol containing medium is associated with increased proliferation rate, ERα content, and ER transcriptional activity, while no effects were seen with ER- breast cancer cells [288-291]. In contrast, we found ethanol concentrations equalivant to that present within our 10^{-6} – 10^{-9} M estradiol solutions were antiproliferative to MCF-7 (data not shown). However, these results may depend on the cell lines investigated, the concentration of ethanol used, the incubation period, and medium composition.

Although we report that both methanol and 1-butanol inhibit MHC class II expression on breast cancer cells, studies suggest that primary alcohols do not always exert similar effects despite similar molecular structure. Etique et al. [292] compared the effects of methanol and 1-butanol to ethanol on MCF-7 cells and found that unlike ethanol, methanol and 1-butanol did not stimulate MCF-7 cell proliferation or increase aromatase mRNA. However, methanol-treated cells displayed increased ERα content, which was not evident in butanol-treated cells [292].

4.4. Cytokine Expression in Breast Cancer Cell Lines

4.4.1. Cytokine mRNA

As our second objective was to analyze the effects of IFN-γ, IL-4, and TGF-β1 combinations on HLA-DR expression on breast carcinoma cell lines, we wondered if the breast cancer cells were transcribing and possible secreting any of these factors. Thus, breast cancer cell lines were assessed using sequence specific primers and RT-PCR (Section 3.6).

Constitutive IFN-γ and IL-4 transcription was not detected in any of the 8 BCCL cultured in either standard or E_2-depleted medium, suggesting that neither IFN-γ nor IL-4 was produced by the breast cancer cell lines themselves.

In contrast, TGF-β1 transcription was detected in 8/8 breast cancer cell lines cultured in standard medium, and 7/8 cultured in E_2-depleted medium. These findings were not surprising as others have shown that TGF-β mRNA is overexpressed in breast cancers and neoplastic breast tissues [293]. TGF-β may also be secreted directly by breast cancer cells and several breast cancer cell lines, including MDA MB 468, MCF-7, T47D, MDA MB 231, BT-20, SKBR3, and Hs578T [244, 294-296]. TGF-β is secreted as a latent protein by tumor cells, but once activated, confers a selective advantage to the tumor *in vivo* by suppressing the cytotoxic activity of infiltrating lymphocytes [297] and antigen presentation by APCs [298, 299], allowing the tumor to proliferate and progress. The activation of latent TGF-β is poorly understood, but is believed to involve certain proteases. Therefore, if breast cancer cell lines are capable of secreting both TGF-β and

protease enzymes, they could activate TGF-β within culture media and possibly mediate an autocrine effect.

We found that medium conditions influence TGF-β1 transcription. TGF-β1 amplification was more intense in BT-474 and T47D cultured in standard medium, while it was more intense in MDA MB 157, MDA MB 231, MDA MB 468, MCF-7 and BT-20 cultured in E_2-depleted medium (Figure 3.13). TGF-β1 amplification was also detected in SKBR3 cultured in standard medium but not E_2-depleted medium. These results suggested that estradiol influenced TGF-β1 transcription in breast carcinoma cells. Since TGF-β1 transcription did not correlate with estrogen receptor status, which is in agreement with previous reports [300], we suggest that estradiol modulation occurs in a cell line specific-manner.

Although we found that TGF-β1 transcription was slightly increased in MCF-7 cultured in E_2-depleted medium, it appears that some controversy exists over this issue in the literature. Herman and Katzenellenbogen [301] assessed long-term effects of steroid deprivation and reported that mRNA for TGF-β1 was transiently increased at 2 to 10 weeks of steroid deprivation. On the contrary, others have reported that estrogen did not modulate TGF-β1 mRNA levels in MCF-7 at 48 hour or 7 day time points [302, 303], which are more similar to our study.

4.4.2. Cytokine Receptor Concerns

This study did not examine the expression of IFN-γ, IL-4, or TGF-β receptors on the surface of breast cancer cell lines. However, our laboratory has previously showed that IFN-γ significantly up-regulated HLA-DR on all breast cancer cell lines examined in this study [210]. Thus, these cell lines must possess IFN-γ receptors and components of the IFN-γ-signaling pathway as they displayed sensitivity to IFN-γ stimulation.

Reports also indicate that the IL-4R is highly expressed on human melanoma and ovarian carcinomas compared to normal cells [136]. More relevant to this study, IL-4 receptors are overexpressed on breast cancer cell lines, and breast carcinomas relative to normal breast epithelium [136]. Displacement analysis of ^{125}I-IL-4 binding to breast cancer cells has revealed that MCF-7, BT-20, ZR-75-1, and MDA MB 231 express high affinity IL-4Rs, with receptor numbers varying among cell lines [120]. Thus, we can be fairly confident that three of the cell lines we used expressed IL-4Rs.

