HLA CLASS II EXPRESSION ON BREAST CANCER CELLS

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by

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ABSTRACT

Presentation of tumor peptides by HLA molecules is required for immune system recognition and eradication of tumors. This study characterized 11 breast cancer cell lines (BCCLs) as potential antigen presenting cells, assessed expression of CIITA, a regulator of HLA class II and co-chaperone transcription, and examined expression of HLA-DR allelic products in these cells.

Gene transcription was determined by RT-PCR. Protein expression was demonstrated using monoclonal antibodies and flow cytometry and immunocytochemistry.

Unexpectedly, constitutive HLA class II and/or co-chaperone protein expression was detected in 5 of 11 BCCLs. Following IFN-γ treatment, most cell lines up-regulated HLA class II and co-chaperones and the co-stimulatory molecule CD40 suggesting a capacity for HLA class II mediated antigen presentation.

Several BCCLs constitutively expressed HLA class II and co-chaperones in the absence of CIITA suggesting CIITA independent regulation of these genes in BCCLs.

Some cell lines selectively up-regulated certain HLA-DR allelic products. Since all HLA-DR alleles were transcribed following IFN-γ treatment, post-transcriptional events may be implicated in this selective expression.

Failure to express HLA class II antigens and co-stimulatory molecules may allow tumors to escape immune recognition.

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APC(s):	Antigen presenting cell(s)
Bob-1:	B cell octamer-binding protein 1
cAMP:	Cyclic adenosine monophosphate
CAT:	Chloramphenicol acetyltransferase
CBP:	CREB-binding protein
cDNA:	Complementary DNA
CELISA:	Cellular enzyme-linked immunoassay
CIITA:	Class II transactivator
CLIP:	Class II-associated invariant chain peptide
CREB:	cAMP responsive element binding protein
CTLs:	Cytotoxic T lymphocytes
DAB:	3,3'-diaminobenzidine
DEPC:	Diethyl pyrocarbonate
DMEM:	Dulbecco's Modified Eagle Medium
DNA:	Deoxyribonucleic acid
dNTPS:	Deoxynucleotide triphosphates
DTT:	Dithiothreitol
EGF:	Epidermal growth factor
ER:	Endoplasmic reticulum
FACS:	Fluorescence activated cell sorter
G3PDH:	Glyceraldehyde 3-phosphate dehydrogenase
GAS:	IFN-gamma activation sequence
GM-CSF:	Granulocyte macrophage-colony stimulating factor
HLA:	Human leukocyte antigen
IC:	Immunocytochemistry
IFN-β:	Interferon-beta
IFN-γ:	Interferon gamma
IHW:	International Histocompatibility Workshop
Ii:	Invariant chain
IL-1α:	Interleukin-1-alpha
IL-1β:	Interleukin-1-beta
IL-4:	Interleukin-4
IL-10:	Interleukin-10
IMDM:	Iscove's Modified Dulbecco's Medium
IRF-1:	Interferon regulating factor-1
Jak:	Janus kinase
mAbs:	Monoclonal antibodies
Mb:	Megabases
MHC:	Major histocompatibility complex

mRNA:	Messenger ribonucleic acid
NF-Y:	Nuclear factor binding to the Y box
OBS:	Octamer binding site
Oct-1 or-2:	Octamer binding transcription factor-1 or -2
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
RFLP:	Restriction fragment length polymorphism
RFX5:	Regulatory factor that binds the X box
RFXAP:	RFX-associated protein
RFXANK:	RFX-associated protein with ankyrin repeats
RNA:	Ribonucleic acid
rRNA:	Ribosomal RNA
RT-PCR:	Reverse transcriptase polymerase chain reaction
SSP:	Sequence specific primers
STAT-1:	Signal transducer and activator of transcription-1
STR:	Short tandem repeats
TAFs:	TATA-binding protein-associated factors
TAP:	Transporter in antigen processing
TBE:	Tris borate EDTA (Ethylenediaminetetraacetic acid)
TBP:	TATA box-binding protein
TFIIB/TFIID:	Transcription factor-IIB or -IID
TFs:	Transcription factors
TGF-β:	Transforming growth factor-beta
TNF-α:	Tumor necrosis factor-alpha
TPA:	12-o-tetradecanoylphorbol-13-acetate
USF-1:	Upstream stimulatory factor-1
UV:	Ultraviolet
X2BP:	X2-specific binding protein

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CHAPTER 1. INTRODUCTION

According to the immunosurveillance theory, the immune system is instrumental in eradicating spontaneously arising epithelial neoplasms (Shankaran et al., 2001). HLA class I (HLA-A, -B, -C) and class II (HLA-DR, -DP, -DQ) molecules, by virtue of their ability to present tumor antigens to T lymphocytes, are critical in activating the cellular tumor antigen-specific immune response (Accolla et al., 1995, Germain, 1994). Since the immune system does not prevent the development or progression of all cancers, it seems apparent that immune surveillance can be evaded. One mechanism by which tumors escape immune recognition is to alter their HLA phenotype (reviewed by Marincola et al., 2000). Much has been reported on HLA class I down-regulation by human cancers but little is known about HLA class II alterations.

This thesis, continuing and expanding research initiated by a Bachelor of Science Honour's student, Jamie Tibbo, investigates the selective up-regulation of HLA-DR genes by breast cancer cell lines. Potentially, tumors not expressing a particular HLA-DR type proficient at tumor antigen presentation may not be recognized nor eliminated by CD4+ T cells. The possibility that breast tumor cells themselves may act as HLA class II restricted antigen presenting cells was addressed by examining HLA class II, class II co-chaperone and co-stimulatory molecule expression. Further, since CIITA is believed to be a critical activator of class II and co-chaperone transcription in non-neoplastic cells, the expression of this factor and its correlation with class II and class II co-chaperone expression in breast cancer cells was studied. Therefore, this chapter reviews pertinent literature on HLA class

II and its involvement in the anti-cancer immune response.

1.1. The Major Histocompatibility Complex (MHC)

The major histocompatibility complex (MHC), termed human leukocyte antigen (HLA) complex in humans, is a group of highly polymorphic genes whose products are essential to the immune response (Kaufman et al., 1984). The two predominant classes of HLA molecules, class I (HLA-A, -B and -C) and class II (HLA-DR, -DP and -DQ), are expressed on the cell surface of multiple cell types (Klein & Sato, 2000). T cell recognition of foreign antigen mainly occurs when antigen is presented by HLA molecules (Zaleski, 1991 and Germain, 1994).

1.1.1. Genomic Organization of the MHC

The MHC in humans is located on the short arm of chromosome six, 6p21.3, and spans 4 Mb (Beck & Trowsdale, 1999, van den Elsen et al., 1998). There are over 200 genes in this complex, of which only 10-20% have recognized roles in class I and class II antigen presentation (Klein & Sato, 2000). Three genetic regions have been identified within the MHC: the class I region, the class II region and the class III region (Figure 1.1).

The class III region is located between the class I and class II regions and spans approximately 1 Mb (Campbell & Trowsdale, 1993). It contains approximately 75 genes encoding a variety of different proteins including the complement components (C2, C4A, C4B and Factor B) (Marsh et al., 2000). The class III region does not encode proteins involved in antigen presentation.

The class I region is located nearest the telomere and spans approximately 1.5 Mb

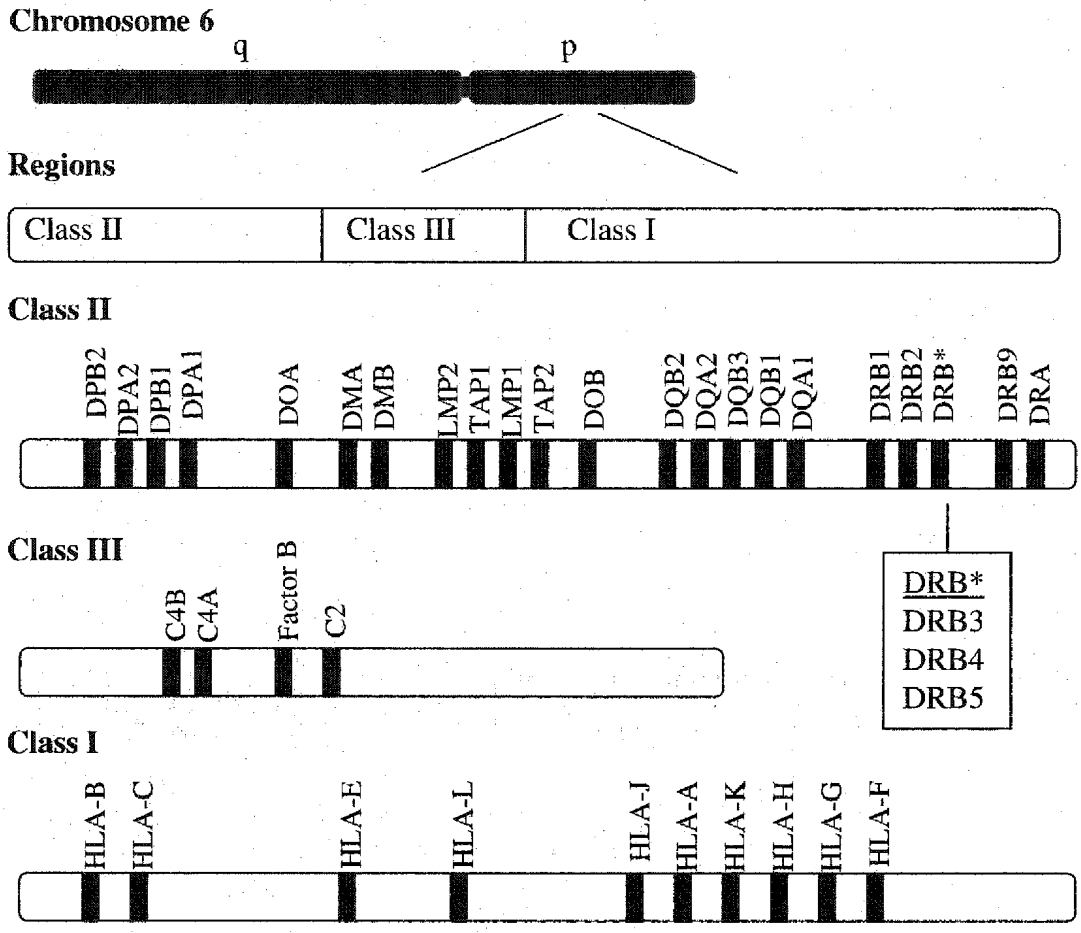


Figure 1.1. Organization of the HLA complex on chromosome six. The HLA complex is divided into three regions: class I, class II and class III. The class I and class II regions encode genes involved in antigen processing and presentation but the class III region does not. Among other functions, class III genes encode complement proteins. Adapted from Klein & Sato, 2000, Abbas et al., 1997 and Marsh et al., 2000.

(Roitt et al., 1998). The principal class I genes encode heavy chains of six class I molecules: HLA-A, -B, -C, -E, -F and -G. HLA-A, -B, -C are termed classical class I molecules while HLA-E, -F and -G are known as class I-like molecules. Additionally, there are four nonfunctional pseudogenes termed HLA-H, -J, -K and -L. The gene encoding the common class I light chain, β_2-microglobulin, is located on chromosome 15 (Marsh et al., 2000).

The class II region is positioned nearest the centromere and extends over 1 Mb (Beck & Trowsdale, 1999). These genes code for five main class II proteins: HLA-DR, -DQ, -DP, -DM and -DO. Class II molecules are heterodimers consisting of an α-chain and a β-chain, both of which are encoded by genes in the class II region. All of the class II genes, with the exception of HLA-DO, are arranged in related pairs of α and β genes *i.e.* HLA-DQA1 next to HLA-DQB1 (Beck & Trowsdale, 1999). The genes encoding HLA-DQ α and β chains are HLA-DQA1 and HLA-DQB1. There are three nonfunctional HLA-DQ pseudogenes: HLA-DQA2, HLA-DQB2 and HLA-DQB3. HLA-DP α and β chains are encoded by HLA-DPA1 and HLA-DPB1. HLA-DPA2 and HLA-DPB2 are pseudogenes (Marsh et al., 2000). HLA-DM α and β chains are encoded by HLA-DMA and HLA-DMB genes while HLA-DO α and β chains are encoded by HLA-DOA and HLA-DOB genes (Marsh et al., 2000).

The HLA-DR locus is more complex than the other class II loci. HLA-DR α and β chains are encoded by HLA-DRA1 and HLA-DRB genes. The number of expressed HLA-DRB genes depends on the HLA-DRB haplotype inherited (Figure 1.2). These HLA-DRB haplotypes are HLA-DR1, HLA-DR51, HLA-DR52, HLA-DR53 and HLA-DR8. All HLA-DRB haplotypes have a functional HLA-DRB1 gene and may have one other functional

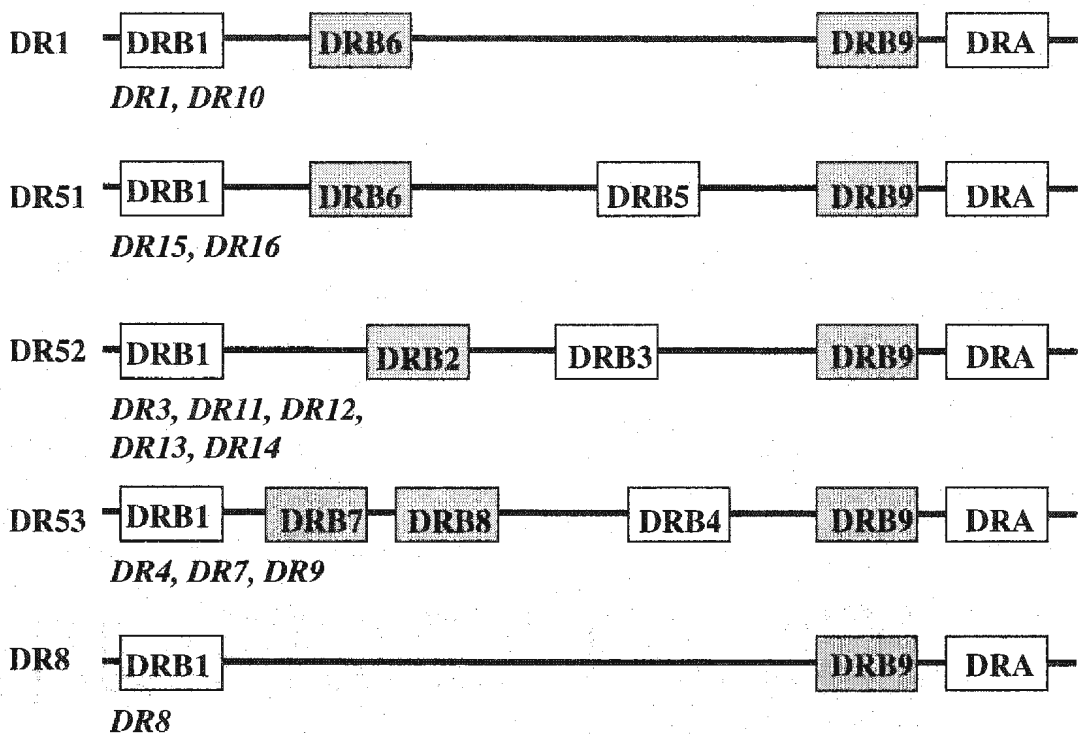


Figure 1.2. Arrangement of HLA-DRB genes in different HLA-DRB haplotypes. There are five HLA-DRB haplotypes: DR1, DR51, DR52, DR53 and DR8. Pseudogenes are indicated by shaded boxes and expressed genes are indicated by open boxes. The serological specificity of the DRB1 encoded gene is indicated in italics. The DRB5, DRB3 and DRB4 genes encode the DR51, DR52 and DR53 antigens. Adapted from Marsh et al., 2000.

HLA-DRB gene (HLA-DRB3, -DRB4 or -DRB5). All HLA-DRB haplotypes also have nonfunctional DRB pseudogenes. The number of pseudogenes depends on the HLA-DRB haplotype (Marsh et al., 2000).

The class II region also contains genes critical for class I antigen processing including proteasome genes and transporter in antigen processing (TAP) 1 and 2 genes (Beck & Trowsdale, 1999). The proteasome is involved with generating antigenic peptides while TAPs transport peptides into the endoplasmic reticulum where they bind class I molecules (Klein & Sato, 2000).

Recombination between HLA-DR and HLA-DQ is rare (Beck & Trowsdale, 1999). Thus, HLA-DR and HLA-DQ genes are in linkage disequilibrium *i.e.* certain HLA-DQ alleles are inherited together with certain HLA-DR alleles (Abbas et al., 1997). In contrast, linkage disequilibrium between HLA-DR/HLA-DQ and HLA-DP is weaker allowing higher rates of recombination between these loci (Begovich et al., 1992, Howell et al., 1993, Baisch & Capra, 1993).

1.1.2. HLA Class II Nomenclature

Class II molecules were identified in the 1970's when it was observed that lymphocytes from two genetically different individuals combined *in vitro* proliferated in response to their distinct cell surface antigens (Marsh et al., 2000). There was limited success in characterizing class II molecules using the lymphocyte activation model so identification based on serology was attempted *i.e.* the recognition of different class II molecules by antibodies. This method defined several HLA-DR and HLA-DQ antigens but

was not useful for the identification of HLA-DP antigens (Marsh et al., 2000). HLA class II serological specificities are listed in Table 1.1.

Molecular biological methods such as restriction fragment length polymorphism (RFLP), nucleotide sequencing and polymerase chain reaction (PCR) enabled the identification of numerous HLA class II alleles (Marsh et al., 2000). Genes encoding α-chains are designated A (*i.e.* HLA-DRA) while genes encoding β-chain genes are labeled B (*i.e.* HLA-DRB). In the example HLA-DRB1*0401, HLA-DRB1 refers to the B1 gene encoding the β-chain of the HLA-DR molecule. 04 refers to the serological specificity *i.e.* HLA-DR4 and 01 identifies the allelic variant of the HLA-DRB1*04 gene (Klein & Sato, 2000, Marsh et al., 2000).

1.1.3. HLA Class II Genetic Polymorphism

Within the population, there is extensive HLA class II genetic variation *i.e.* multiple class II allelic variants (Beck & Trowsdale, 1999). This is not surprising given that the physiological role of class II molecules is to bind foreign antigenic peptides. Accordingly, much of the genetic variation within HLA class II genes occurs at positions encoding the peptide binding pockets. HLA class II genes not directly involved in peptide binding, HLA-DM and HLA-DO, have minimal genetic variation (Beck & Trowsdale, 1999). The ability to display numerous pathogen derived peptides is both advantageous to an individual and to a population as it increases the odds of warding off deadly pathogens.

Depending on the HLA class II isotype, both the α-chain and the β-chain may contribute to class II polymorphism. The HLA-DR α-chain has limited polymorphism as .

Table 1.1. HLA-DR and HLA-DQ serological specificities. Numbers in parenthesis indicate the original serological classification of a particular class II antigen. Adapted from Marsh et al., 2000.