On the other hand, loss of TGF-β response due to dysregulation of TGF-β receptors type I (TβRI) and type II (TβRII) is well known to contribute to oncogenesis in a variety of cancer cells [304], including breast carcinoma [305]. Several ER+ breast cancer cell lines are known to be defective of TβRII [240], complicating analysis of TGF-β-mediated effects in breast cancer cell lines. In contrast, ER- BCCL generally retain expression of TβRII but may show a low level sensitivity to TGF-β [239]. The only reported exception to this dogma is that early passage MCF-7 cells, which were initially sensitive to TGF-β, gain resistance after long term passage [239, 245, 296].

Sensitivity to TGF-β may be restored in ER+ BCCL through stable transfection of TβRII [240, 247, 306]. Mutations of TβRI are reported to occur in a low percentage of breast cancers [306], and restoration of TGF-β signaling in breast cancer cell lines with silenced TGFβI may occur through use of histone deacetylase inhibitors [306].

4.5. Cytokine Modulation of HLA-DR Expression on Breast Cancer Cell Lines

To our knowledge, this is the first study to report selective up-regulation of HLA-DR alleles on human cancer cell lines in response to combined cytokine treatment, and the first to examine differential HLA-DR allelic expression in E_2-depleted medium. We decided that cells would be treated with 100 units/ml IFN-γ, 500 units/ml IL-4, and 10 ng/ml TGF-β1 based on preliminary research, cytokine titrations, and previous published studies. The 96 hour incubation period was utilized as it was previously shown to be optimal for IFN-γ induction of HLA-DR on breast cancer cell lines [210].

4.5.1. Assay limitations

HLA-DR specific antibodies, used to assess HLA-DR expression, were shown to bind strongly to HLA-DR allelic products on appropriate B cell line controls (Table 2.4). As HLA-DR molecular conformations may differ on breast carcinoma cells compared to B cell controls, our laboratory has previously established that these mAbs neither recognized cell-type restricted epitopes nor crossreacted with additional DRB allotypes based on examination of various cell types with known DR allotypes. These included IFN-γ-treated ovarian and breast cancer cell lines, peripheral blood mononuclear cells

(from local donors), B cell lines (10^{th} IHW), mouse L-cell fibroblast lines transfected with HLA-DR molecules (11^{th} IHW), and synovial tissue sections [249]. Thus, mAbs used in this assay were selected because they bound HLA-DR molecules on many cell types. Furthermore, each mAb was also previously shown to bind one or more breast cancer cell lines [210], indicating their suitability for binding HLA-DR on breast carcinoma cells. Based on this, failure to detect a particular HLA-DR product is likely due to lack of expression rather than weak antibody reactivity.

Preliminary experiments indicated that despite equivalent detection of DRβ1*04 on B cell line controls (Table 2.4), 359-13F10 was much better than NFLD.D1 for detecting DRβ1*04 on BT-20 (Figure 3.2). This difference could pertain to the epitopes recognized by these antibodies as 359-13F10 binds the HLA-DRβ1*04 β1 domain [307] and NFLD.D1 binds the β2 domain [222]. Thus, glycosylation near the cell surface could inhibit accessibility to the NFLD.D1 epitope and be responsible for such findings. Consistent with this, NFLD.D1 was shown to bind strongly to intracellular DRβ1*04 [210]. Unfortunately, the supply of 359-13F10 was limited so NFLD.D1 was utilized for detection of DRβ1*04 on breast carcinoma cell lines.

We were unable to study expression of DRβ4 or DRβ5 HLA-DR types on breast carcinoma cells due to lack of specific mAbs. Therefore, it is possible that additional HLA-DR types were also modulated in response to various cytokine treatments.

Finally, cytokine-mediated modulation of HLA-DR expression was detected by both flow cytometry and CELISA; however, in some cases discrepancies were evident between flow cytometry and CELISA analysis (Tables 3.3 and 3.4). This may be due to

the ability to remove dead cells through gating in flow cytometry analysis, but inability to do similar removal by CELISA analysis. In this regard, primary antibodies often stick to dead cells and could skew CELISA optical densities, providing inaccurate results. We have also identified sensitivity differences between these two assays as constitutive and modulated expression was often detected by flow cytometry but not CELISA.

4.5.2. Constitutive HLA-DR Expression

As summarized in Tables 3.3 and 3.4, constitutive HLA-DR expression was detected on MDA MB 231 and inconsistently detected on BT-20 using the pan anti-HLA-DR antibody L243. Examination of DRβ1*07 and DRβ3 allelic products on MDA MB 231 revealed that constitutive expression was predominately DRβ3. Examination of DRβ1*04, DRβ1*13, and DRβ3 allelic products on BT-20 failed to reveal any constitutive expression suggesting that constitutive expression detected by L243 was either DRβ4, or that individual allotypic expression was below the limits of detection of allele specific mAbs. This finding also confirmed a previous report on BT-20 [210].

Constitutive expression on BT-20 was also inconsistent as it was detected by flow cytometry during preliminary experiments (Section 3.1) and during analysis of cytokine modulation of HLA-DR (Section 3.8.1.4), but was not detected by CELISA during the medium comparison study (Section 3.2). Thus, our results indicate sensitivity differences between the two assays and suggest that flow cytometry was more sensitive than CELISA for detection of HLA-DR on breast cancer cell lines.

4.5.3. IFN-γ

IFN-γ induced generic and allelic HLA-DR to varying degrees on all breast cancer cell lines (Tables 3.3 and 3.4), confirming previous findings [210, 213, 308]. Up-regulation of nearly all HLA-DR molecules on breast carcinoma cells was not surprising as IFN-γ is the most potent inducer of MHC class II expression [212]. However, differential induction of HLA-DR alleles on MDA MB 157, BT-20, MCF-7, and MDA MB 231 is intriguing as it suggested selective up-regulation of HLA-DR molecules on breast carcinoma cells in response to IFN-γ.

It is tempting to speculate that poor IFN-γ induction of DRβ1*04 in both MDA MB 157 and BT-20 is inherent to DRβ1*04 as other alleles in these cell lines were expressed. However, since only two cell lines were analyzed, it is impossible to draw such conclusions. Another allotype, DRβ3, which was poorly up-regulated on BT-20 and not up-regulated at all on MCF-7, was strongly up-regulated on MDA MB 231, illustrating the problem of drawing conclusions on the basis of a few cell lines. Similarly, DRβ1*07 was poorly up-regulated on MDA MB 231, a line which strongly expresses HLA-DR, but moderately up-regulated on SKBR3, a line which expresses considerable less DR than MDA MB 231. Based on the small number of cell lines examined in this study, we suggest that allotypic expression is regulated internally in a cell line-specific manner.

We suggest that DRβ3 and DRβ1*03 are poorly expressed by MCF-7 as neither allele was up-regulated by IFN-γ. These findings may be simply explained by loss of heterozygosity, as DRβ3 and DRβ1*03 are commonly expressed as a haplotype.

Furthermore, our results are in contrast to a previous study in which 500 units/ml IFN-γ provoked substantial up-regulation of DRβ1*03 and DRβ3 on MCF-7 cultured in standard medium [210]. While our study differed in terms of both IFN-γ concentration and medium composition, we have previously indicated that the concentration of IFN-γ (Section 3.7.1) and the presence / absence of estradiol (Section 3.2) had minimal effects on generic HLA-DR expression on MCF-7. Thus, future research could examine if our cell line has lost the MHC complex on one of its chromosome 6.

4.5.4. IL-4

IL-4 did not modulate constitutive HLA-DR expression on MDA MB 157, MDA MB 231, SKBR3, BT-20 or T47D. However, IL-4 induced HLA-DR and DRβ1*15 on MCF-7 according to flow cytometry analysis (Table 3.4). Overall, addition of IL-4 did not affect IFN-γ induced HLA-DR on MDA MB 157, but suppressed IFN-γ induced HLA-DR on MDA MB 231 and T47D. In contrast, IFN-γ + IL-4 augmented HLA-DR on SKBR3, and generic HLA-DR and DRβ1*15 on MCF-7. Taken together, the results suggest that IL-4 alone and in combination with IFN-γ, selectively modulated HLA-DR on breast carcinoma cell lines. These effects also appeared to occur in a cell line-specific manner.

IL-4 mediated modulation of HLA-DR has been previously reported in human monocytes [130], B cells [126, 127], melanoma cells [134], and renal cell carcinomas [135]. Such effects are believed to be dependent on JAK1/JAK3 activation of the STAT-

6 pathway [122, 131-133]. Whether IL-4 mediated induction of HLA-DR on MCF-7 is also STAT-6-dependent is presently unclear and may warrant further investigation.

Enhanced HLA-DR expression on MCF-7 and SKBR3 with IL-4 was modest at best, and the ability to detect these modest levels was inconsistent. However, the combination of IFN-γ and IL-4 is known to augment MHC class II expression in primary microglial cells, melanoma carcinoma cells, and breast carcinoma cells [84, 136]. These findings are in contrast to other studies describing IL-4 suppression of IFN-γ-induced CIITA accumulation, and in turn, MHC class II expression on monocytes, murine microglial cell lines, and rat astrocytes [84, 130, 137-139]. Such studies indicated that IL-4 did not inhibit phosphorylation of STAT-1 or USF-1 of the IFN-γ signaling pathway, suggesting interaction with cis-acting elements in the CIITA promoters [84]. Nevertheless, the discrepancy identified here may be due to the different cell types examined.

The role of CIITA in IL-4 induction of MHC class II on B cells has been extensively studied. Itoh-Lindstrom et al. [80] demonstrated using $CIITA^{-/-}$ mice that CIITA is required for IL-4 induction of MHC class II transcription in murine $B220^{+}$ B cells and Mac-1^{+} cells. However, a more recent report indicated that while IL-4 caused a 4-fold increase in MHC class II expression on $B220^{+}$ murine splenic B cells, IL-4 did not affect mRNA levels of MHC class II or CIITA as determined by quantitative real-time RT-PCR [309]. Thus, post-transcriptional and post-translational mechanisms may contribute to increased MHC class II expression in response to IL-4.