HLA-DR	HLA-DQ
DR1	DQ1
DR2	DQ2
DR3	DQ3
DR4	DQ4
DR5	DQ5 (1)
DR6	DQ6 (1)
DR7	DQ7 (3)
DR8	DQ8 (3)
DR9	DQ9 (3)
DR10	
DR11 (5)	
DR12 (5)	
DR13 (6)	
DR14 (6)	
DR15 (2)	
DR16 (2)	
DR17 (3)	
DR18 (3)	
DR51	
DR52	
DR53	

there are only 2 DRA alleles. Thus, HLA-DR polymorphism is derived from DRB genes. There are 221 HLA-DRB1 alleles, 19 HLA-DRB3 alleles; 9 HLA-DRB4 alleles and 14 HLA-DRB5 alleles currently defined at the population level. Both HLA-DQA and HLA-DQB genes are polymorphic. In fact, there are 19 HLA-DQA1 alleles and 39 HLA-DQB1 alleles. Genes encoding the HLA-DP α-chain and the HLA-DP β-chain are also polymorphic. There are 15 HLA-DP1 alleles and 84 HLA-DPB1 alleles. As indicated, HLA-DM and HLA-DO exhibit little polymorphism. Presently, there are 4 HLA-DMA alleles, 5 HLA-DMB alleles, 8 HLA-DOA alleles and 3 HLA-DOB alleles (Marsh et al., 2000).

1.2. HLA Class II Structure

HLA class II molecules are cell surface glycoproteins which are formed by the non-covalent association of two transmembrane polypeptide chains: an α-chain (33-35kDa) and a β-chain (27-29kDa) (Kaufman et al., 1984, Germain, 1994). Each class II α and β chain has four domains: a peptide binding domain (~90 amino acids), an immunoglobulin-like domain (95 amino acids), a transmembrane domain (~25 amino acids) and a cytoplasmic domain (variable length) (Figure1.3A) (Kaufman et al., 1984, Abbas et al., 1997). The two α chain extracellular domains are termed α_1 and α_2 while the two β chain extracellular domains are called β_1 and β_2. The peptide binding site is formed by the association of the α_1 domain and the β_1 domain (Klein & Sato, 2000).

The organization of exons and introns within class II α- and β-chain genes is depicted in Figure 1.3B.

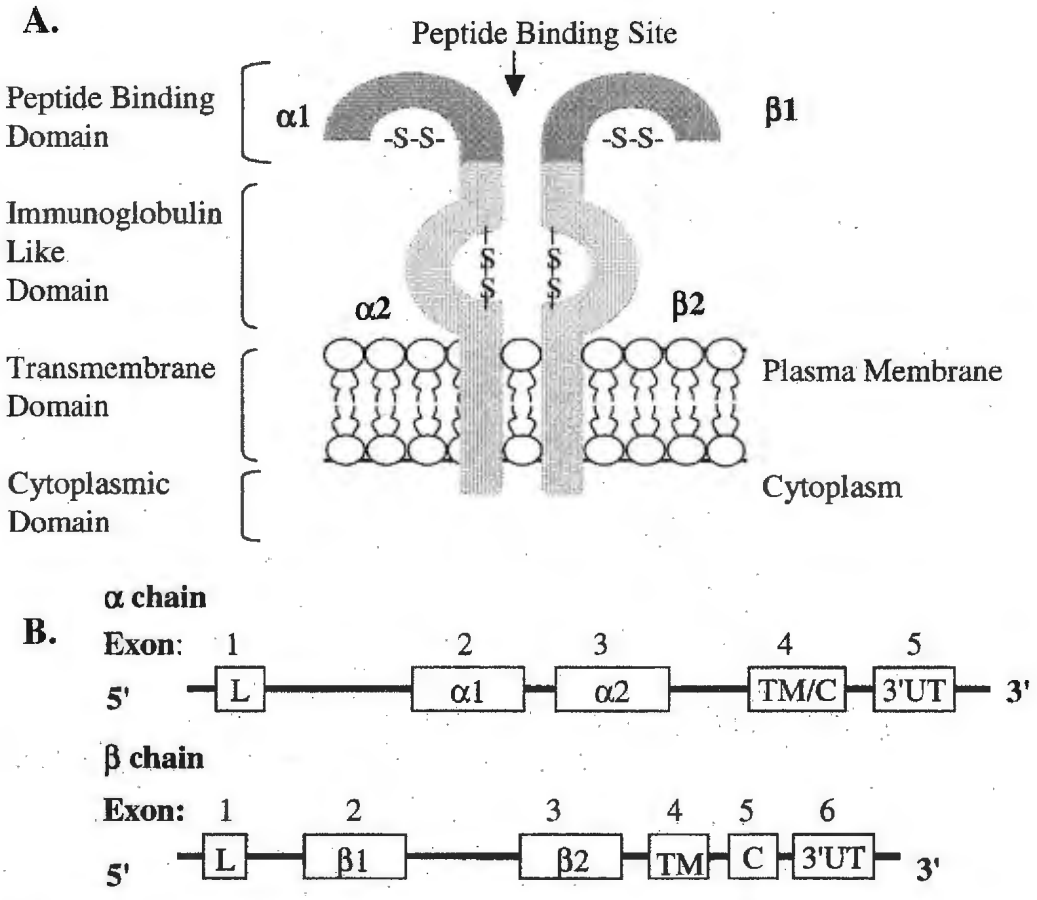


Figure 1.3. Structure and exon-intron organization of HLA class II. A) Structure of the HLA class II molecule. Class II molecules are cell surface heterodimers composed of an α–chain and a β–chain. Each α and β chain has four domains: a peptide binding domain, an immunoglobulin-like domain, a transmembrane domain and a cytoplasmic domain. The peptide binding site is formed by the association of the α1 domain and the β1 domain. Adapted from Tizzard, 1995 and Abbas et al., 1997. B) Exon-intron organization of the HLA class II α–chain and β–chain. The α chain is organized as follows: exon 1 encodes the leader peptide (L), exon 2 encodes the α1 domain, exon 3 encodes the α2 domain, exon 4 encodes the transmembrane region (TM) and the cytoplasmic tail (C) and exon 5 encodes the 3' untranslated region (UT). The β chain is organized similarly. Exon 1 encodes the leader peptide (L), exon 2 encodes the β1 domain, exon 3 encodes the β2 domain, exon 4 encodes the transmembrane region (TM), exon 5 encodes the cytoplasmic tail (C) and exon 6 encodes the 3' untranslated region (UT). Notably, exon 2 exhibits extensive sequence variability among class II alleles since it encodes peptide binding domains. Adapted from Marsh et al., 2000.

1.3. HLA Class II Expression

1.3.1. Cellular Distribution of HLA Class II Molecules

Unlike HLA class I molecules which are expressed on most nucleated cells, constitutive HLA class II expression is restricted to conventional antigen presenting cells (APCs) such as dendritic cells, macrophages, B cells, thymic epithelial cells and activated T cells (Klein & Sato, 2000). However, class II expression is easily induced on a variety of cell types using immunomodulators such as IFN-γ (Boehm et al., 1997). Other immunomodulators may also alter class II expression in a cell type dependent manner. For example, class II expression may be suppressed by IL-10, TNF-α, TGF-β, IL-1β or IFN-β (Lu et al., 1995, Koopleman et al., 1997, Lee et al., 1997, Han et al., 1999, Ito et al., 1999, Rohn et al., 1999). In contrast, class II expression may be induced by IL-4, GM-CSF, TNF-α, IL-1α, retinoic acid or prolactin (Bernard et al., 1986, Gerrard et al., 1990, Sedlak et al., 1992, de Waal Malefyt et al., 1993, Gardner & Walker, 1993, Speiser et al., 1993).

1.3.2. Co-dominant Expression of HLA Class II Molecules

HLA molecules are co-dominantly expressed *i.e.* all maternally and all paternally inherited functional class II genes are expressed as cell surface proteins (Figure 1.4). The complete set of HLA alleles present on each chromosome is called the HLA haplotype (Abbas et al., 1997). Therefore, assuming heterozygousity, each individual inherits two haplotypes and thus will express at least six and as many as eight distinct HLA class II molecules *i.e* HLA-DR7, -DR53, -DR1, -DQ2, -DQ5, -DP1, -DP3. The reason for the variable number of functionally expressed HLA class II molecules is that some HLA-DRB1

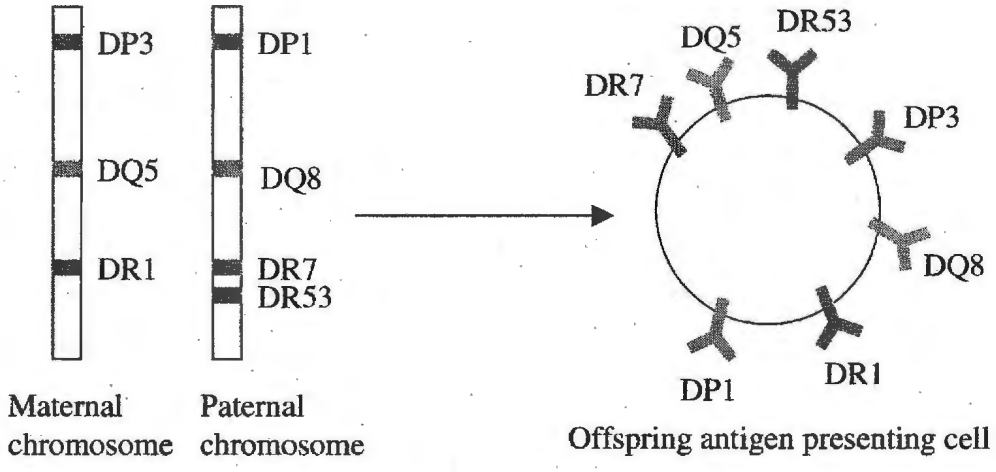


Figure 1.4. HLA class II molecules are co-dominantly expressed. All functional HLA class II alleles inherited from both parents are expressed as cell surface proteins.

genes are expressed with an additional HLA-DRB gene (Marsh et al., 2000) (Figure 1.2).

1.4. HLA Class II Regulation

Since constitutive class II expression has restricted cellular distribution and many cell types may be induced to express class II, strict regulation of these genes is implied. The class II regulatory region is located approximately 150 base pairs upstream of the transcription initiation site (Boss, 1997). Within this promoter, there is a conserved set of *cis*-acting regulatory elements (van den Elsen et al., 1998) (Figure 1.5). These include (5' to 3'): the seven nucleotide S box that is part of a larger, approximately 20 nucleotide, W box; the 15 nucleotide X1 box which overlaps by two nucleotides with the eight nucleotide X2 box; and the 10 nucleotide Y box (Abbas et al., 1997). Proximal to the transcription initiation site is a TATA motif (Singal et al., 1993). The HLA-DRA promoter contains an additional regulatory element termed the octamer binding site (OBS) located between the Y and TATA boxes (Sherman et al., 1989, Voliva et al., 1995).

W, X and Y boxes are characteristic of class II (HLA-DR, -DQ and -DP) and class II co-chaperone (Ii, HLA-DM and HLA-DO) promoters (Boss, 1999, Taxman et al., 2000). This infers coordinate regulation of these genes. However, Ii and HLA-DM promoters contain additional regulatory elements which may trigger their expression in the absence of class II expression (Westerheide et al., 1997, Moore et al., 1998, Tai et al., 1999). HLA-DO expression, in particular, infrequently correlates with expression of other class II genes (Taxman et al., 2000).

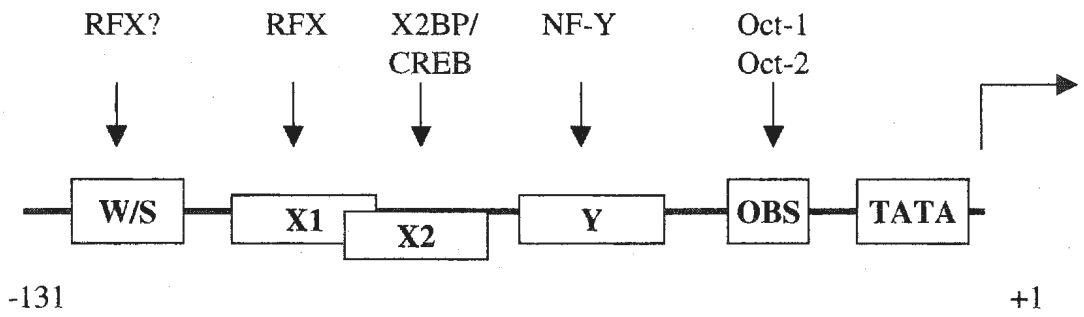


Figure 1.5. Regulatory elements in the HLA class II promoter and their respective transcription factors. The regulatory region of all class II promoters and class II co-chaperones (the invariant chain, HLA-DM and HLA-DO) contains the W/S, X1, X2, Y and TATA box elements. The HLA-DRA promoter, depicted above, also contains the octamer binding site (OBS). Adapted from Boss, 1997 and Harton & Ting, 2000.

1.4.1. Transcription Factors Binding HLA Class II Regulatory Elements

The class II regulatory elements and the ubiquitously expressed DNA binding proteins which bind these elements have been extensively investigated (van den Elsen et al., 1998, Boss, 1999). These are illustrated in Figure 1.5.

The W box contains the S and Z subregions (Boss, 1997). Factors which bind the W box are not well defined since this region has poor affinity for DNA binding proteins (Boss, 1999). However, Jabrane-Ferret et al. (1996) demonstrated that RFX binds this regulatory element.

The X box is composed of two elements, X1 and X2, that bind distinct transcription factors (TFs). X1 is bound by RFX which consists of three subunits: RFX5 (regulatory factor that binds the X box), RFXAP (RFX-associated protein) and RFXANK (RFX-associated protein with ankyrin repeats) (Fontes et al., 1999b). Activation of class II transcription requires the association of all three subunits (Boss, 1999).

The X2 box shares homology with TPA (12-o-tetradecanoylphorbol-13-acetate) and cAMP responsive elements (Boss, 1997). This regulatory element is bound by a homodimer of X2BP (X2-specific binding protein) (Boss, 1999). Recently, Moreno et al. (1999) demonstrated that X2BP is CREB (cAMP responsive element binding protein).

The Y box is an inverted CCAAT box (van den Elsen et al., 1998) which is bound by NF-Y (nuclear factor binding to the Y box). NF-Y is a heterotrimer consisting of NF-YA, NF-YB and NF-YC (Boss, 1999). NF-YA and NF-YC share homology with histone fold domains. Thus, NF-Y subunits may be involved in DNA bending which would allow direct

contact between NF-Y and RFX (Sinha et al., 1996, Boss, 1999).

The OBS is found exclusively in HLA-DRA promoters (Sherman et al., 1989). In B lymphocytes, this regulatory region is bound by octamer-binding TFs, Oct-1 and Oct-2. This binding is enhanced in the presence of the B cell octamer-binding protein 1 (Bob-1) (Strubin et al., 1985, Gstaiger et al., 1995).

The presence of all regulatory elements is necessary for optimal class II expression. In fact, deletion or replacement of the X or Y boxes dramatically decreases class II expression while S box deletion is not as catastrophic (Tsang et al., 1988, Koch et al., 1989, Tsang et al., 1990, Janitz et al., 1997).

1.4.2. The Class II Transactivator (CIITA)

Even when all appropriate TFs have bound the W, X1, X2 and Y regulatory elements, class II transcription is normally not initiated. Another protein, termed the class II transactivator (CIITA), is required for efficient constitutive and IFN-γ inducible class II expression (Steimle et al., 1993, Steimle et al., 1994). CIITA also activates Ii, HLA-DM and HLA-DOA transcription (Chang et al., 1999, Taxman et al., 2000). HLA-DOB expression is not controlled by CIITA (Taxman et al., 2000).

1.4.2.1. CIITA Regulation

Four independent promoters control CIITA expression in a cell-type specific manner. Promoter I controls constitutive class II expression in dendritic cells, promoter III controls constitutive class II expression in B cells and promoter IV controls IFN-γ inducible

expression in a variety of class II negative cells. Promoter II function is not yet determined (Muhlethaler-Mottet et al., 1997). Promoter III may also trigger IFN-γ inducible expression (Piskurich et al.,1999).

Mechanisms of post-translational control of this transactivator have been suggested although none have been identified (Mach, 1999).

1.4.2.2. CIITA Interacts with Class II Promoter Bound Transcription Factors

CIITA does not directly bind DNA (Steimle et al., 1993). However, it interacts with TFs bound to regulatory elements of the HLA class II promoter and components of the basal transcription machinery (Figure 1.6) (Fontes et al., 1999a, Harton & Ting, 2000, Zhu et al., 2000). Zhu et al. (2000) demonstrated that CIITA directly interacts with the RFX complex, specifically RFX5 and RFXANK and with the NF-Y complex, specifically NF-YB and NF-YC. Interactions between CIITA and CREB have been described. However, further studies are required to elucidate whether CREB directly binds CIITA or binds indirectly through interaction with another TF (Zhu et al., 2000).

CIITA recruits CREB-binding protein (CBP). This histone acetytransferase promotes chromatin remodeling and thus is recognized as a co-activator of class II transcription (Fontes et al., 1999a). Another co-activator of class II transcription, Bob-1, is also recruited by CIITA (Fontes et al., 1996). This factor contains activation domains different from those of CIITA and thus may interact with additional general TFs (Fontes et al., 1996).

Interactions between CIITA and components of the basal transcription complex have been identified (Fontes et al., 1999b). Specifically, TATA-binding protein-associated factors

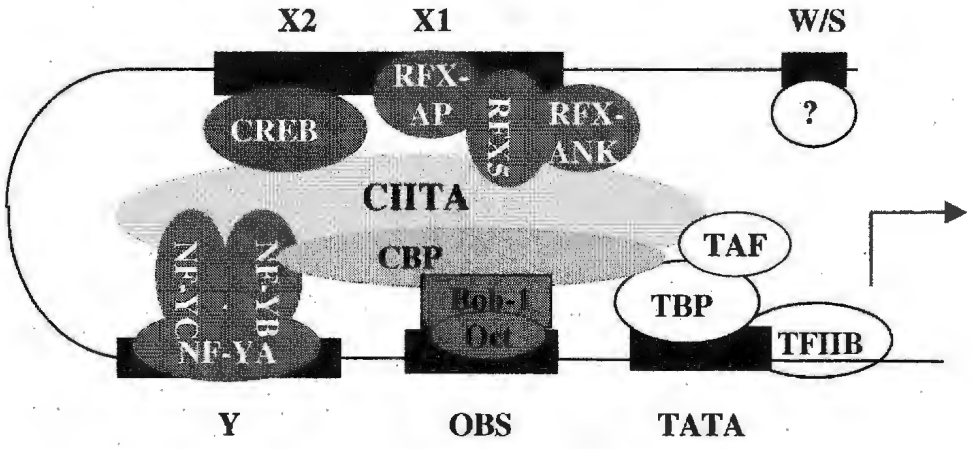


Figure 1.6. HLA class II and class II co-chaperone promoters have common regulatory elements: the W/S box, the X box, the Y box and the TATA box. CIITA interacts with transcription factors bound to these regulatory elements and components of the basal transcriptional machinery to activate class II transcription. The above diagram depicts the DRA promoter in B cells. Adapted from Fontes et al., 1999b, Zhu et al., 2000 and Harton & Ting, 2000.

(TAFs) or TFIIB, a basal transcription factor, may bridge TATA box-binding protein (TBP) to CIITA (Mahanta et al., 1997). As well, $TAF_{II}32$ which is a subunit of the basal transcription complex TFIID interacts with CIITA (Fontes et al., 1997).