Since we found that IL-4 modulated IFN-γ-induced HLA-DR expression on MDA MB 231, SKBR3, BT-20, MCF-7, and T47D, this indicated that these cell lines were responsive to IL-4 and likely expressed IL-4Rs, confirming previous studies [120]. Based on our observations, only MDA MB 157 is questionable for IL-4 sensitivity, as there was minimal modulation of HLA-DR expression and affects on cell numbers were inconsistent (Appendix A). On this note, the differential effects mediated by IL-4 addition on breast carcinoma cells may depend on the number of IL-4Rs present of the cell surface. Future research could examine whether an association exists between the quantity of IL-4Rs on breast cancer cells and the differential responses to individual and combined IL-4 treatments.

4.5.5. TGF-β1

We found that TGF-β1 almost completely eradicated constitutive generic and allelic HLA-DR expression on MDA MB 231 and BT-20 breast carcinoma cells (Tables 3.3 and 3.4). In combination with IFN-γ, TGF-β1 inhibited IFN-γ induction of HLA-DR on MDA MB 157 and MBA MB 231, but surprisingly augmented IFN-γ induced generic and allelic HLA-DR on BT-20 and SKBR3. TGF-β1 did not modulate IFN-γ induced HLA-DR on MCF-7 or T47D, agreeing with previous studies that ER+ breast cancer cell lines lose responsiveness to TGF-β stimulation [239, 240, 245, 296]. More interestingly, results on BT-20 suggested that TGF-β1 may act differently when combined with IFN-γ as TGF-β alone inhibited constitutive HLA-DR, but with IFN-γ it increased HLA-DR.

TGF-β1 induction of HLA-DR on particular breast carcinoma cells is in contrast to previous reports describing TGF-β-mediated suppression of CIITA and IFN-γ-induced MHC class II expression in various cell types, including astrocytes [86], monocytes [87], macrophages [310], microglial cells [84], fibrosarcoma cells [88, 100, 101], epithelial cells [311], melanoma cells [312], and synovial cells [153]. In such studies, TGF-β inhibited constitutive and IFN-γ inducible CIITA mRNA accumulation, and resulting MHC class II expression through interaction with CIITA pIII and pIV [86, 101]. However, this transcriptional inhibition was not due to destabilization of CIITA mRNA [88].

Piskurich et al. [100] revealed that TGF-β suppression of pIII was more complete than that of pIV in 2fTGH human fibrosarcoma cells. It is unknown whether TGF-β1 mediates similar suppression of pIII and pIV in breast carcinoma cells, but this may correlate with the divergent patterns of TGF-β1 suppression on HLA-DR in breast carcinoma cell lines. Furthermore, as TGF-β did not completely abolish activity from either promoter [100], this provides a possible explanation as to why TGF-β did not completely eliminate constitutive or IFN-γ induced HLA-DR expression on breast carcinoma cells. Future studies could examine breast carcinoma cell lines for differential usage of pIII and pIV under various physiological conditions.

Inhibition of IFN-γ induced CIITA transcription upon exposure to TGF-β1 in breast carcinoma cells could result from a number of cellular modifications, including alterations in the number and/or affinity of IFN-γ receptors and/or interference with IFN-

γ-induced signaling events. Previous studies on astrocytes and monocytes showed that TGF-β treatment did not affect IFN-γ induced tyrosine phosphorylation of JAK1, JAK2, and STAT-1, or IFN-γ-induced IRF-1 expression [86, 87, 154]. Thus, the effects of TGF-β on CIITA can not be explained by altered signaling through the JAK-STAT-1 pathway. However, this does not exclude the possibility that TGF-β may still interfere with the binding of STAT-1, IRF-1, and USF-1 to their respective elements on the CIITA promoters.

It is possible that TGF-β inhibition of CIITA expression may be mediated by proteins that interact with the transcription factors binding the CIITA promoter. A number of reports have shown that TGF-β-activated Smads interact with transcriptional coactivators such as CBP and p300 [313-317]. CBP and p300 have histone acetyl transferase activity and may act directly by recruiting the RNA polymerase II to the promoter. Thus, while TGF-β does not alter the ability of STAT-1 to bind DNA [154], it may affect the ability of STAT-1 binding to induce transcription.

TGF-β activated Smads have been shown to recruit transcriptional corepressors and strongly inhibit transcription from TGF-β-responsive promoters by binding to specific Smad binding elements (SBE) [318-321]. Analysis using Smad3-deficient mice and overexpression of Smad3 protein in murine astrocytes has demonstrated that Smad3 is essential in mediating TGF-β negative regulation of CIITA [86]. This suggests that Smad3 is a potential effector for TGF-β regulation of MHC class II expression. However, examination of the -70 to -50 bp sequence within the CIITA pIV region did not reveal a

SBE or TGF-β inhibitory element [86], suggesting that Smad3 complexes do not directly inhibit CIITA transcription by binding to this promoter. It is tempting to speculate that TGF-β1 mediates similar action in breast carcinoma cells, but to the best of our knowledge these studies have not been conducted.