Therefore, CIITA has been characterized as a transcriptional scaffold that mediates recruitment and organization of transcription factors and basal transcriptional machinery to ultimately activate HLA class II expression (Harton & Ting, 2000, Zhu et al., 2000).

1.4.3. Transcriptional Control of HLA Class II Expression

HLA class II expression is predominantly regulated at the transcriptional level (Chang et al., 1999).

1.4.3.1. CIITA as a Rate Limiting Factor

CIITA has been described as the primary rate limiting factor for class II expression (Otten et al., 1998). In fact, Otten et al. (1998) demonstrated a quantitative correlation between the level of CIITA mRNA and HLA-DRA mRNA. Eventually, increasing CIITA did not further increase HLA-DRA mRNA but this occurred at levels of HLA-DRA expression far exceeding that observed in control cells.

1.4.3.2. HLA Class II Promoter Polymorphism

Polymorphism within the class II promoter may alter the ability of TFs to bind regulatory elements and thus alter class II transcription. For example, sequence variation in the regulatory element X1 allows RFX to bind class II promoters in B cells with differing affinities as follows: DRA>DPA>>DQA=DQB>>>DRB and DPB (reviewed by Beaty et al., 1999).

Additionally, polymorphism within regulatory elements influences class II allele expression (reviewed in Beaty et al., 1999). For example, sequencing of numerous HLA-DRB allele promoters has revealed sequence variation within X and Y boxes and spacing differences between these elements (Perfetto et al., 1993, Emery et al., 1993, Singal et al., 1993, Louis et al., 1994, Singal & Qiu, 1995).

Several studies have characterized B cell line HLA-DRB promoter transcriptional activity using chloramphenicol acetyltransferase (CAT) reporter assays (Leen et al., 1994, Louis et al., 1994, Singal & Qiu, 1994, Singal & Qiu, 1995). Singal & Qiu (1994,1995) found that the transcriptional activity of DRB1 promoters in the DR1/DR51 haplotype groups was higher than that of DRB1 promoters in the DR52/DR8 and DR53 haplotype groups. DRB1 promoters in the DR53 haplotype were slightly more active than DRB4 promoters while DRB1 promoters in the DR52 haplotype were slightly more active than DRB3 promoters. It was found that differences in HLA-DRB transcriptional activities were the result of sequence and spacing variations within HLA-DRB promoters (Singal & Qiu, 1995).

In contrast, Leen et al. (1994) showed that the transcriptional activities of HLA-DRB1*1501, HLA-DRB1*0701 and HLA-DRB5*0101 promoters were similar. However, the DRB1*1501 promoter was slightly more transcriptionally active than the DRB1*0701 promoter and the DRB5*0101 promoter. Conflicting data may be explained by several differences in experimental protocol. For instance, Leen et al. (1994) cloned a larger fragment of the HLA-DRB promoter into the CAT reporter plasmid than Singal & Qiu (1994,1995). Additionally, different methodologies were utilized to assess CAT activity.

Louis et al. (1994) observed that DRB1 promoters in the DR52 haplotype displayed the highest transcriptional activity. DRB1 promoters of the DR1/DR51 and DR8 haplotypes also showed high activity. All DRB1 promoters of the DR53 haplotype showed low transcriptional activity. DRB3 promoters showed the same level of transcriptional activity as DRB1 promoters of the DR52 group while DRB4 promoters displayed lower transcriptional activity than DRB1 promoters of the DR53 haplotype. These data differ from those reported by Singal & Qiu (1994,1995) and Leen et al.(1994). There are several explanations for these discrepant findings. The CAT plasmid used by Louis et al. (1994) *i.e.* pCAT3 was different than that used in the other studies *i.e.* pCAT. Louis et al. (1994) transfected CAT constructs into the B cell line BM92 while the other studies used the B cell line Raji (Singal & Qiu, 1994, Singal & Qiu,1995 and Leen et al., 1994). As well, different methodologies were used to measure CAT activity.

Vincent et al. (1996) attempted to correlate HLA-DRB promoter transcriptional activities with levels of HLA-DRB transcripts. This group extracted RNA from peripheral blood B cells. Using competitive polymerase chain reaction (PCR), they found that DRB1 transcripts from the DR52 haplotype were 2.5 to 3.5 times more prevalent than DRB1*01 transcripts, 1.5 to 2 times more abundant than DRB1*04 transcripts and 7 times more abundant than DRB1*08 transcripts. DRB1 transcripts from the DR52 haplotype were quantitatively identical to DRB3 transcripts. The levels of HLA-DRB transcripts assessed by Vincent et al. (1996) best correlated with HLA-DRB promoter transcriptional activity measured by Louis et al. (1994) with one exception. Even though DRB1*04 transcriptional

activity was lower than that of DRB1*01 and DRB1*08 (Louis et al., 1994), HLA-DRB1*04 transcripts were detected in greater abundance (Vincent et al., 1996). It was suggested that post-transcriptional modifications enhanced stability of DRB1*04 mRNA (Vincent et al., 1996).

1.4.4. Post-Transcriptional Control of HLA Class II Expression

Post-transcriptional regulation of class II expression has been described (reviewed in Del Pozzo et al., 1999). For example, Leen & Gorski (1996) demonstrated that HLA-DRB1*07 pre-mRNA levels were 3 to 4 fold higher than DRB4 pre-mRNA levels in B cell lines. In contrast, DRB1*07 mRNA was 7 fold higher than that of DRB4 suggesting post-transcriptional regulation (Leen & Gorski, 1996).

Maffei et al. (1989) reported the existence of labile proteins involved in stabilizing class II mRNA. When B cells were treated with cycloheximide, an inhibitor of protein synthesis, a dramatic decrease in class II mRNA was observed. It was postulated that cycoheximide inhibited the expression of class II mRNA stabilizing proteins (Maffei et al., 1989). Del Pozzo & Guardiola (1996) argued that cycloheximide did not inhibit synthesis of class II mRNA stabilizing proteins. Instead, it was suggested that in the absence of class II translation, class II mRNA was rapidly degraded. Thus, class II translation stabilized class II mRNA (Del Pozzo & Guardiola, 1996).

Gonalons et el. (1998) reported that IFN-γ increased class II surface expression on mouse B cells without a concomitant increase in class II mRNA. They showed that IFN-γ increased binding of class II mRNA to ribosomes thus enhancing translation. More recently,

Cullell-Young et al. (2001) demonstrated that regulation of IFN-γ induced class II expression in mouse macrophages occurs at different levels. Specifically, IFN-γ increased class II transcription, ribosome loading, protein synthesis, protein half life and level of class II expression on the cell surface.

Caplen et al. (1992) suggested that post-transcriptional mechanisms regulated class II cell surface expression in peripheral blood T cells. Class II mRNA was detected in resting T cells but class II surface protein was not. Following T cell activation, class II expression was rapidly detected on the cell surface. This indicated that class II was already transcribed, processed and transported to the cytoplasm. It was speculated that a post-transcriptional negative regulator inhibited class II surface expression in resting T cells (Caplen et al., 1992).

1.5. IFN-γ Induction of HLA Class II Expression

IFN-γ is the most potent inducer of HLA class II expression (Jabrane-Ferret et al., 1990). Initially, the exact mechanism of how IFN-γ induced class II expression remained elusive as research failed to identify an IFN-γ response element within the class II promoter (Piskurich et al., 1999). However, the identification of CIITA as an activator of class II expression and its ability to be induced by IFN-γ quickly resolved this quandary (Chang et al., 1999). IFN-γ induction of class II expression is depicted in Figure 1.7.

CIITA promoter IV has three *cis*-acting regulatory elements which are crucial for IFN-γ induction: the GAS (IFN-γ-activating sequence) element, the E box and the IRF-1 (interferon regulating factor-1) binding site (Muhlethaler-Mottet et al., 1998, Reith et al.,

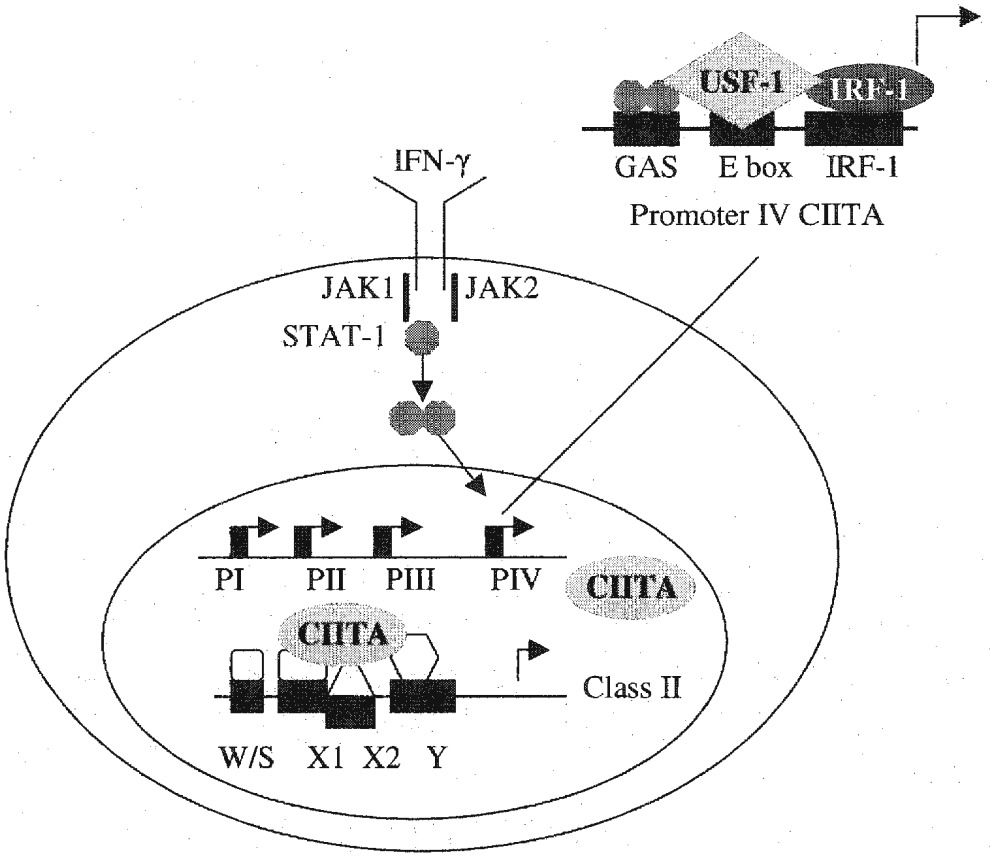


Figure 1.7. IFN-γ activates transcription of HLA class II and class II co-chaperone genes. IFN-γ interacts with its receptor which activates JAK-1 and JAK-2. Subsequently, JAK-1 and JAK-2 activate STAT-1. STAT-1 dimerizes and transclocates to the nucleus where its binds to the GAS element in CIITA promoter IV(PIV). Binding of IRF-1 to the IRF-1 response element and USF-1 to the E box activates CIITA transcription. CIITA has three additional promoters: promoter I (PI) involved in CIITA regulation in dendritic cells, promoter III involved in CIITA regulation in B cells and promoter II whose function is not known. CIITA interacts with transcription factors bound to regulatory elements on the class II promoter thereby activating class II transcription. Adapted from Mohlethaler-Mottet et al., 1998 and Piskurich et al., 1999.

1999). IFN-γ interacts with its cell surface receptor. This phosphorylates the protein tyrosine kinases Jak1 and Jak2. The Jak kinases phosphorylate the latent cytoplasmic protein, STAT-1 (signal transducer and activator of transcription). Activated STAT-1 dimerizes and translocates into the nucleus (Darnell et al., 1994, Darnell, 1997, Reith et al., 1999).

STAT-1 binds the GAS element within promoter IV of CIITA. Binding of the ubiquitously expressed transcription factor USF-1 (upstream stimulatory factor-1) to the E box enhances stability of STAT-1 binding to the GAS element (Muhlethaler-Mottet et al., 1998). Following STAT-1 induced activation of IRF-1 expression, IRF-1 interacts with the IRF-1 binding site within promoter IV (Piskurich et al., 1999). Interaction of STAT-1, USF-1 and IRF-1 with their respective regulatory elements induces CIITA expression. CIITA is then able to activate class II expression (Reith et al., 1999).

CIITA promoter III is also induced by IFN-γ in a number of cell types but its activity is weaker than that of promoter IV (Piskurich et al., 1999).

1.6. HLA Class II Antigen Processing and Presentation

HLA class II molecules are distinguished for their ability to present extracellular antigens to CD4+ T cells (Pieters, 1997). A description of the various components of the HLA class II presentation pathway (Figure 1.8) will ensue.

1.6.1. The Invariant Chain

Assembly of newly synthesized HLA class II α- and β-chains occurs in the endoplasmic reticulum (ER). Here, class II molecules associate with the invariant chain (Ii) or CD74 (Wubbolts & Neefjes, 1999). Specifically, three αβ heterodimers interact with three

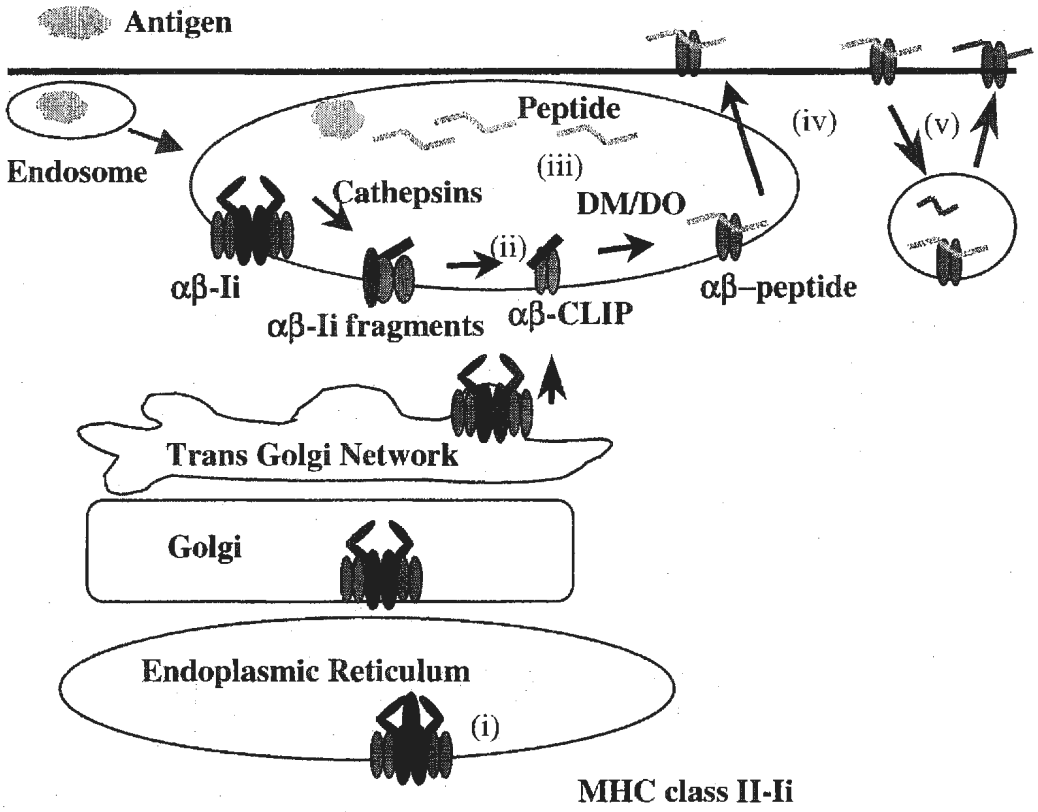


Figure 1.8. The HLA class II antigen processing and presentation pathway. i) In the ER, class II α and β chains associate with the Ii. The Ii has several important functions *i.e.* to prevent premature antigenic peptide loading, to stabilize class II molecules and to target class II to the endosomal compartments. Three Ii and three αβ complexes travel to the endocytic compartments. ii) Along the way, the Ii is degraded until only a portion of the Ii, CLIP, occupies the peptide binding site. iii) HLA-DM exchanges antigenic peptides for CLIP and also acts as a peptide editor. HLA-DO associates with HLA-DM and regulates DM activity in certain cell types. iv) The class II-peptide complex is transported to the cell surface. v) Class II molecules are recycled. This process allows cell surface class II to be internalized to endosomal compartments where antigenic peptide exchange occurs. Adapted from Pieters, 1997.

Ii subunits to form a nonamer (Cresswell, 1994). In the ER, correct class II protein folding and class II stabilization are mediated by the Ii. Additionally, the Ii, specifically a fragment termed CLIP (class II-associated invariant chain peptide), prevents peptide binding to the class II peptide binding groove in the ER (Wubbolts & Neefjes, 1999). Sorting motifs within the Ii and the class II molecule target the class II-Ii complex to endocytic compartments (Bakke & Nordeng, 1999). As this complex traverses the endocytic pathway, the Ii is degraded by proteases until only CLIP remains. CLIP occupies the class II peptide binding site until exchange with antigenic peptide (Pieters, 1997).

1.6.2. Extracellular Antigen Degradation

Antigens can access endocytic compartments by several means including receptor mediated endocytosis, phagocytosis, macropinocytosis and autophagy (Villadangos et al., 1999). As antigens progress through the endocytic route, the environment within these vesicles becomes increasingly more acidic and more proteolytically active. Thus, antigens are gradually degraded (Villadengos et al., 1999). Antigenic peptides 12-24 residues in length (Germain, 1994) are bound by class II molecules in acidic endosomal/lysosomal compartments (Jensen et al., 1999).

1.6.3. Antigenic Peptide Binding to HLA Class II Molecules: Role of HLA-DM and HLA-DO

HLA-DM associates with class II molecules in endosomal/lysosomal compartments and catalyzes the exchange of CLIP for antigenic peptides (Denzin & Cresswell, 1995, Sherman et al., 1995, Sloan et al., 1995). HLA-DM facilitated CLIP removal is class II allele

dependent. Those alleles which bind CLIP with high affinity require HLA-DM activity for efficient CLIP removal while those alleles with low affinity for CLIP do not (Kropshofer et al., 1999, Sette et al., 1995, Stebbins et al., 1996). Furthermore, CLIP may promote its own dissociation from class II molecules in HLA-DM deficient cells (Kropshofer et al., 1995).

HLA-DM is believed to function by transiently binding to the class II-CLIP complex. HLA-DM facilitates CLIP removal and remains associated with empty class II molecules until binding of an antigenic peptide. Thus, DM acts to stabilize empty class II molecules (Kropshofer et al., 1999, Busch et al., 2000, van Ham et al., 2000). DM also functions as a peptide editor. It displaces peptides with low affinity from the class II peptide binding groove in favor of high affinity peptides (Jensen et al., 1999, Krophshofer et al., 1999).

Another class II co-chaperone, HLA-DO, modifies the peptide binding repertoire of class II molecules. HLA-DO is primarily expressed in B cells and thymic epithelial cells, although it has been detected in a subset of dendritic cells (Alfonso et al., 1999). HLA-DO associates with HLA-DM and this association is required for its exit from the ER (Liljedahl et al., 1996). DO may inhibit DM activity since its expression reduces DM facilitated CLIP release from class II antigen binding sites (Denzin et al., 1997). This modulation of HLA-DM activity may be pH dependent. For example, in lysosomes (pH 5.0) HLA-DO reduces HLA-DM activity while in early endosomes (pH 6.0) DO inhibits DM function altogether (van Ham et al., 2000). DO's function as a negative regulator of DM activity makes sense given its involvement in B cell antigen presentation. In B cells, antigens are taken up by receptor mediated endocytosis. Release of antigen from these receptors requires low pH.