Others have shown that IFN-γ inhibits TGF-β-induced phosphorylation of Smad3, the association of Smad3 with Smad4, the accumulation of Smad3 in the nucleus, and the activation of TGF-β-responsive genes [322]. Acting through JAK1 and STAT-1, IFN-γ was also shown to induce Smad7 in human fibrosarcoma and monocytic leukemia cells [322] which antagonized TGF-β1 signaling [323, 324]. Thus, these studies suggest that the differential effects mediated by TGF-β1 on HLA-DR on breast carcinoma cells may be attributed to differences in Smad-signaling components. We have addressed this issue in terms of Smad2 and Smad3, but future studies could investigate if IFN-γ induces Smad7 in breast carcinoma cell lines which inhibit TGF-β1 phosphorylation of Smad3.

Consequently, our study showing that addition of TGF-β1 augmented IFN-γ-induced HLA-DR expression on BT-20 and SKBR3 is novel. We do not believe that this is due to lack of estrogen in culture medium as Pouliot and Labrie [145] examined the regulation of Smad mRNA expression by estrogen and antiestrogens in ZR-75-1 ER+ BCCL and reported that neither estradiol nor dihydrotestosterone affected Smad2, 3, or 4 mRNA levels in ZR-75-1 cells, suggesting that Smad expression is not regulated by sex steroids. Instead, our findings could be explained by differences in TGF-β1 sensitivity or differences in TGF-β1 signaling components. Alternatively, such effects may be

mediated through a Smad-independent signaling pathway, preventing activation of Smad3 and its interaction with CIITA.

4.5.6. IL-4 + TGF-β1

Modulation of HLA-DR by IL-4 + TGF-β1 was only observed on MDA MB 231 and MCF-7. In the case of MDA MB 231, IL-4 + TGF-β1, like TGF-β1 alone, mediated suppression of constitutive HLA-DR. This was not unexpected since IL-4 alone did not modulate HLA-DR on MDA MB 231. In the case of MCF-7, IL-4 + TGF-β1 mediated similar effects as IL-4 alone, as HLA-DR expression was slightly increased. This finding also agreed with observations that MCF-7 was insensitive to TGF-β1.

IL-4 + TGF-β1 inhibited IFN-γ-induced HLA-DR on MDA MB 157, MDA MB 231, and T47D. As generic and allelic expression on MDA MB 157 was equivalent following treatment with IFN-γ + IL-4 + TGF-β1 and IFN-γ + TGF-β1, this further suggested that HLA-DR modulation on MDA MB 157 was insensitive to IL-4. Furthermore, as expression levels on MDA MB 231 were similar to that following IFN-γ + TGF-β1 treatment, we suggest that the effects mediated by combined cytokine stimulations are due to the quantity and functioning of specific cytokine receptors on the cell surface.

Despite some discrepancies between flow cytometry and CELISA in expression of particular alleles on BT-20, SKBR3, and MCF-7, IL-4 + TGF-β1 appeared to augment IFN-γ induction of HLA-DR on each of these cell lines. It was noted that the effects of

IFN-γ + IL-4 + TGF-β1 were similar to IFNγ-TGF-β1 for both MCF-7 and T47D, again suggesting that ER+ cell lines had lost sensitivity to TGF-β1. It was also noted that flow cytometry data indicated HLA-DR expression to be highest on BT-20 following IFN-γ + IL-4 + TGF-β1, suggesting that all three cytokines may function in synergy to induce HLA-DR on specific cell lines. With exclusion of MDA MB 157, MCF-7, and T47D where cell lines displayed insensitivity to either IL-4 or TGF-β1, our results indicate that IFN-γ + IL-4 + TGF-β1 mediated differential effects on HLA-DR expression on breast carcinoma cells, and these effects occurred in a cell line specific manner.

4.6. Factors Potentially Mediating Differential HLA Class II Expression by Breast Carcinoma Cell Lines

We found selective up-regulation of HLA-DR allelic products on human breast cancer cell lines in an E_2-depleted environment in response to individual and combined cytokine treatments. These findings correlate with a previous study in our laboratory indicating differential expression of HLA-DRB alleles on tumor cells in breast carcinoma [249]. In that study, 40% of HLA-DR$^+$ tumors failed to express one or more of their DR allotypes [249]. Biologically, these findings may be significant as failure to express a particular HLA-DR allele on the cell surface may provide a selective advantage to the tumor if antigens on tumor cells are presented by HLA-DR for recognition by CD4$^+$ tumor specific T cells. In this regard, and in accordance with earlier studies, our laboratory has established that HLA-DR expression on breast tumor cells is significantly associated with T-cell infiltration [249, 325], suggesting that HLA-DR is induced on

tumor cells in response to the cytokine milieu. Furthermore, others have shown that $CD4^+$ T cells are capable of recognizing autologous tumor-specific antigens in an HLA class II restricted manner [326-328]. Thus, qualitative and quantitative alterations in the expression of HLA-DR molecules may dramatically affect the onset and maintenance of an immune response involving tumor rejection.