Thus, DO inhibits premature DM mediated peptide exchange until release and processing of receptor bound antigens in lysosomal compartments (reviewed in Alfonso et al., 1999). Further, HLA-DO is a peptide editor (Kropshofer et al., 1998). The significance of DO expression in cells other than B cells is not known.

In the absence of HLA-DO, HLA-DM still retains all function (van Ham et al., 1997, Kropshofer et al., 1998). In fact, class II molecules in H2-O deficient mice present an array of peptides similar to that of wild type mice (Liljedahl, et al., 1998).

1.6.4. Cell Surface Transport of HLA Class II Molecules

Following class II peptide loading, lysosomal/endosomal vesicles fuse with the plasma membrane depositing mature class II molecules and other vesicle components including HLA-DM and HLA-DO on the cell surface. Most vesicle contents, with the exception of mature class II molecules, are rapidly retrieved via clathrin-mediated internalization (Wubbolts & Neefjes, 1999).

1.6.5. Alternative HLA Class II Antigen Presentation Pathways

Class II molecules expressed on the cell surface may recycle to early endosomes (Pinet et al., 1998). This recycling is mediated by endosome specific sorting signals in class II cytoplasmic tails (Pinet et al., 1995). Once internalized, HLA-DM may facilitate peptide exchange on these class II molecules (Arndt et al., 2000, Pathak et al., 2001).

Finally, it is important to note that even though class II molecules are primarily known for their ability to present exogenous antigens, these molecules frequently present endogenously derived antigen *i.e.* cytoplasmic, nuclear, mitochondrial and membrane

associated (Sant, 1994, Armstrong et al., 1997, Lich et al., 2000, Qi et al., 2000). Endogenous antigens may gain access to antigenic peptide receptive class II molecules in endosomal/lysosomal compartments by several means including chaperone-mediated trafficking from the cytosol, autophagy from the cytosol and direct trafficking from the Golgi (Loss et al., 1993).

1.7. HLA Class II and Cancer

1.7.1 HLA Class II May Confer Susceptibility to or Protection from Cancer

Given the critical role of HLA in the immune response, it is perhaps not surprising that an individual's HLA genotype influences whether a person develops particular types of cancer (Bateman & Howell, 1999, Little & Stern, 1999). For example, DR1 may be associated with the development of multiple basal cell carcinomas (Bateman & Howell, 1999). Iarygin et al. (1991) reported that HLA-DRB1*04 is correlated with a poor clinical prognosis in breast carcinoma. Not all HLA associations with neoplastic disease are negative. For example, Chaudhuri et al. (2000) reported that HLA-DRB1*11 and DQB1*03032 are protective in early onset breast cancer.

1.7.2. HLA Class II Expression by Breast Carcinomas

To investigate class II expression in normal and neoplastic breast tissue, immunohistochemistry and monoclonal antibodies capable of recognizing class II specific determinants have been employed (Newman et al., 1980, Whitwell et al., 1984, Bartek et al., 1987, Zuk & Walker, 1988, Koretz et al., 1989, Moller et al., 1989, Garrido et al., 1993,

Concha et al., 1995, Maiorana et al., 1995).

Variable class II expression was observed on normal breast tissue (Newman et al., 1980, Bartek et al., 1987, Moller et al., 1989, Garrido et al., 1993). Class II was not expressed on normal non-lactating breast epithelium but was expressed during late pregnancy and lactation implying hormonal regulation of class II expression (Newman et al., 1980, Bartek et al., 1987, Moller et al., 1989). In fact, prolactin has been implicated in class II up-regulation on normal mammary tissue (Klareskog et al., 1980, Newman et al., 1980).

HLA class II expression on breast neoplasms has been investigated (Whitwell et al., 1984, Bartek et al., 1987, Zuk & Walker, 1988, Koretz et al., 1989, Moller et al., 1989, Concha et al., 1995, Maiorana et al., 1995, Vitale et al., 1998). These studies revealed variable class II expression on breast cancers. For example, Concha et al. (1995) found that 30% of breast carcinomas expressed class II. Zuk & Walker (1988) showed that 39% of breast tumors were class II positive. Moller et al. (1989) reported that 55% of breast tumors expressed class II.

The biological significance of class II expression on breast tumors is controversial. While some studies have correlated class II expression with good tumor differentiation and favorable clinical prognosis (Zuk & Walker, 1988, Brunner et al., 1991, Concha et al., 1991b, Concha et al., 1995) others have shown no such correlation (Moller et al., 1989, Wintzer et al., 1990, Maiorana et al., 1995). Since the biological function of class II molecules is to present antigenic peptides to CD4+ T cells, several studies have attempted to correlate class II positive tumors with infiltrating immune cells. In many cases, there was

no correlation between the extent of infiltrating immune cells and class II positive breast tumors (Whitwell et al., 1984, Bartek et al., 1987, Zuk & Walker, 1988, Concha et al., 1995). In contrast, Gottlinger et al. (1985) and Zuk & Walker (1987) have shown a positive correlation between the extent of tumor infiltrates and class II expression on breast tumors.

1.7.3. The HLA Class II Restricted Anti-Tumor Immune Response

The presentation of tumor antigen by class II molecules is essential for generating a CD4+ T cell tumor antigen-specific immune response. A putative model of this immune response is depicted in Figure 1.9. Class II positive tumor cells may migrate to lymphoid tissue where they encounter and activate T cells. Tumors that remain outside secondary lymphatic tissues are ignored by T cells (Ochsenbein et al., 2001). As well, conventional APCs present tumor antigen in the context of class II after acquiring shed tumor debris. This process is termed cross-priming. These cells then traffic to lymphoid tissue and activate tumor specific CD4+ T cells (Armstrong et al., 1998b, Hung et al., 1998, Pardoll & Topalian, 1998).

Assuming tumor antigen presentation by class II, activation of tumor specific T cells may not occur since many tumor antigens are recognized as self. Thus, tumors may be immunologically ignored (Costello et al., 1999, Marincola et al., 2000).

Nevertheless, there is evidence suggesting that an HLA class II restricted immune response is elicited. For example, class II restricted CD4+ tumor infiltrating lymphocytes have been isolated from multiple neoplasms including breast cancer (Schwartzentruber et al.,

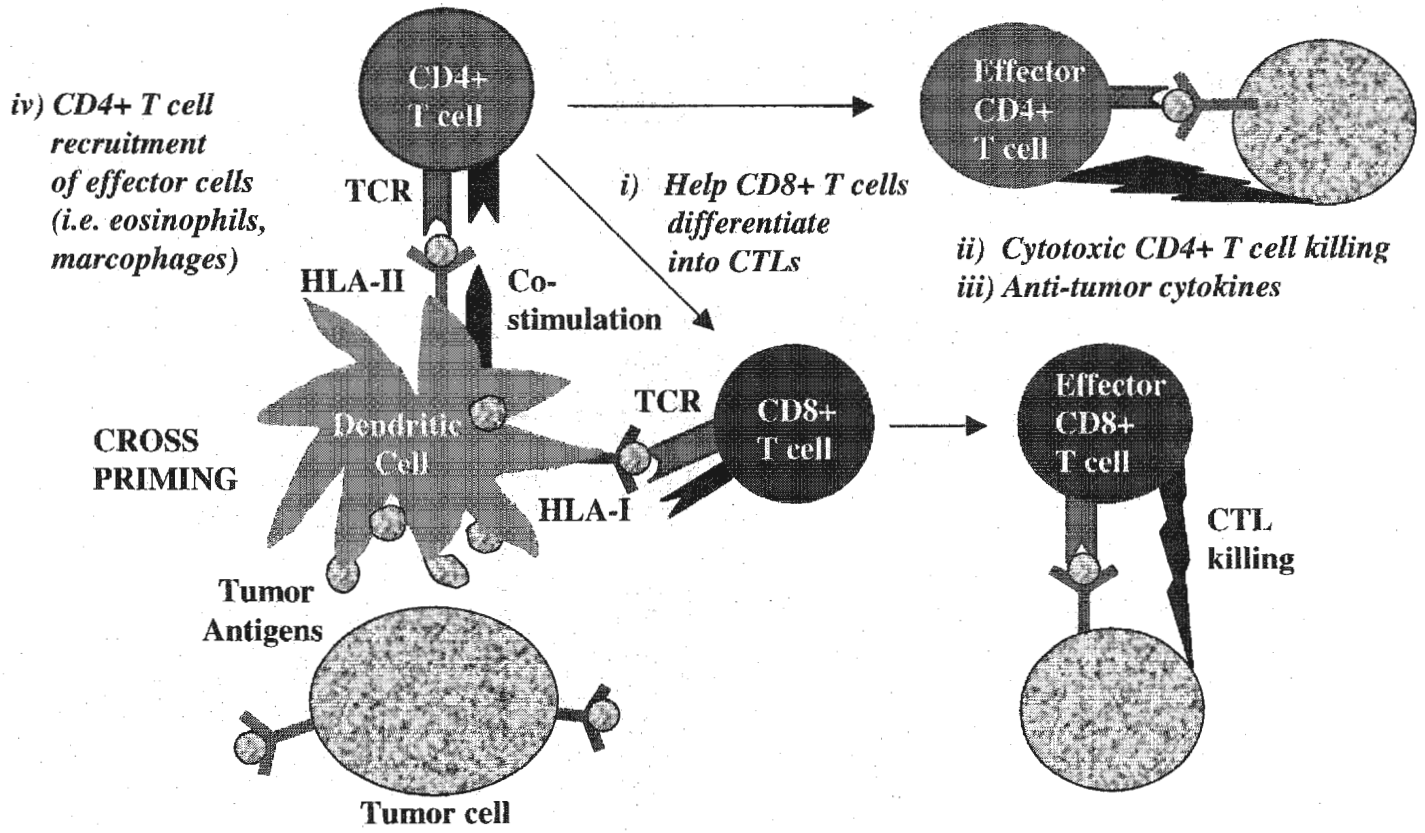


Figure 1.9. The role of CD4+ T cells in the anti-tumor immune response. Dendritic cells may capture shed tumor antigen and present antigenic tumor peptides to CD4+ and CD8+ T cells. This process is termed cross priming. Activated effector T cells may then destroy tumor cells. Tumor cells may also activate T cells (not shown). CD4+ T cells are essential in the tumor antigen-specific immune response. CD4+ T cells may i) help CD8+ T cells differentiate into potent tumor antigen-specific CTLs ii) eliminate cancer cells in a manner similar to CTL killing or generate cytokines which may iii) destroy tumor cells iv) recruit tumoricidal effector cells (*i.e.* macrophages, eosinophils). Adapted from Pardoll & Topalian, 1998.

1992, Dadmarz et al., 1995, Robbins & Kawakami, 1996, Hiltbold et al., 1998, Tuttle et al., 1998). Whether tumor cells or conventional APCs mediated this CD4+ T cell activation is not known. Some studies have identified the class II molecule involved with tumor antigen presentation (Fujita et al., 1998, Hiltbold et al., 1998, Tuttle et al., 1998, Manici et al., 1999, Kobayashi et al., 2000, Zarour et al., 2000). For example, Hiltbold et al. (1998) reported that MUC1 was presented by HLA-DRB1*03 and that this complex activated CD4+ T cells.

Despite induction of a tumor antigen-specific immune response, tumor growth and dissemination may not be suppressed. This suggests that tumors evade immune recognition. Indeed, several immune evasion strategies have been recognized. Down-regulation of all class I molecules or selective down-regulation of class I loci or class I alleles is frequently observed in tumors. Class I loss may be attributed to mutations in β2-microglobulin, mutations in TAP proteins or chromosomal alterations or deletions (Moller et al., 1992, Cabrera et al., 1996, Vitale et al., 1998, Gilboa, 1999, Hicklin et al., 1999, Seliger et al., 2000). Secretion of cytokines such as IL-10 or TGF-β may act to hinder an effective immune response by suppressing cell mediated immunity (Costello et al., 1999, Geertsen et al., 1999). Additionally, malignant cells expressing FasL may interact with T cells expressing Fas, thus causing apoptotic cell death in T cells (Nagata, 1996, Costello et al., 1999). Furthermore, lack of expression of appropriate adhesion or co-stimulatory molecules on tumor cells may prevent T cell activation or induce T cell deletion or anergy (Costello et al., 1999).

1.9. Hypotheses

CIITA has been characterized as a master regulator of class II transcription (Steimle et al., 1993). While CIITA has also been implicated in regulating class II co-chaperone (invariant chain, HLA-DM and HLA-DOA) expression, several studies have demonstrated CIITA independent co-chaperone regulation (reviewed in Chang et al., 1999 and Harton & Ting, 2000). As such, we hypothesize that CIITA is expressed in correlation with class II but is not necessarily expressed in concert with class II co-chaperones. Thus, we expect that CIITA is not expressed constitutively by breast cancer cell lines but is up-regulated following IFN-γ treatment.

Studies indicate that class II positive tumor cells may present antigenic tumor peptide to CD4+ T cells thereby eliciting tumor specific immunity (Ostrand-Rosenberg, 1994, Armstrong et al., 1998b, Armstrong et al., 1998c). Given that constitutive HLA class II expression is normally restricted to conventional APCs, we predict that class II molecules (HLA-DR, -DP and -DQ) and class II co-chaperones (invariant chain and HLA-DM) will not be expressed constitutively by breast cancer cell lines but will be up-regulated in response to IFN-γ. HLA-DO expression is not expected since this co-chaperone has only been identified in B cells, thymic epithelium and a subset of dendritic cells (Alfonso et al., 1999). We further predict that CD80 and CD86 are not expressed by breast cancer cell lines given that these co-stimulatory molecules are normally found on conventional APCs and cells of hematopoietic origin (Li et al., 1996) but that CD40 is expressed since this co-stimulatory molecule is detected on many cell types (Alexandroff et al., 2000).

As mentioned in section 1.7.2, discrepant findings were reported regarding HLA-DR expression on human breast carcinomas and clinical prognosis (Zuk & Walker, 1988, Moller et al., 1989, Wintzer et al., 1990, Brunner et al., 1991, Concha et al., 1991, Concha et al., 1995, Maiorana et al., 1995). Despite years of research, this controversy has not been adequately resolved. Since these studies assessed HLA-DR expression using antibodies which recognized all HLA-DR types, it is unknown whether breast carcinomas up-regulated all HLA-DR types or select HLA-DR types. It is proposed that class II positive tumors not associated with a good clinical outcome did not express a particular HLA-DR allele that was particularly adept at presenting tumor antigen. This proposal is supported by the observation that selective loss of HLA class I alleles is frequently observed in human cancers (reviewed in Marincola et al., 2000).

1.10. Objectives

i) To examine CIITA expression in breast cancer cell lines and to correlate expression of this transactivator with class II and class II co-chaperone transcription and protein expression.

ii) To characterize breast cancer cell lines as potential HLA class II restricted antigen presenting cells by analyzing expression of HLA class II molecules, class II co-chaperones and co-stimulatory molecules.

iii) To investigate selective HLA-DR up-regulation by breast cancer cell lines.

CHAPTER 2. METHODOLOGY

2.1. Culture and Maintenance of Cell Lines

Eleven human breast cancer cell lines (Table 2.1), generous gifts from Dr. Shou-Ching Tang and Dr. Alan Pater (Memorial University of Newfoundland, St. John's, NF) and Dr. Janice Blum (Indiana University, Indianapolis, IN) were acquired. These cells were grown as adherent cultures in 25 cm^2 tissue culture flasks (Corning Incorporated, Corning, NY). MDA-MB-468 was cultured in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) (GibcoBRL, Grand Island, NY) while all other cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (GibcoBRL). B cell lines, used as positive controls throughout the project (Table 2.2), were cultured in IMDM. These cells were grown as suspension cultures in 25 cm^2 tissue culture flasks. All media were supplemented with 10% inactivated fetal bovine serum (GibcoBRL), 2 mM L-glutamine (GibcoBRL) and Antibiotic-Antimycotic (GibcoBRL) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. Cell lines were maintained in a 37°C humidified atmosphere containing 6.5% CO_2.

When breast cancer cell line growth was 80-100% confluent, as ascertained by phase contrast microscopy, cells were harvested and subcultured. This was done by removing medium from the tissue culture flask and by washing the adherent cells once with PBS (phosphate buffered saline-see Appendix A). Cells were detached from the flask by adding 0.25% trypsin (GibcoBRL) diluted in PBS and by incubating at 37°C for 2-5 minutes. When

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

Breast Cancer Cell Line	**ATCC * Identification**	**Morphology**	**Description**
MDA-MB-435**	HTB-129	Spindle-shaped	Ductal carcinoma
T-47D	HTB-133	Epithelial	Ductal carcinoma
BT-20	HTB-19	Epithelial	Adenocarcinoma
MDA-MB-231	HTB-26	Epithelial	Adenocarcinoma
MCF7	HTB-22	Epithelial	Adenocarcinoma
SK-BR-3	HTB-30	Epithelial	Adenocarcinoma
Hs578T	HTB-126	Epithelial	Ductal carcinoma
MDA-MB-436	HTB-130	Pleomorphic	Adenocarcinoma
MDA-MB-157	HTB-24	Epithelial	Medulla carcinoma
BT-474	HTB-20	Epithelial	Ductal carcinoma
MDA-MB-468	HTB-132	Epithelial	Adenocarcinoma

* American Type Culture Collection (Manassas, VA)

** Using DNA microarrays, MDA-MB-435 was found to express genes associated with melanoma (Ross et al., 2000). Thus it is possible that this cell line is not a breast cancer cell line but instead a melanoma cell line.

Table 2.2. B cell lines used as controls in this study.

B Cell Line	IHW* Identification	HLA-DR Type
JESTHOM	IHW 9004	DRB1*0101
MGAR	IHW 9014	DRB1*1501, DRB5*0101
COX	IHW 9022	DRB1*0301, DRB3*0101
SAVC	IHW 9034	DRB1*0401, DRB4*0101
MT14B	IHW 9098	DRB1*0404, DRB4*0101
HAS115	IHW 9298	DRB1*0405, DRB4*0101
OMW	IHW 9058	DRB1*1301, DRB3*0101
CB6B	IHW 9060	DRB1*1301, DRB3*0202
HO301	IHW 9055	DRB1*1302, DRB3*0301
PLH	IHW 9047	DRB1*0701, DRB4*0101

* International Histocompatibility Workshop

cells no longer adhered to the flask, the cell suspension was transferred to a 15 ml centrifuge tube and trypsin activity was terminated by adding an equivalent volume of complete media. Cells were centrifuged at 300 g for 7 minutes after which the supernatant was decanted and the cell pellet was re-suspended in 5 ml complete media. To maintain cell cultures, $3x10^5$-$5x10^5$ cells were dispensed into 25 cm^2 tissue culture flasks in a final volume of 7 ml complete media.

B cell lines were maintained by removing approximately one third of mixed cell suspension and by adding the same volume of fresh media. This was done twice a week.

2.2. IFN-γ Treatment of Breast Cancer Cell Lines

To up-regulate expression of HLA class II and class II co-chaperone genes, breast cancer cell lines were treated with 500 units/ml human recombinant interferon-gamma (IFN-γ) (Pharmingen, San Diego, CA). Twenty-four hours prior to IFN-γ treatment, cell lines were subcultured, as described above, into 25 cm^2 tissue culture flasks such that each flask received $2x10^5$-$5x10^5$ cells in 7 ml complete media.

At the time of IFN-γ treatment, media was aspirated from cells and replaced with complete media containing 500 units/ml IFN-γ. To ascertain constitutive gene expression, a second flask containing the same cell line received complete media without IFN-γ. Most experiments were incubated for 96 hours but to determine the optimal time for induced expression some experiments were incubated for various times, from 6 hours to 168 hours.

2.3. HLA Class II Typing of Breast Cancer Cell Lines

2.3.1. DNA Extraction

Adherent breast cancer cells were harvested using 0.25% trypsin (see section 2.1) and washed once with PBS. The cell pellet was re-suspended in 1 ml of PBS and transferred to a 1.5 ml Eppendorf tube. Cells were centrifuged at 12,000 g for 1 minute and the supernatant was drained. Cells were lysed by re-suspending in 1 ml DNAZOL reagent (GibcoBRL) for 3 minutes. DNA was precipitated by adding 0.5 ml 100% ethanol (Sigma, St. Louis, MO). Following centrifugation at 12,000 g for 30 seconds, the supernatant was decanted and the DNA pellet was washed twice using 1 ml 95% ethanol. For each wash, the Eppendorf tube was inverted several times and centrifuged at 12,000 g for 30 seconds. Following the second ethanol wash, DNA was air dried for10 minutes and re-suspended in 50-100 µl 8 mM NaOH, depending on the amount of DNA extracted. To facilitate DNA solubilization, DNA was heated at 65°C for 5 minutes. Finally, DNA was quantified using UV spectrophotometry. A A_{260}/A_{280} ratio ranging from 1.6-1.9 was used as an indication of DNA purity. Since A_{260} = OD_{260} = 1 for a 50 µg/ml solution of double stranded DNA (Ryan et al., 1997), the following calculation was used to determine the concentration of DNA:

$(OD_{260} - OD_{320})$ x 50 µg/ml x Dilution Factor = DNA Concentration (µg/ml).

The OD_{320} was subtracted as background.

2.3.2. HLA Class II DNA Typing of Breast Cancer Cell Lines for HLA-DRB and HLA-DQB Genes

To identify the HLA-DRB and the HLA-DQB1 genes in each breast cancer cell line,

the Micro SSP Generic HLA Class II DNA Typing kit from One Lambda (Canoga Park, CA) was used. When this kit was unsuccessful in determining HLA-DQB1 types, the Fastype DQ Low Resolution PCR-SSP Typing kit (Bio-Synthesis, Louisville, TX) was employed. Cell lines typing positive for HLA-DRB1*01, DRB1*04, DRB1*13 and DRB3 were subtyped to identify HLA-DRB alleles. The following commercially available kits were used: the Micro SSP HLA-DRB1*04 Allele Specific Class II DNA Typing Tray (One Lambda), the Fastype DNA-SSP DRB1*01 Subtyping kit (Bio-Synthesis), the Fastype DNA-SSP DRB1*13 Subtyping kit (Bio-Synthesis) and the Fastype DNA-SSP DRB3 Subtyping kit (Bio-Synthesis). The DRB1*01 and DRB1*13 typing kits were generously provided by the Health Sciences Centre Clinical Immunology Laboratory (St. John's, NF). Detailed instructions describing usage of each typing kit were provided by the manufacturer. For this reason, methodologies will not be repeated. However, a brief description of typing kits will ensue.

Pre-aliquoted sequence specific primers capable of amplifying class II alleles or groups of alleles by polymerase chain reaction (PCR) were supplied in DNA typing trays. As a control for successful PCR amplification, primers capable of amplifying housekeeping genes *i.e.* β-globin or glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were included. Specially formulated mixes containing PCR buffer and dNTPS were provided. Taq DNA polymerase (5 units/μl) (GibcoBRL) was required but not provided. Class II genes were amplified in the Lab-Line Programmable Thermal Blok II (Melrose Park, IL) or the Biometra Temperature Gradient (Montreal Biotech Inc., Kirkland, QC) as per manufacturer's instructions.

2.3.3. Electrophoresis of PCR Products

To visualize PCR products, each PCR reaction (10 µl) was loaded into wells of a 1.5% agarose gel. Electrophoresis dye was not required since cresol red was included in reagents in the typing kits. To determine the size of amplified PCR products, ~0.19 µg of a 100bp DNA ladder (GibcoBRL) was mixed with ~1 µl loading buffer (see Appendix A) and electrophoresed at the same time. The gel was made by dissolving 0.6 g agarose (GibcoBRL) in 40 ml 0.5x TBE buffer (Tris borate EDTA buffer-see Appenidx A) containing 0.5 µg/ml ethidium bromide. PCR products were electrophoresed at 120V for 7-15 minutes in the Mini Sub DNA Cell electrophoresis chamber (BioRad, Mississauga, ON) containing 0.5x TBE buffer. The gel was photographed under UV light and interpretation of HLA class II typing results was aided by the provided worksheets.

2.4. Flow Cytometry

2.4.1 Primary Antibodies

A description of primary antibodies used to detect expression of HLA class II (HLA-DR, -DP, -DQ) and class II co-chaperones is presented in Table 2.3. NFLD.M67 was obtained locally. L243 (HB-55) and W6/32 (HB-95) were obtained from the American Type Culture Collection while the remaining antibodies were obtained commercially. Primary antibodies used to determine expression of HLA-DR allelic products are described in Table 2.4. NFLD.D1, NFLD.D7, NFLD.D2, NFLD.D10 and NFLD.M1 were obtained

Table 2.3. Primary antibodies used to detect HLA class II and class II co-chaperone protein expression by flow cytometry and immunocytochemistry.

Antibody	Isotype	Specificity	Concentration Flow	IC*	Reference/ Source
L243 (Supernatant)	Mouse IgG2a	All HLA-DR	5µg/ml 19µg/ml	2.4µg/ml, 19µg/ml	Lampson & Levy, 1980
SPV-L3	Mouse IgG2a	All HLA-DQ	1/20	NT**	Novocastra (Newcastle upon Tyne, UK)
NFLD.M67 (Supernatant)	Mouse IgG1	All HLA-DP	Neat	NT	Inoko et al., 1992
CD74 Clone LN2	Mouse IgG1	Invariant chain	NT	1/5	Biotest Diagnostics (Denville, NJ)
CD74 Clone LN2	Mouse IgG1	Invariant chain	10µg/ml	NT	PharMingen International (San Diego, CA)
HLA-DM Clone MaP.DM1	Mouse IgG1	HLA-DM	5µg/ml	5µg/ml	PharMingen International
CD40 Clone 5C3	Mouse IgG1	CD40	5µg/ml	5µg/ml	PharMingen International
CD80 Clone DAL-1	Mouse IgG1	CD80	20µg/ml	20µg/ml	Cedarlane Laboratories (Hornby, ON)
CD86 Clone BU63	Mouse IgG1	CD86	20µg/ml	20µg/ml	Cedarlane Laboratories

* Immunocytochemistry ** Not tested

Table 2.4. Primary antibodies used to detect expression of HLA-DR allelic products by flow cytometry and by immunocytochemistry.

Antibody	Isotype	Specificity	Concentration		Reference/ Source
			Flow	IC*	
NFLD.D1 (Purified)	Mouse IgG1	All DR4	39µg/ml	10µg/ml	Drover et al., 1992a, Drover et al., 1994
NFLD.D7 (Supernatant)	Mouse IgG1	DR4, DR15, DR16, DR52	Neat	1/100	Drover et al., 1992a, Drover et al., 1994
NFLD.D10 (Purified)	Mouse IgG1	DR1, DR4 except B1*0402, DR9, DR10, DR14, DR15, DRB5*0201	2.5µg/ml	2.5µg/ml	Drover et al., 1992a, Drover et al., 1994
NFLD.D2 (Purified)	Mouse IgG1	DR4 except B1*0402, DR1, DR15, DR14, DRB5*0201	5µg/ml	5µg/ml	Drover et al., 1992a, Drover et al., 1994
NFLD.M1 (Supernatant)	Mouse IgG1	DR4, DR8, DR52	Neat	1/100	Drover et al., 1985, Fu et al., 1995
JS-1 (Supernatant)	Mouse IgG2a	DR1 except B1*0103, DR3, DR4 except B1*0402, DRB1*1402, DR52	1/100	1/1000	Sachs et al., 1986 11th IHW
UK8.1 (Supernatant)	Mouse IgG2b	DR3, DR11, DR13, DR14 except B1*1404	1/100	1/1000	Bodmer et al., 1985, 11th IHW
7.3.19.1 (Supernatant)	Mouse IgG2b	DR3, DR52	1/100	1/1000	Koning et al., 1984, 11th IHW

Table 2.4. Continued.

Antibody	Isotype	Specificity	Concentration Flow	IC*	Reference/ Source
NDS13 (Supernatant)	Mouse IgG1	DR3, DR11, DR12, DR13, DR14, DR8, DR52	1/100	1/1000	Fuggle et al., 1987, 10th IHW
PL3 (Supernatant)	Mouse IgG3	DR7, DR9, DR53	1/100	1/500	Horibe et al., 1994, 11th IHW
SFR16.DR7M (Supernatant)	Rat IgG2b	DR7	1/100	NT**	Radka et al., 1984, 11th IHW

* Immunocytochemistry ** Not tested

locally while the remaining HLA-DR specific antibodies were obtained through the 10th and the 11th International Histocompatibility Workshops (IHW). Negative isotype control antibodies used for flow cytometry were generous gifts from Terra Nova Biotechnology (St. John's, NF).

2.4.2. Secondary Antibody

To detect primary antibody binding, a fluorescent labeled secondary antibody, R-phycoerythrin conjugated AffiniPure $F(ab')_2$ fragment goat anti-mouse IgG, Fc fragment specific (Jackson ImmunoResearch, West Grove, PA) was used.

2.4.3. Extracellular Flow Cytometry

To detect expression of cell surface antigens, extracellular flow cytometry was performed. Twenty-five microlitres of primary monoclonal antibody diluted to a pre-determined concentration (Table 2.3 and Table 2.4) using FACS buffer were added to each polystyrene12x75 mm tube (Fischer Scientific, Nepean, ON). FACS buffer contained 0.2% fetal bovine serum (GibcoBRL) and 0.02% sodium azide (BDH Chemicals, Poole, England) in PBS. IFN-γ treated and untreated adherent breast cancer cell lines were removed from culture using 0.25% trypsin (see section 2.1). Non-adherent B cells, used as positive controls, were also removed from culture. Cells were washed once with PBS and re-suspended in an appropriate volume of FACS buffer such that each tube received $1x10^5$-$3x10^5$ cells in a final volume of 100 µl. The cell suspension and primary antibody were mixed and incubated for 30 minutes at 4°C in the dark. After the incubation, the cells were washed twice. For each wash, 2 ml FACS buffer were added to each tube and the cells were

centrifuged at 300 g at 4°C for 7 minutes. Following the second wash, the supernatant was decanted and the lip of each tube blotted on gauze. Twenty-five microlitres of secondary antibody, diluted 1/20 in FACS buffer, were placed in each tube. The cells and secondary antibody were incubated for 30 minutes at 4°C in the dark. Cells were washed twice using FACS buffer as described above. Finally, cells were re-suspended in 150-200 µl 1% paraformaldehyde (see Appendix A). Tubes were stored at 4°C for approximately 24 hours until FACS analysis using the FACS Star Plus (Becton-Dickinson, San Jose, CA).

2.4.4. Intracellular Flow Cytometry

To assess expression of intracellular antigens, intracellular flow cytometry was performed. This methodology is similar to flow cytometry for cell surface antigens with the notable exceptions that the cells were fixed and permeabilized.

After breast cancer cell lines and B cell lines were removed from culture and washed once with PBS, cells were fixed in 5 ml 2% paraformaldehyde for 15 minutes at 4°C. To quench fixation, 8 ml complete media were added to the cells and the cells were centrifuged at 300 g for 7 minutes at 4°C. Cells were washed once with 10 ml PBS and cells were centrifuged at 300 g for 7 minutes at 4°C. To permeabilize cells, cells were re-suspended in 5 ml PBS containing 0.2% saponin (Polysciences, Warrington, PA) for 15 minutes at 4°C. Cells were centrifuged at 300 g for 7 minutes at 4°C and re-suspended in an appropriate volume of wash buffer. Wash buffer contained 0.5% bovine serum albumin (Sigma) and 0.2% saponin (Polysciences).

The remainder of the assay proceeded as described for extracellular flow cytometry (section 2.4.3) except that the primary antibodies and the secondary antibody were diluted in wash buffer and the cell washes were completed using wash buffer. This was done to ensure that the cells remained permeabilized throughout the assay.

2.4.5. Interpretation of Flow Cytometry Data

The degree of expression of a particular antigen was determined as follows: Test Mean Fluorescence Intensity/ Background Mean Fluorescence Intensity. Scores of 2.0 and below were considered negative. Scores of 2.1-5.0 were considered weakly positive. Scores of 5.1-10 were considered moderately positive while scores of 10 and above were considered strongly positive. This arbitrary analysis was adapted from Tibbo et al. (1998).

When more than one experiment was performed, data was averaged and standard deviations between experiments were determined.

2.5. Immunocytochemistry

2.5.1. Primary Antibodies

As described in section 2.4.1, primary antibodies used to assess HLA class II and class II co-chaperone expression are provided in Table 2.3. Similarly, expression of HLA-DR allelic products was ascertained using antibodies described in Table 2.4. Negative control isotype antibodies used for immunocytochemistry were as follows: mouse IgG1 isotype control (Clone DAK-GO1) (DAKO), mouse IgG1 isotype control (clone 15H6) (Southern Biotechnology, Birmingham, AL), mouse IgG2a isotype control (clone HOPC-1)

(Southern Biotechnology), mouse IgG2b isotype control (clone A-1) (Southern Biotechnology) and mouse IgG3 isotype control (clone B10) (Southern Biotechnology).

2.5.2. Secondary Antibodies

DAKO EnVision peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins (Ig) (DAKO, Carpinteria, CA) or peroxidase conjugated AffiniPure $F(ab')_2$ fragment goat anti-mouse IgG + IgM (H+L) (Jackson ImmunoResearch) were used to detect primary antibody binding.

2.5.3. Substrate

To detect primary and secondary antibody complexes, Sigma Fast 3,3'-diaminobenzidine (DAB) (Sigma) was used. This substrate was made by dissolving one DAB tablet and one urea hydrogen peroxide (H_2O_2) tablet in 1 ml distilled water. H_2O_2 reacts with peroxidase conjugated to the secondary antibody liberating O_2 and water. O_2 reacts with DAB and an insoluble, brown-black precipitate is formed. Thus cells positive for a particular protein were colored brown-black.

2.5.4. Immunocytochemistry using Cytocentrifuge Preparations

2.5.4.1. Cytocentrifuge Preparations

Adherent IFN-γ treated and untreated breast cancer cell lines were removed from culture using 0.25% trypsin (see section 2.1). Non-adherent B cell lines, used as positive controls, were additionally collected. Cells were washed twice with PBS. For each wash, cells were re-suspended in approximately 6 ml 1x PBS and centrifuged at 300 g for 7 minutes. To permanently adhere cells to 3" x 1" microscope slides, cytocentrifuge

preparations were made. A filter card (Shandon Inc., Pittsburgh, PA) was assembled between the cytocentrifuge (Shandon, Inc.) sample chamber and the microscope slide. Cells ($4x10^4$-$5x10^4$), re-suspended in 0.5 ml PBS, were added to the cytocentrifuge sample chamber. Cells were cytocentrifuged at 200 g for 5 minutes. Prior to immunocytochemistry, cells were air dried for at least 24 hours.

2.5.4.2. Immunocytochemistry

Cells were fixed with acetone for 15 minutes at -20°C and air dried for 1 hour. After washing cells with PBS, cells were treated with 200 µl 0.9% H_2O_2 diluted in PBS to release cellular endogenous peroxidase. Cells were washed once with PBS and once with wash buffer containing 0.5% bovine serum albumin (Sigma) and 0.05% Tween-20 (Fischer Scientific, Nepean, ON). The washes were performed by expelling a stream of PBS or wash buffer from a wash bottle. To prevent non-specific antibody binding, cells were treated with approximately 200 µl blocking buffer, 15% goat serum diluted in PBS, for 1 hour. After removal of excess blocking buffer, 50 µl primary antibody, diluted to a pre-determined concentration (Table 2.3 and Table 2.4) in wash buffer, were placed on cells for 1 hour. Cells were then washed by a stream of wash buffer and also by immersing cells in wash buffer for 20 minutes. Fifty microliters of secondary antibody, DAKO EnVision peroxidase labeled polymer (DAKO), were added to cells for 30 minutes. Cells were then washed with wash buffer as described above except following immersion in wash buffer, slides were immersed in PBS for 15 minutes. The substrate, Sigma Fast 3,3'-DAB (Sigma), was added

to cells for 7-10 minutes. Cells were washed with distilled water and counterstained with Mayer's hematoxylin (see Appendix A) for 10 minutes. After washing cells in tap water to remove excess hematoxylin, cells were immersed in Scott's tap water (see Appendix A) for 1 minute. Cells were dehydrated by immersion in 70% ethanol, 95% ethanol, 2 changes of 100% ethanol for 1 minute each and in 2 changes of xylene for 2 minutes each. Cells were permanently mounted in DPX (BDH Chemicals, Toronto, ON) or Micromount (SurgiPath, Winnipeg, MB) and examined by light microscopy.

2.5.5. Immunocytochemistry using Chamber Slides

2.5.5.1. Chamber Slide Set-Up

Breast cancer cell lines were re-suspended in complete media containing IFN-γ or in complete media without IFN-γ. Complete media (200 µl) containing $5x10^4$-$1x10^5$ cells were distributed into a 16-well Lab-Tek chamber slide (Nalge Nunc, Naperville, IL) such that 8 wells received IFN-γ treated cells and 8 wells received untreated cells. Chamber slides were incubated for 96 hours after which media was aspirated and cells were washed twice with PBS. For each wash, each well of the chamber slide was filled with PBS and then carefully aspirated using a Pasteur pipette. Prior to immunocytochemistry, cells were air dried for at least 24 hours.

2.5.5.2. Immunocytochemistry

Cells were fixed with acetone for 15 minutes at -20°C and air dried for 1 hour. Cells were washed twice using wash buffer containing 0.5% bovine serum albumin (Sigma) and

0.05% Tween-20 (Fisher Scientific) in PBS. For each wash, each well of the chamber slide was filled with wash buffer and then carefully aspirated. Next, 100 µl blocking buffer, 15% human AB serum in PBS, were added to cells for 1 hour. Blocking buffer was aspirated from cells and approximately 65 µl primary antibody diluted to the appropriate concentration (Table 2.3 and Table 2.4) in wash buffer were added to cells for 1 hour. After washing cells 3 times with wash buffer, 100 µl secondary antibody diluted 1/500 in wash buffer, were added to cells for 30 minutes. The secondary antibody was from Jackson ImmunoResearch (see section 2.5.2). Cells were washed twice with wash buffer and once with PBS. The substrate, Sigma Fast 3,3'-DAB (Sigma), was added to cells for 7-10 minutes. Cells were washed with distilled water and counterstained with Mayer's hematoxylin and dehydrated as described in section 2.5.4.2. Finally, cells were permanently mounted in DPX mount (BDH Chemicals) and examined by light microscopy.