We consistently found DRβ1*04 was poorly expressed on MDA MB 157 and BT-20, while DRβ1*07 was weakly expressed on MDA MB 231, DRβ1*03 was weakly expressed on BT-20, and DRβ3 was weakly expressed on MCF-7. These weak or differential expression of DRB genes by tumor cells may be due to promoter polymorphisms [329-332]. Expression of DRB genes is regulated mainly at the level of transcription and is characterized by the presence of S-X-Y motifs within the promoter region. Polymorphisms within X and Y box motifs have been shown to control the level of HLA-DRB1 gene expression and affect the recruitment of transcription factors and transcriptional activity [329, 332]. Furthermore, comparison of differential HLA-DRB promoter transcriptional activity with HLA-DRB mRNA levels indicated that differential DRB gene expression is regulated at both transcriptional and post-transcriptional levels [333, 334]. Whether breast carcinoma cells do not express, or weakly express, particular HLA-DR allotypes as a result of natural promoter polymorphisms, or genetic or epigenetic changes resulting from tumorgenesis is unknown. Thus, future research could examine DRB genes not up-regulated by IFN-γ for possible nucleotide variations within promoter regions. Future work could also examine potential loss of heterozygosity and epigenetic alterations such as hypermethylation within CIITA promoter regions [335].

IFN-γ, IL-4, and TGF-β receptor expression could also have drastically differed between cell lines, promoting differences in modulation of HLA-DR expression. In theory, one would speculate that cells with copious receptors would be more sensitive to cytokine treatment, and therefore up-regulate / down-regulate MHC class II expression to greater degree than cell lines with few cytokine receptors. On this note, we found selective and broad ranges of growth inhibition following all cytokine treatments (Appendix A) suggesting that cell lines differed in their sensitivity and response to particular cytokine(s). Thus, future studies could attempt to correlate differential class II expression with the expression of IFN-γ, IL-4 and TGF-β receptors on the cell surface.

In some cases, flow cytometry analysis indicated modulation of the intensity of HLA-DR expression, but comparable modulation was not indicated in the percentage of positive cells. This suggests that particular cytokine combinations increase the stability of HLA-DRB mRNA or the steady state expression of HLA-DR molecules, allowing DR proteins to be expressed longer on the cell surface. Alternatively, since HLA-DR molecules are contained in intracellular vesicles before transport and expression on the cell surface, these findings suggest that cytokines modulate the mobilization of MHC class II molecules in breast carcinoma cells. In addition, cytokine(s) may affect the expression of proteins involved in MHC class II antigen presentation, which could subsequently modulate class II expression on the cell surface. Our laboratory has previously shown that Ii and HLA-DM were up-regulated by IFN-γ in some breast carcinoma cells expressing high amounts of HLA-DR [210]. Future studies could conduct time course studies examining HLA-DR mRNA and protein expression, as well as

examine if addition of IL-4 and/or TGF-β1 to IFN-γ modulates expression of Ii, HLA-DO, and HLA-DM in breast carcinoma cells.

4.7 Analysis of the TGF-β Signaling Pathway in Breast Cancer Cell Lines

As reviewed in Section 1.4.3, TGF-β1 signaling is mediated predominately via TGF-β receptors and their substrates, the Smad proteins. We analyzed the Smad signaling pathway to determine if differences in TGF-β1 sensitivity or signaling components could explain the differential effects mediated by IFN-γ + TGF-β1 on HLA-DR expression. Smad proteins themselves do not display enzymatic activity, therefore, this signaling pathway is not amplified as it progresses. Thus, the relative level of Smad2 and Smad3 in a given cell can not only affect its ability to respond to TGF-β, but also influence the nature of the response.

We found 8/8 breast cancer cell lines expressed Smad2, while 6/8 expressed Smad3 (Table 3.5). However, of cell lines expressing Smad2/3, equivalent levels of Smad2 and Smad3 were detected between untreated and TGF-β1 treated samples. As Smad3 was not detected in BT-474 or T47D, this suggested that Smad3 was differentially expressed in breast cancer cell lines. In contrast, Smad2 appeared to be universally expressed.

Constitutive P-Smad3 in MCF-7, MDA MB 157, MDA MB 468 and SKBR3 (Figure 3.24) was unexpected as Smad3 is phosphorlyated in response to TGF-β, and thus, these findings suggested autocrine activation of the Smad signaling pathway. As we have indicated that SKBR3 did not transcribe TGF-β1 when cultured in E_2-depleted

medium based on mRNA analysis (Section 3.6), and that MCF-7 was insensitive to TGF-β1 based on lack of HLA-DR modulation (Section 3.8.2.1), these results did not agree with previous findings. Furthermore, as no constitutive P-Smad2 was detected in any breast cancer cell line, this suggested that only selective components on the Smad signaling pathway were constitutively active.

Since activated Smad3 is reported to interact with CIITA to inhibit HLA-DR expression [86], it was noted that constitutive P-Smad3 expression correlated with lack of constitutive HLA-DR on these particular cell lines. Consistent with this, constitutive HLA-DR expression on MDA MB 231 and BT-20 also correlated with the absence of constitutively active Smad2 and Smad3. However, no correlation was found for T47D, and thus the lack of constitutive expression of this cell line is unclear.