2.5.6. Interpretation of Immunocytochemistry Data

Immunocytochemistry was analyzed independently by two different individuals. When possible, the percentage of positive cells was determined and data was interpreted as follows: <20% positive cells was considered negative; 21-40% positive cells was considered weakly positive; 41-60% positive cells was considered moderately positive; 61-80% positive cells was considered strongly positive and 81-100% positive cells was considered very strongly positive. Staining intensity was also noted. This arbitrary analysis was adapted from Drover et al. (1994a) and Tibbo et al. (1998).

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

2.6.1. RNA Extraction

IFN-γ treated and untreated adherent breast cancer cells, grown in 25 cm^2 tissue culture flasks, were harvested using 0.25% trypsin (section 2.1) and washed once with PBS. B cells, used as controls, were removed from suspension culture and washed once with PBS. After centrifugation at 300 g for 7 minutes, the cell pellet was re-suspended in 1ml PBS and transferred to a 1.5 ml Eppendorf tube. Cells were centrifuged at 12,000 g for 1 minute and the supernatant was drained. Cells were lysed by re-suspending in 1 ml TRIZOL reagent (GibcoBRL) for 3 minutes. Following centriguation at 12,000 g for 10 minutes, the TRIZOL mixture was transferred to a 1.5 ml Eppendorf tube. Chloroform (0.2 ml) (Sigma) was added to the tube and the tube was shaken vigorously for 20 seconds. After an incubation of 3 minutes, the mixture was centrifuged at 12,000 g for 15 minutes. The upper aqueous layer, containing the RNA, was transferred to a 1.5 ml Eppendorf tube and then combined with 0.5 ml isopropanol (Sigma). RNA was precipitated overnight at -20°C.

RNA was centrifuged at 12,000 g for 10 minutes after which the supernatant was drained from the tube. RNA was washed once with 1 ml 75% ethanol dissolved in DEPC (diethyl pyrocarbonate) water (see Appendix A). Following centrifugation at 12,000 g for 5 minutes, the supernatant was removed and the RNA was air dried for 10 minutes. RNA was re-suspended in 10-30 µl DEPC water depending on the amount of RNA extracted. To facilitate RNA solubilization, RNA was heated at 65°C for 5 minutes. Finally, RNA was quantified using UV spectrophotometry. A A_{260}/ A_{280} ratio ranging from 1.6-1.9 was used

as an indication of RNA purity. Since the $A_{260} = OD_{260} = 1$ for a 40 μg/ml solution of RNA (Mullner & Garcia-Sanz, 1997), the following calculation was used to determine the RNA concentration:

$(OD_{260} - OD_{320})$ x 40 μg/ml x Dilution Factor = RNA Concentration (μg/ml).

The OD_{320} was subtracted as background.

Alternatively, adherent breast cancer cells, grown in 25 cm^2 tissue culture flasks, were washed once with PBS. After aspiration of PBS from cells, 1 ml TRIZOL reagent was added directly to cells in the tissue culture flask. The remainder of the RNA extraction followed the same protocol as described above.

2.6.2 RNasin Treatment of RNA

To protect RNA from ribonuclease activity, 30 Units RNasin (Promega, Madision, WI), a ribonuclease inhibitor, was added to each RNA sample.

2.6.3. DNase Treatment of RNA

In some instances, it was necessary to remove contaminating DNA from RNA using DNase. Critically, RNA extracted from cells exposed to trypsin was not suitable for DNase treatment. An explanation will be provided in section 3.2. Thus, RNA for treatment with DNase was extracted by adding TRIZOL directly to adherent cells in a tissue culture flask.

DNA-free DNase treatment and removal system from Ambion (Austin, TX) was used to remove contaminating DNA from RNA samples. Manufacturers instructions were followed in detail. Briefly, DNase I (2 units) and DNase I buffer containing 10 mM Tris-HCl, 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$ were combined with RNA in a 0.2 ml thin walled

micro tube (Gordon Technologies, Mississauga, ON). This mixture was incubated at 37°C for 25 minutes. Following this incubation, DNase Inactivation Reagent was added to this RNA preparation and the tube was incubated at room temperature for 2 minutes. Centrifugation at 12,000 g for 1 minute was required to pellet the DNase Inactivation Reagent. DNase treated RNA was stored at -70°C.

2.6.4 cDNA Synthesis

The synthesis of cDNA from RNA samples was accomplished using the First Strand cDNA Synthesis kit form Pharmacia Biotech (Baie d'Urfe, QC). A 1.5 µg sample of RNA was placed in a 0.2 ml thin walled micro tube (Gordon Technologies) and DEPC water was added to the RNA such that the final volume was 8 µl. RNA was heated at 65°C for 10 minutes and then quick chilled on ice.

For each reaction, 5 µl bulk first-strand cDNA reaction mix, 1 µl DTT (dithiothreitol) solution and 1 µl Not I-d $(T)_{18}$ primer (diluted 1/25 in DEPC water) were combined and added to the denatured RNA. The sample was then incubated in the Lab-Line Programmable Thermal Block II (Melrose Park, IL) or the Biometra Temperature Gradient (Montreal Biotech Inc., Kirkland, QC) at 37°C for 1 hour and at 70°C for 10 minutes. cDNA was stored at -70°C until required.

2.6.5. Primers for RT-PCR

Bio-Synthesis (Louisville, TX) primer mixes, mainly intended for use in HLA-DR DNA typing, were used to amplify DRB1, DRB3 and DRB5 mRNA in PCR. Olerup and

Zetterquist (1992) provide a detailed description of these primers. Primers used to analyze gene transcription are listed in Table 2.5. For the most part, these primer sequences are published. However, sequences of β-actin primers were kindly provided by Drs. Laura Gillespie and Gary Paterno (Memorial University of Newfoundland, St. John's, NF). HLA-DQA and HLA-DQB primers were provided by Dr. David Haegart (Memorial University of Newfoundland, St. John's, NF). HLA-DMA, HLA-DMB, HLA-DOA and HLA-DOB primers were designed using sequences acquired from Genbank. The accession numbers of the gene sequences used to construct these primers were: X76775 for DMA, X76776 for DMB, M26039 for DOA and L29472 for DOB.

2.6.5.1. Primer Design

Primer sequences, 20-22 bases in length, were devised using the Genbank sequences listed above (section 2.6.5). These sequences were entered in the *Amplify* program (Engels, 1993). This software analyzes primers to ensure that primer pairs do not form dimers and to ensure that primer affinity to the gene of interest is suitable. As well, primer sequences were analyzed using *OligoTech Analysis* software (Wilsonville, OR) to determine the primer G+C content and melting temperature. Once primer sequences were selected, primers were synthesized by GibcoBRL.

2.6.6. PCR Amplification of HLA-DRB1, DRB3, DRB4 and DRB5 mRNA

For each reaction, 15 µl primer mix (Bio-Synthesis) (see section 2.6.5) were added to each 0.2 ml thin walled polypropylene PCR tube (Gordon Technologies). Additionally, PCR buffer (GibcoBRL) containing 20 mM Tris-HCl (pH 8.4) and 50 mM KCl, 0.2 mM

Table 2.5. Primers used to detect HLA class II and class II co-chaperone transcription by RT-PCR.

Primer	Primer Sequence (5' to 3')	Reference/Source
DRA Sense DRA Antisense	GTTCTGCTGCATTGCTTTTGCGCA CGAGTTCTATCTGAATCCTGACCA	Nandan & Reiner, 1997
DRB Sense DRB Antisense	CCCCACAGCACGTTTCTTG CCGCTGCACTGTGAAGCTCT	Kimura & Sasazuki, 1992
DRB1*0404 Sense DRB1*0404 Antisense	GTTTCTTGGAGCAGGTTAAACA CTGCACTGTGAAGCTCTCAC	Olerup & Zetterquist, 1992
DRB1*0401/0405 Sense DRB1*0401/0405 Antisense	GTTTCTTGGAGCAGGTTAAACA CTGCACTGTGAAGCTCTCCA	Olerup & Zetterquist, 1992
DRB1*07 Sense DRB1*07 Antisense	CCTGTGGCAGGGTAAGTATA CCCGTAGTTGTGTCTGCACAC	Olerup & Zetterquist, 1992
DRB4 Sense DRB4 Antisense	GAGCGAGTGTGGAACCTGA CTGCACTGTGAAGCTCTCCA	Olerup & Zetterquist, 1992
DQA Sense DQA Antisense	ATGGTGTAAACTTGTACCAGT TTGGTAGCAGCGGTAGAGTTG	Kimura & Sasazuki, 1992
DQB Sense DQB Antisense	CATGTGCTACTTCACCAACGG CTGGTAGTTGTGTCTGCACAC	Kimura & Sasazuki, 1992
DPA Sense DPA Antisense	GCGGACCATGTGTCAACTTAT GCCTGAGTGTGGTTGGAACG	Kimura & Sasazuki, 1992

Table 2.5. Continued.

Primer	Primer Sequence (5' to 3')	Reference/Source
DPB Sense DPB Antisense	CCAGCTCCATGATGGTTC CTGGACTCGGCGCTGCAGG	Peijnenburg et al., 1995
CIITA Sense #1 CIITA Antisense #1	CCTGATGCACATGTACTGGGC GGTCTTCCACATCCTTCAGGG	Chin et al., 1994
CIITA Sense #2 CIITA Antisense #2	CAAGTCCCTGAAGGATGTGGA ACGTCCATCACCCGGAGGGAC	Tai et al., 1999
Invariant chain Sense Invariant chain Antisense	TCCCAAGCCTGTGAGCAAGATG CCAGTTCCAGTGACTCTTTCG	Chang & Flavell, 1995
DMA Sense DMA Antisense	CCAATGTGGCCAGATGACCTGC GCGTGAACACTTCAGCGATAG	See Section 2.6.5.1
DMB Sense DMB Antisense	GCAGAAGTGACTATCACGTGG CCGCCAGCTGATCACACCAAG	See Section 2.6.5.1
DOA Sense DOA Antisense	CCACATGGGCTCCTACGGACC GGCAGGTAGTGGAACTTGCGG	See Section 2.6.5.1
DOB Sense DOB Antisense	CTGCACTGCTCTGTGACAGGC GGACCTTAGCATGACTGAGG	See Section 2.6.5.1
β-actin Sense β-actin Antisense	ATCTGGCACCACACCTTCTACAATGAGCTGCG CGTCATACTCCTGCTTGCTGATCCACATCTGC	Paterno et al., 1998

dNTPs (GibcoBRL), 1.5 mM $MgCl_2$ (GibcoBRL), 1 unit of Taq DNA polymerase (GibcoBRL) and 1 µl cDNA or 1 µl RNA were combined in a final volume of 25 µl. An RNA sample was subjected to PCR to ensure that RNA was not inadvertently contaminated with DNA. Samples were placed in the Lab-Line Programmable Thermal Blok II or the PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA). PCR was performed for 1 cycle at 94°C for 5 minutes and 34 cycles of 94°C for 30 seconds, 57°C for 1 minute and 72°C for 1 minute. A final extension at 72°C for 5 minutes followed.

HLA-DRB1*04, B1*07 and DRB4 (DR53) primers were synthesized commercially by GibcoBRL from sequences obtained from Olerup & Zeitterquist (1992) (see Table 2.5). The reaction mixes required for amplification of these HLA-DRB transcripts were the same as that described above with some notable differences. The final volume of these reactions was 50 µl. Primers were used at a concentration of 20 pmol and 1.25 mM $MgCl_2$ was required in the DRB1*04 reaction mix. PCR amplification conditions are given in Table 2.6.

2.6.7. PCR Amplification of HLA Class II, Class II Co-chaperone and CIITA mRNA

PCR was performed in a final volume of 50 µl using: PCR Buffer (GibcoBRL) consisting of 20 mM Tris-HCl (pH 8.4) and 50 mM KCl, 0.2 mM dNTPs (GibcoBRL), 1Unit of Taq DNA polymerase (GibcoBRL) and 1 µl of cDNA or RNA. $MgCl_2$ (GibcoBRL) was required at a concentration 2 mM for DRB, DQB, DPA reactions and 1.5 mM for all other reactions. Concentrations of primers varied depending on the gene being amplified. Specifically, 10 pmol DMA and DMB primers were required, 25 pmol DRB, DQA, DQB

Table 2.6. PCR conditions used to amplify HLA class II and class II co-chaperone mRNA. The expected size of the PCR product is also given.

Primer	Size of PCR Product (bp[a])	PCR Conditions
DRA	643	94°C for 1 minute, 55°C for 1 minute, 72°C for 3 minutes for 30 cycles.
DRB	274	95°C for 1 minute, 63°C for 1 minute, 72°C for 1 minute for 30 cycles. A final extension at 72°C for 5 minutes.
DRB1*04	260	94°C for 1 minute and 68°C for 1 minute for 2 cycles. 94°C for 1 minute, 65°C for minute and 72°C for 1 minute for 30 cycles.
DRB1*07	232	94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute for 30 cycles.
DRB4	213	94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute for 30 cycles.
DQA	229	95°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute for 30 cycles. Final extension at 72°C for 5 minutes
DQB	214	95°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute for 30 cycles. Final extension at 72°C for 5 minutes.
DPA	240	95°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute for 30 cycles. Final extension at 72°C for 5 minutes.
DPB	376	94°C for 1 minute, 63°C for 1 minute, 72°C for 1 minute for 30 cycles.

Table 2.6. Continued.

Primer	Size of PCR Product (bp[a])	PCR Conditions
CIITA Set #1	266	94°C for 1 minute, 60°C for 1 minute, 72°C for 3 minutes for 30 cycles.
CIITA Set #2	470	94°C for 1 minute, 60°C for 1 minute, 72°C for 3 minutes for 30 cycles.
Invariant chain	340	94°C for 1 minute, 55°C for 1 minute, 72°C for 3 minutes for 30 cycles.
DMA	303	94°C for 1 minute, 60°C for 1 minute, 72°C for 3 minutes for 30 cycles.
DMB	296	94°C for 1 minute, 60°C for 1 minute, 72°C for 3 minutes for 30 cycles.
DOA	446	94°C for 1 minute, 55°C for 1 minute, 72°C for 3 minutes for 30 cycles.
DOB	406	94°C for 1 minute, 55°C for 1 minute, 72°C for 3 minutes for 30 cycles.

[a] base pairs

and DPA primers were required and 20 pmol of all remaining primers were required. PCR tubes were placed in the Lab-Line Programmable Thermal Block II, the Biometra Temperature Gradient (Montreal Biotech Inc.) or the PTC-100 Programmable Thermal Controller (MJ Research Inc.). Conditions employed to amplify cDNA are displayed in Table 2.6.

2.6.8. Electrophoresis of PCR products

To visualize PCR products, 5 µl of each PCR reaction were mixed with ~1 µl loading buffer (see Appendix A) and loaded into wells of a 1.5% agarose gel. At the same time, ~0.19 µg of a 100bp DNA ladder (GibcoBRL) was mixed with loading buffer and loaded into wells of the gel. The gel was made by dissolving 0.6 g agarose (GibcoBRL) in 40 ml 0.5x TBE buffer containing 0.5 µg/ml ethidium bromide. PCR products were electrophoresed at 120V for 20-30 minutes in the Mini Sub DNA Cell electrophoresis chamber (BioRad) containing 0.5x TBE buffer with 0.5 µg/ml ethidium bromide. The gel was photographed under UV light and results were interpreted.

2.6.9. Analysis of RT-PCR Data

Gene transcription was described as present or absent. No distinction between weak positives or strong positives was made.

CHAPTER 3. RESULTS

3.1. Establishment of Optimal Conditions for Investigating Expression of HLA Genes in Breast Cancer Cell Lines

3.1.1. Interferon-gamma (IFN-γ) Culture Conditions

IFN-γ is the most potent inducer of HLA expression (Jabrane-Ferret et al., 1990). For this reason, this agent is commonly used to up-regulate class II and class II co-chaperone expression in a variety of cell types (Collins et al., 1984, Gastl et al., 1985, Boyer et al., 1989, Jabrane-Ferrat et al., 1990, Muczynski et al., 1998, Kooy et al., 1999). IFN-γ culture conditions reported in the literature vary from study to study. Previous research in our laboratory determined that a concentration of 500 units/ml IFN-γ was optimal for class II induction in synovial cells (Drover, unpublished data). Additionally, Tibbo (1998, Honor's thesis) showed that the optimal IFN-γ exposure time for breast cancer cell lines was 96 hours.

3.1.1.1. Concentration of IFN-γ

To confirm that 500 units/ml IFN-γ was suitable for induction of HLA class II genes in breast cancer cell lines, T-47D was exposed to different IFN-γ concentrations, 100 units/ml, 250 units/ml and 500 units/ml, for 96 hours. Cell surface HLA-DR expression was measured using the antibody L243 and flow cytometry. The results, reported in Figure 3.1, show that HLA-DR up-regulation by T-47D cells was virtually the same for each concentration of IFN-γ tested. The concentration of 500 units/ml IFN-γ was chosen to up-

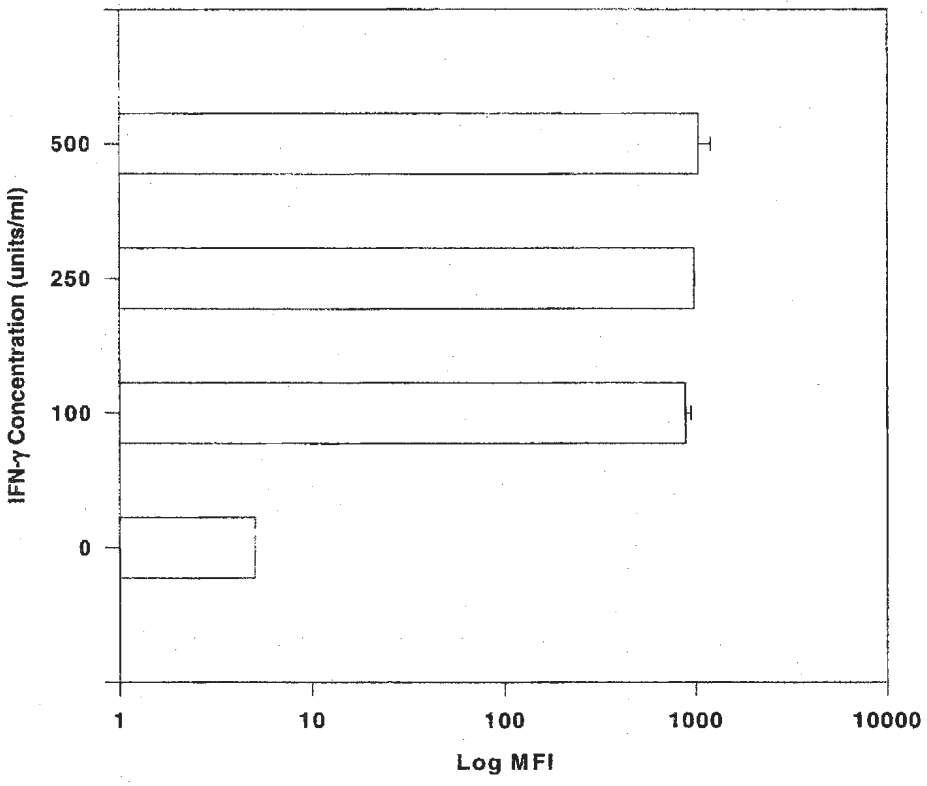


Figure 3.1. Effect of different concentrations of IFN-γ on the up-regulation of cell surface HLA-DR on the breast cancer cell line T-47D. Cells were cultured in the presence or absence of IFN-γ for 96 hours and assayed for HLA-DR expression using the antibody L243 and flow cytometry. Expression is shown as test mean fluorescence intensity (MFI) minus negative control MFI. Standard deviations between two experiments are indicated.

regulate HLA class II and class II co-chaperones in breast cancer cell lines since it was well above saturation.

3.1.1.2. Exposure Time of IFN-γ

Previous work in our laboratory showed that 96 hours was optimal for up-regulating cell surface HLA class II expression on two breast carcinoma cell lines, BT-20 and MCF-7 (Tibbo, 1998). To confirm these findings, two additional cell lines, MDA-MB-435 and MDA-MB-231, were treated with 500 units/ml IFN-γ for various times. For the cell line MDA-MB-435, cell surface HLA-DR expression was measured every 24 hours for 120 hours using the antibody L243 and flow cytometry (Figure 3.2A). HLA-DR expression was first observed at 24 hours, it increased 10 fold from 24 hours to 48 hours, remained steady from 48 to 72 hours, increased 3 fold from 72 to 96 hours and declined from 96 to 120 hours. This experiment confirmed Tibbo's (1998) findings that IFN-γ treatment for 96 hours was optimal for HLA-DR up-regulation on breast cancer cell lines.

Contrasting these findings, another experiment showed that an IFN-γ exposure time of 96 hours was not always optimal. MDA-MB-231 was treated with 500 units/ml IFN-γ and cell surface HLA-DR expression was measured every 24 hours for 168 hours except 120 hours using L243 and flow cytometry. It was found that HLA-DR expression increased with increasing IFN-γ exposure *i.e.* it did not plateau at 96 hours as previously observed (Figure 3.2A). Optimally, each cell line should have been analyzed for their optimal IFN-γ incubation time for induction of maximum HLA class II expression. However, for convenience, all breast cancer cell lines were treated with IFN-γ for 96 hours to induce or up-

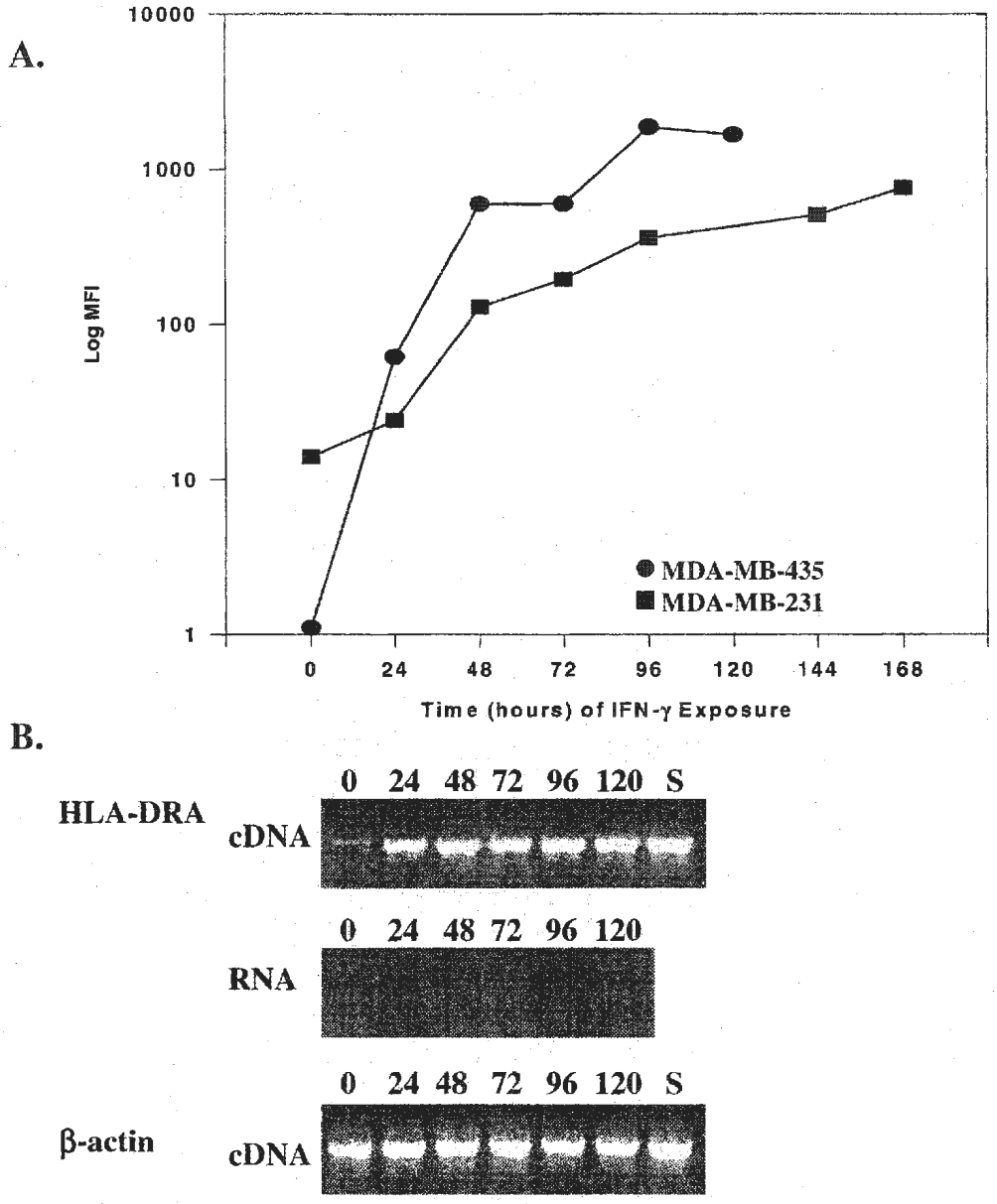


Figure 3.2. Effects of IFN-γ exposure time on HLA-DR expression in breast cancer cell lines. A) HLA-DR cell surface expression on MDA-MB-435 and MDA-MB-231. Cells were cultured in the presence of 500 U/ml IFN-γ and then assayed for HLA-DR expression using the antibody L243 and flow cytometry. Results are reported as mean fluorescence intensity (MFI) minus the background. B) HLA-DRA transcription in MDA-MB-435 as assessed by RT-PCR for 30 cycles. β-actin was amplified as a control. cDNA was synthesized using non-DNase treated RNA from trypsinized cells. RNA was subjected to PCR to ensure the absence of DNA contamination. IFN-γ exposure time is listed along the top. The B cell line SAVC (S) was used as a positive control.

regulate gene expression. It was reasoned that a longer period of time was not suitable since most cell lines required harvesting after 96 hours in culture.

Additionally, RNA was extracted from MDA-MB-435 prior to IFN-γ treatment and every 24 hours during the 120 hour IFN-γ time response. HLA-DRA and β-actin mRNA were amplified using sequence specific primers and RT-PCR (see section 2.6). As seen in Figure 3.2B, HLA-DRA and β-actin transcripts were detected at all intervals throughout the IFN-γ time response. No DNA contamination was detected in this reaction (Figure 3.2B). Clearly, less HLA-DRA mRNA was detected in untreated cells *i.e.* at 0 hours. Quantitatively, there is little variation in the amount of DRA mRNA detected between 24 hours and 120 hours of IFN-γ treatment. The main reason for assessing class II transcription at various times of the IFN-γ time response was to ensure that class II mRNA was detectable following 96 hours of IFN-γ treatment. This allowed the analysis of class II protein expression and class II transcription at the same time using the same passage of cells in certain experiments.

3.1.2. Effects of Trypsin Treatment on Breast Cancer Cell Lines

3.1.2.1. Cell Surface Expression

To test whether trypsin treatment of cells reduced the level of HLA molecules, a B cell line, MT14B, which constitutively expresses HLA class II , was used. Cells (5×10^6) were re-suspended in 5ml PBS containing 0.25% trypsin for 2 minutes and for 5 minutes or in 5ml PBS for 5 minutes at 37°C. These times were chosen as they are the times typically required to detach adherent breast cancer cell lines. After adding 5ml complete media to

quench trypsin activity, cells were washed once with PBS and cell surface HLA-DR, -DP and -DQ expression were determined using monoclonal antibodies (mAbs) and flow cytometry (see section 2.4). The results, displayed in Figure 3.3, show that class II was only slightly modulated by trypsin treatment. Cells treated for 2 minutes and 5 minutes showed a 5% and a 14% decrease in HLA-DR cell surface expression, respectively, while HLA-DQ expression increased by 18% and 8% with trypsin treatment for 2 and 5 minutes respectively. HLA-DP expression increased by 2% with 2 minutes of trypsin treatment and decreased by 7% with 5 minutes of trypsin exposure. Since trypsin treatment insignificantly altered HLA class II cell surface expression, all assays to determine cell surface expression of class II molecules on breast cancer cell lines were carried out using trypsin to remove adherent cells.

3.1.2.2. RNA Quality and RT-PCR

While trypsin induced changes in cell surface antigenic determinants have been addressed in the literature, no information was found regarding trypsin induced alterations of mRNA. At the start of this project, experiments were set-up such that IFN-γ induced and non-induced cell surface protein and mRNA were examined from the same cell culture. Thus, adherent breast cancer cell lines were treated with 0.25% trypsin to facilitate their removal from cell culture. A portion of these cells was used for flow cytometry while the remainder was stored at -70°C for RNA extraction at a later date. RT-PCR performed using cDNA synthesized from these RNA samples yielded satisfactory amplification of class II mRNA.

Unfortunately, DNA contamination was detected in certain RNA samples. This was

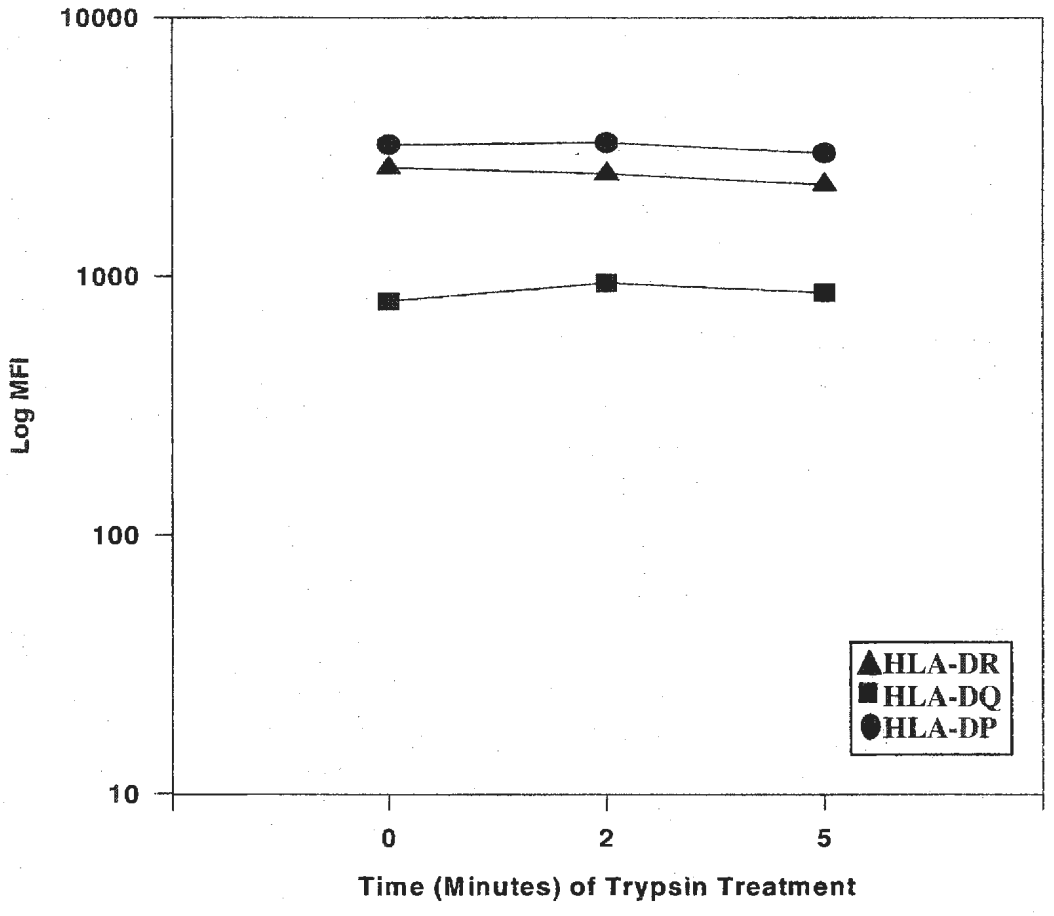


Figure 3.3. Trypsin treatment minimally affected cell surface expression of HLA class II molecules on a B cell line, MT14B. The B cell line, MT14B was treated with 0.25% trypsin for 2 minutes and 5 minutes or not treated. Cell surface HLA class II expression was assessed using monoclonal antibodies (Table 2.3) and flow cytometry. Results are depicted as test mean fluorescence intensity (MFI) minus the background MFI.

most noticeable when using primers that amplified genes with several copies in the genome *i.e.* DRB primers. For example, as seen in Figure 3.4, certain RNA samples subjected to RT-PCR for DRB amplification were contaminated with DNA. Reagents were not contaminated since a water control was negative.

To remove contaminating DNA from RNA, RNA was treated with DNase. When cDNA synthesized from DNase treated RNA extracted from trypsinized cells was subjected to RT-PCR, β-actin mRNA amplification was drastically reduced or non-existent (data not shown). Since B cell line RNA treated with DNase and subjected to RT-PCR showed good β-actin amplification, this suggested that the apparent RNA degradation was related to trypsin treatment of breast cancer cell lines. To test this theory, RNA was extracted from MDA-MB-435 cells that were removed from culture using 0.25% trypsin or by adding TRIZOL directly to the tissue culture flask *i.e.* cells were not trypsinized. Equal amounts of both MDA-MB-435 RNA samples were treated with DNase. As a control, RNA was extracted from the B cell line, SAVC, which grows in suspension. A sample of SAVC RNA was treated with DNase as well. To detect RNA degradation, samples of all the above mentioned RNA were electrophoresed for 40 minutes on a 1.5% agarose gel containing ethidium bromide. Two prominent bands, one representing 28s rRNA (~5kb in size) and one representing 18s rRNA (~2kb in size), were expected (product information sheet supplied with TRIZOL). As shown in Figure 3.5, RNA from trypsin treated MDA-MB-435 was degraded with further degradation occurring if this RNA was treated with DNase. In contrast, there was no degradation of RNA from untrypsinized MDA-MB-435 whether

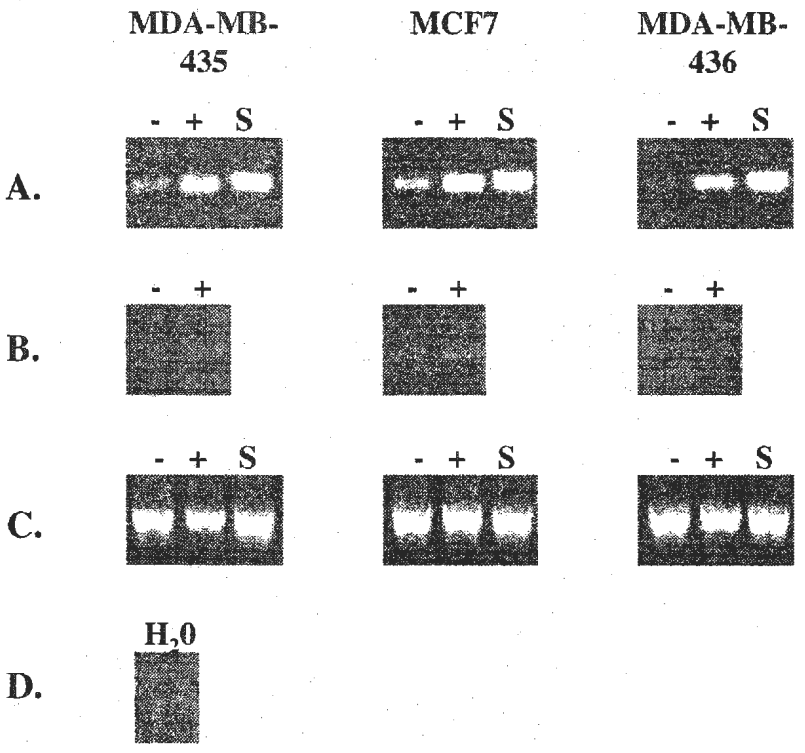


Figure 3.4. DNA contamination in breast cancer cell line RNA. HLA-DRB transcription in non-induced cells (-) and in IFN-γ induced cells (+) was assessed using sequence specific primers and RT-PCR. The B cell line SAVC (S) was the positive control. (A) cDNA synthesized from RNA extracted from trypsinzed cells (not treated with DNase) was subjected to RT-PCR. (B) RNA was subjected to PCR to detect DNA contamination. (C) β-actin was a control for mRNA integrity. Lack of amplification in the water control (D) suggests that amplification in (B) was due to DNA contamination and not due to contamination of PCR reagents.