The differential expression of P-Smad2 and P-Smad3 observed in response to TGF-β1 may partially explain the divergent effects mediated by TGF-β1 and IFN-γ + TGF-β1 on HLA-DR expression in breast cancer cell lines. In this regard, MDA MB 157 and MDA MB 231 both demonstrated sensitivity to TGF-β1 by phosphorylation of Smad2 and Smad3, suggesting such factors could suppress IFN-γ induction of HLA-DR. Similarly, unmodulated IFN-γ-induced HLA-DR expression on T47D following addition of TGF-β1 may be explained by the lack P-Smad2, Smad3, and P-Smad3, suggesting that this cell line is unresponsive to TGF-β1.

MCF-7 did not express P-Smad2, but contained constitutive P-Smad3, with P-Smad3 levels induced by TGF-β1. Hence, both constitutive and TGF-β1 induced P-Smad3 could theoretically interact with CIITA promoters and suppress HLA-DR

expression. Furthermore, induction of P-Smad3 by TGF-β1 in MCF-7 indicated active TGF-β1 signaling, which subsequently contradicts our previous findings that MCF-7 is insensitive to TGF-β1 based on its HLA-DR expression. This discrepancy may denote factors affecting the transport of P-Smad3 to the nucleus, or the interaction of P-Smad3 with CIITA. Mutations in Smad3 appear to occur only rarely [336], but lack of Smad4 expression has been reported in specific breast cancer cell lines, including MDA MB 468, and is essential in the activation of certain transcriptional responses [145, 337]. Dowdy et al. [338] reported up-regulation of Smad7 in endometrial cancers, suggesting that Smad7 may be up-regulated in cancer cells and antagonize Smad3 signaling [339, 340]. In this regard, reports also indicate that Smad7 expression is induced by TGF-β [323, 339]. Based on these studies, future research could examine the expression of Smad4 and Smad7 proteins in MCF-7.

More interesting, lack of P-Smad2 and unmodulated, weak expression of P-Smad3 following TGF-β1 raised the question whether SKBR3 was indeed sensitive to TGF-β1. These results were also in agreement with our findings that TGF-β1 augmented, rather than suppressed, IFN-γ-induced HLA-DR expression on SKBR3. Such findings suggest that TGF-β1-mediated augmentation of IFN-γ-induced HLA-DR expression on SKBR3 occurred in a P-Smad2/3-independent manner. As TGF-β may signal through MAPKs including c-Jun N-terminal kinase [149], p38 MAPK [341], and ERK1/2 kinase [342], preliminary research investigated the role of the ERK signaling using an upstream MEK1/2 kinase inhibitor. These results did not indicate that TGF-β augmentation

occurred through ERK signaling, but did indicate that IFN-γ induction of HLA-DR on SKBR3 is predominantly MEK1/2 dependent (data not shown). Future research could examine the roles of other alternative pathway, including Rho guanosine triphosphates, PI3K / AKT, and protein phosphate A.

The results of BT-20 were also unique. BT-20 expressed both Smad2 and Smad3, but only expressed P-Smad3 in response to TGF-β1 treatment. Thus, theoretically, P-Smad3 could inhibit IFN-γ induction of HLA-DR within this cell line. However, the reverse was found as addition of TGF-β augmented IFN-γ-induced HLA-DR expression on BT-20. To explain such novel findings, we again suggest a Smad-independent signaling pathway in BT-20, similar to that speculated in SKBR3.

4.8. Conclusion

The significance of HLA-DR expression on breast carcinoma cells is presently unclear. MHC class II molecules are not normally present on the epithelial cells of breast, but have been detected on late-pregnant and lactating epithelia, and on a portion of breast carcinomas [343]. Our laboratory has previously demonstrated that breast carcinoma cell lines differentially express HLA-DR molecules, but do not express CD80 and CD86 co-stimulatory molecules typically found on APCs, and selectively express CD40 [210]. Whether the expression level of CD40 would be sufficient to activate $CD4^+$ T cells is unknown, but suggests that breast carcinoma cells may act as non-professional APCs.

In this regard, substantial evidence supports a role for MHC class II antigen expression on tumor cells in antitumor immunity. Armstrong et al. [251] showed that

class II-transfected tumor cells could directly activate tumor-specific $CD4^+$ T cells *in vitro* and *in vivo*. Similarly, transfection of CIITA into MHC class II-negative murine mammary adenocarcinoma cells resulted in expression of MHC class II and tumor rejection requiring both $CD4^+$ and $CD8^+$ T cells [344]. Vaccination with irradiated tumor cells transduced to secrete granulocyte/macrophage colony-stimulating factor was also found to simulataneous induce T_H1 and T_H2 responses in mice [345]. Thus, HLA-DR expression on breast carcinoma cells may also contribute to the tumor immune response *in vivo* by presenting tumor-specific antigens to effector $CD4^+$ T cells.

The association of HLA-DR expression on breast carcinoma cells with a favorable clinical prognosis appears controversial. A number of studies suggested that DR expression was associated with favorable prognostic indicators such as well differentiated tumors [346], and PgR expression [347], while others report no effect [348-351]. However, these studies examined generic HLA-DR expression using pan-HLA-DR antibodies. Therefore, it is unknown if all or just selective HLA-DR allotypes were expressed.

This thesis provided a comprehensive analysis of whether IFN-γ, IL-4, and TGF-β1 individually and collectively regulate the expression of HLA-DR allelic products on the cell surface of breast carcinoma cell lines in an estrogen-depleted environment. To our knowledge, this is the first study to show that breast carcinoma cell lines differentially regulate expression of HLA-DR allotypes in response to particular cytokine combinations. Significant modulation of HLA-DR expression on breast cancer cell lines by IFN-γ, IL-4 and TGF-β1 suggests that these cytokines may have important roles in

natural tumor immune defense mechanisms. When certain tumor-infiltrating lymphocytes are activated they may release IFN-γ, IL-4, or TGF-β1 [108, 109, 352, 353], which may have significant direct effects on growth and modulation of antigen expression on tumor cells. Understanding the roles of cytokines in tumor immune responses should provide opportunities to develop better therapeutic strategies.

Chapter 5. References

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Chapter 6: Appendix A

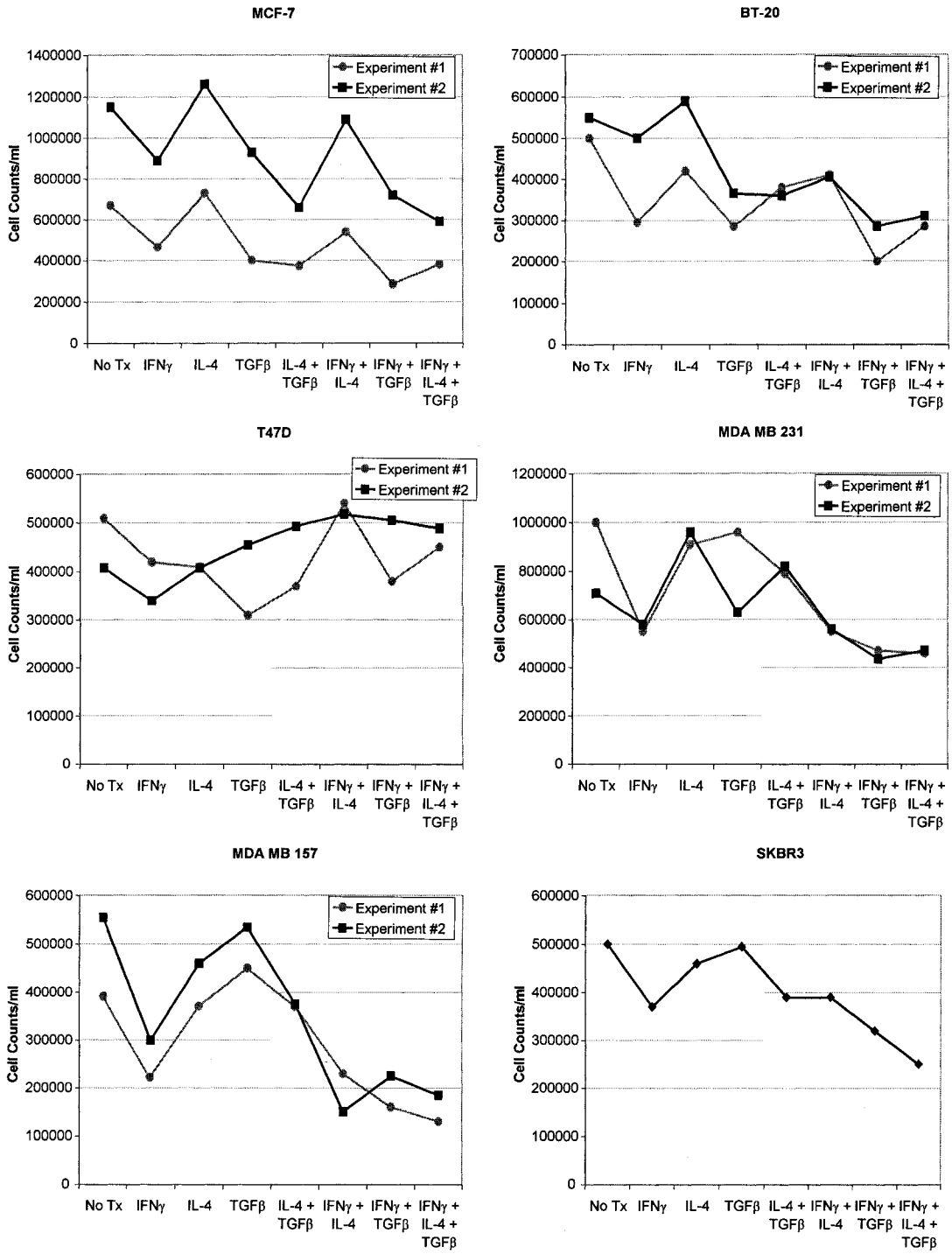

Figure 6.1. The antiproliferative / cytotoxic effects of cytokines on breast cancer cell lines. Counts were conducted using a hemocytometer and phase contrast microscopy.