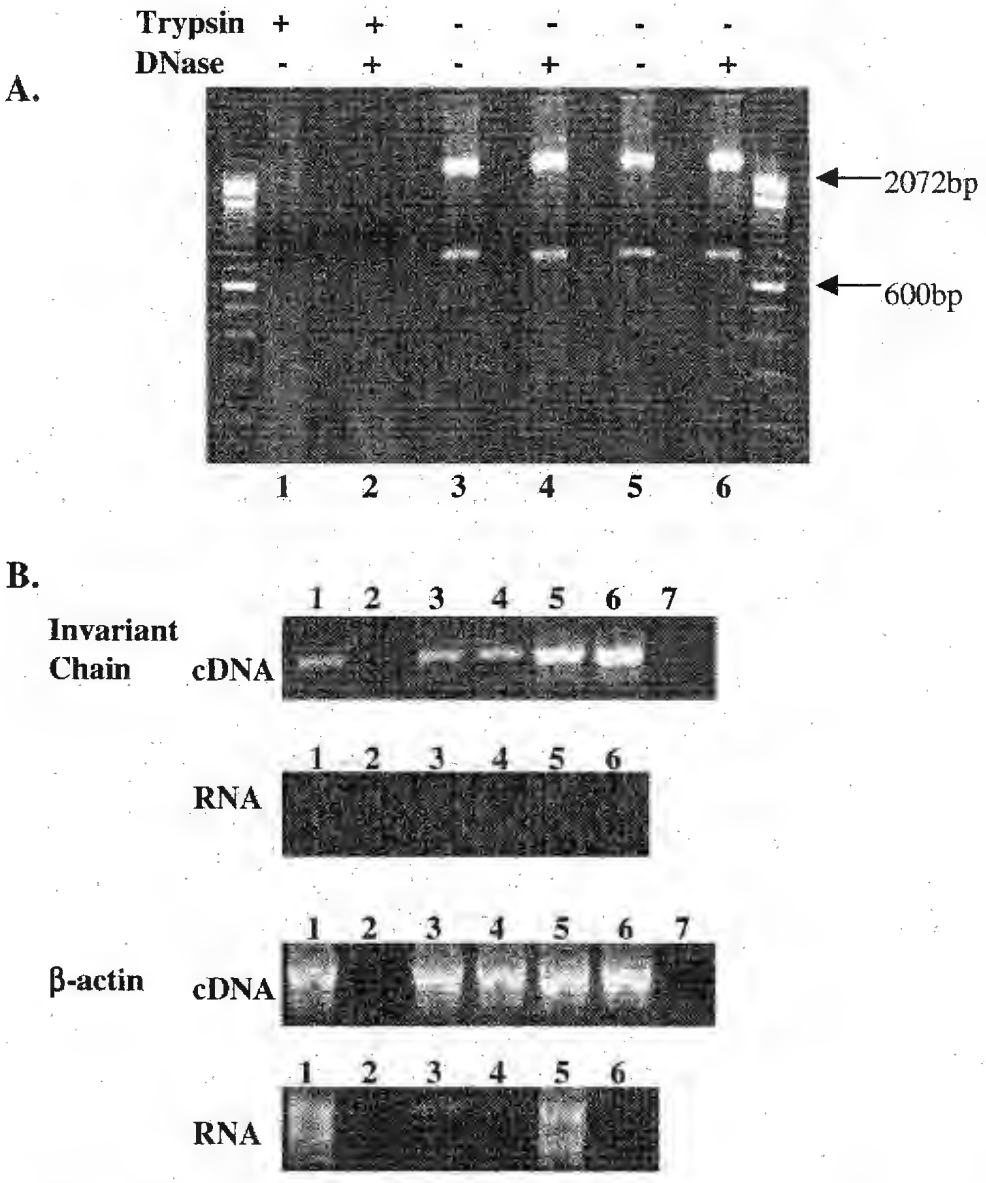


Figure 3.5. Trypsin causes degradation of breast cancer cell line RNA. A) RNA degradation was visualized on a 1.5% agarose gel. (1) RNA from trypsin treated MDA-MB-435 (2) DNase treated RNA from trypsin treated MDA-MB-435, (3) RNA from untrypsinized MDA-MB-435, (4) DNase treated RNA from untrypsinized MDA-MB-435, (5) SAVC RNA and (6) SAVC RNA treated with DNase. A 100bp DNA ladder is displayed along the side. B) Invariant chain and β-actin mRNA were amplified using sequence specific primers and RT-PCR for 30 cycles. Numbers along the top correspond to RNA samples in A. A H_20 control (7) was included to ensure that PCR reagents were not contaminated. RNA was subjected to PCR to detect DNA contamination.

treated with DNase or untreated. As expected, RNA from SAVC, regardless of DNase treatment, was undamaged. cDNA synthesized from the above mentioned RNA samples was subjected to RT-PCR to detect amplification of the invariant chain (Ii) and β-actin. Ii was used since previous experiments found that Ii mRNA was constitutively transcribed at high levels in MDA-MB-435. The results, displayed in Figure 3.5B, show that despite RNA degradation in trypsin treated MDA-MB-435 RNA, Ii and β-actin mRNA were detected. However, these transcripts were not detected after RNA treatment with DNase. In contrast, Ii and β-actin transcripts were observed in untrypsinized MDA-MB-435 and SAVC regardless of DNase treatment. Quantitatively, similar amounts of transcripts were seen in trypsinized MDA-MB-435 and untrypsinized MDA-MB-435 samples that were not treated with DNase. While Ii DNA was not amplified in the RNA control, β-actin DNA was detected in the non-DNase treated RNA. Treatment of RNA with DNase eliminated this amplification.

Notably, the RNA degradation observed in trypsinized MDA-MB-435 and trypsinized MDA-MB-435 treated with DNase is not specific to this cell line. In fact, RNA degradation was observed in all cell lines when RNA was extracted from trypsinized cells (data not shown). Three additional trypsinized cell lines were analyzed for RNA degradation after DNase treatment. Again, RNA degradation was observed (data not shown). In summary, DNase treated RNA from trypsinized breast cancer cell lines was not suitable for RT-PCR but, as the data indicate, RNA from trypsinized breast cancer cell lines that was not DNase treated was still suitable for use in RT-PCR. For the most part, RNA from

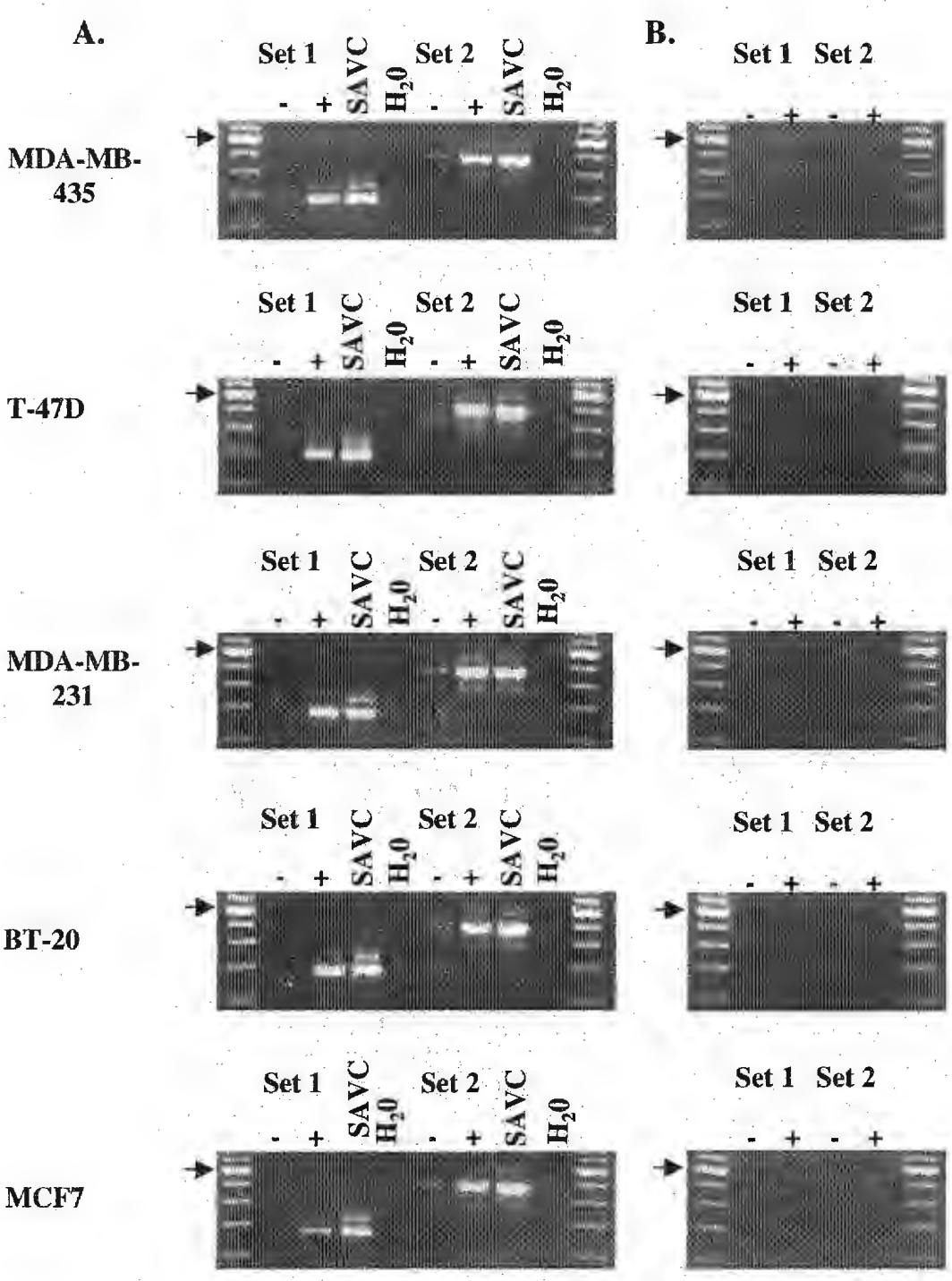
trypsinized breast cancer cells not treated with DNase was used for assessing class II and class II co-chaperone transcription in this study. Any deviations from this protocol will be described in the appropriate section. RNA controls, included in PCR to detect DNA contamination, were compared with cDNA reactions to ensure that amplified products were mRNA and not DNA.

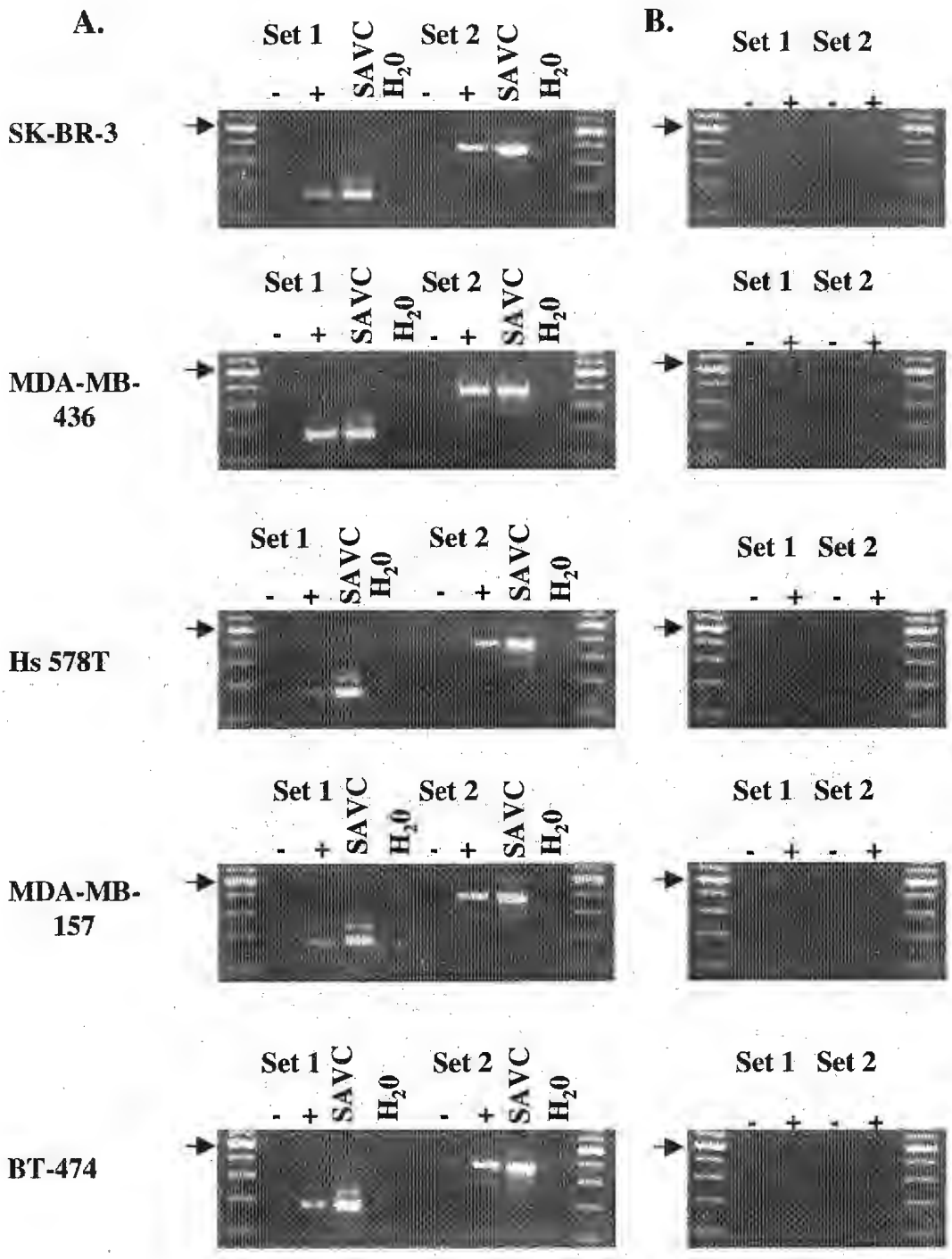
3.2. Transcription of the Class II Transactivator (CIITA) by Breast Cancer Cell Lines

Since the class II transactivator (CIITA) has been deemed the master regulator of HLA class II and class II co-chaperone transcription in APCs (reviewed in Fontes et al., 1999b), breast cancer cell lines were analyzed for CIITA transcripts to elucidate whether this factor regulates class II and class II co-chaperone expression in these cells. CIITA transcription was analyzed using sequence specific primers and RT-PCR (see section 2.6). Two different sets of CIITA primers capable of amplifying different regions of the CIITA transcript were utilized.

3.2.1. Constitutive CIITA Transcription

CIITA transcription in breast cancer cell lines is depicted in Figure 3.6 and summarized in Table 3.1. Constitutive CIITA transcription was detected in six cell lines. CIITA mRNA was observed in MDA-MB-435 and MCF7 using both CIITA primer sets. CIITA mRNA was detected in MDA-MB-231, BT-20, BT-474 and MDA-MB-468 with primer set #2 only. T-47D had an amplified product smaller than the expected 470bp using primer set #2 but none with primer set #1. The significance of the amplification of a smaller


A.
B.
Set 1
Set 2
SAVC
H_2O
MDA-MB-435
T-47D
MDA-MB-231
BT-20
MCF7


A.
B.
Set 1
Set 2
SAVC
H_2O
SK-BR-3
MDA-MB-436
Hs 578T
MDA-MB-157
BT-474

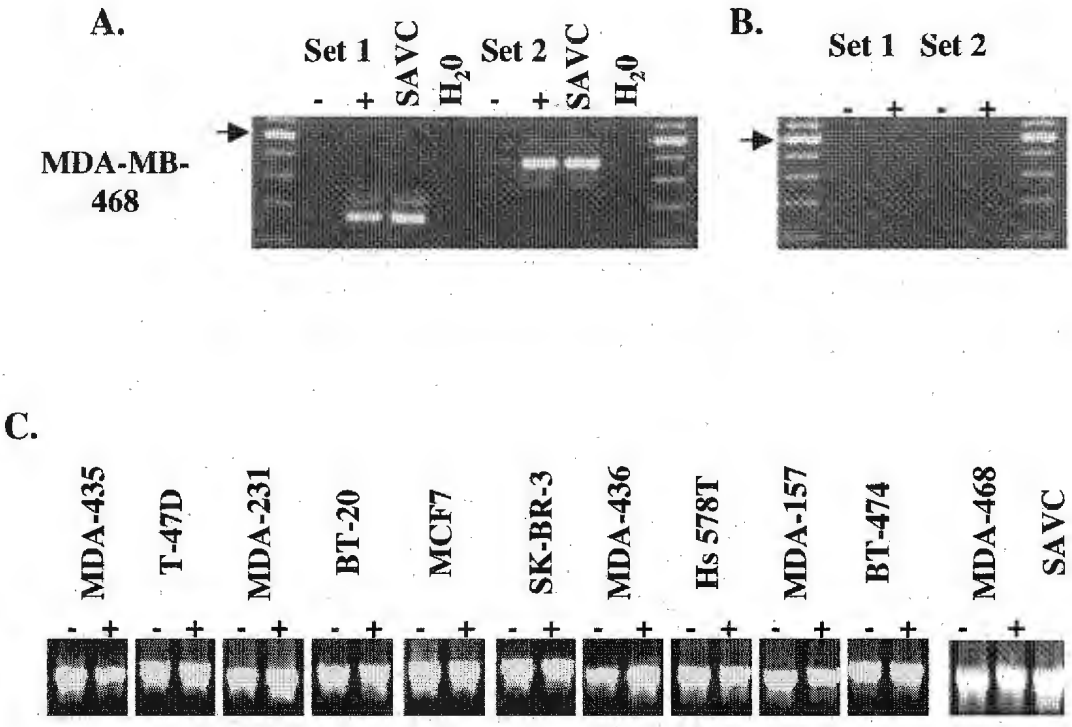


Figure 3.6. Representative gels depicting constitutive (-) and IFN-γ (+) induced CIITA transcription in breast cancer cell lines. A) CIITA transcripts were amplified using two sets of CIITA primers and RT-PCR for 30 cycles. CIITA expression was analyzed at least three times using primer set #1 and once using primer set #2. cDNA was prepared from DNase treated RNA from non-trypsinized cells. The B cell line SAVC was the positive control. A H_2O control was included to ensure that PCR reagents were not contaminated. A 100bp DNA ladder is displayed along the side of each gel with an arrow indicating 600bp. Primer set #1 amplified a transcript of 266bp while set #2 amplified a transcript of 470bp. B) RNA was subjected to RT-PCR to ensure that it was not contaminated with DNA. C) β-actin mRNA amplification, performed at the same time as CIITA amplification, was a control for mRNA integrity.

Table 3.1. CIITA transcription[a] in breast cancer cell lines.

Breast Cancer Cell Lines	Constitutive mRNA		IFN-γ Induced mRNA	
	Primer set #1	Primer set #2	Primer set #1	Primer set #2
MDA-MB-435	+[b]	+	+	+
T-47D	-[c]	-	+	+
MDA-MB-231	-	+	+	+
BT-20	-	+	+	+
MCF7	+	+	+	+
SK-BR-3	-	-	+	+
MDA-MB-436	-	-	+	+
Hs578T	-	-	+	+
MDA-MB-157	-	-	+	+
BT-474	-	+	+	+
MDA-MB-468	-	+	+	+

[a] CIITA mRNA was amplified using two sets of sequence specific primers and RT-PCR. cDNA was prepared from DNase treated RNA from non-trypsinized cells.
[b] CIITA was transcribed
[c] CIITA mRNA was not detected

CIITA product is not known but could suggest a mutation in the CIITA gene. CIITA mRNA was also not detected in SK-BR-3, MDA-MB-436, Hs578T and MDA-MB-157. Differences in CIITA mRNA amplification by the two primer sets are most likely explained by differences in their affinities for the CIITA transcript *i.e.* primer set #2 has a higher melting temperature than primer set #1. In some instances, weak bands that are visible in gel photographs are not as apparent in Figure 3.6. All RNA samples were negative for CIITA amplification indicating that CIITA mRNA was amplified and not contaminating DNA.

3.2.2. IFN-γ Induced CIITA Transcription

Following IFN-γ treatment, all breast cancer cell lines transcribed CIITA (Figure 3.6, Table 3.1).

3.3. Selective Expression of HLA Class II Isotypes (HLA-DR, -DQ and-DP) by Breast Cancer Cell Lines

To characterize breast cancer cell lines as potential HLA class II restricted APCs, it was obviously important to determine whether class II was constitutively expressed or could be induced by treatment with IFN-γ. Transcription of class II genes was determined using sequence specific primers and RT-PCR (see section 2.5). Cell surface class II protein expression was measured using mAbs and flow cytometry (see section 2.4). All flow cytometry assays were done at least twice. The B cell line SAVC served as a positive control in flow cytometry and in RT-PCR.

3.3.1. HLA-DR Expression

3.3.1.1 HLA-DRA and -DRB Transcription

DRA and DRB transcription in breast cancer cell lines are summarized in Table 3.2 and representative RT-PCR gels depicting DRA and DRB transcription are shown in Figure 3.7. In some instances, weak bands that are visible in gel photographs are not as apparent in Figure 3.7. Constitutive HLA-DRA transcription was detected in all cell lines except Hs578T, MDA-MB-157, BT-474 and MDA-MB-468. HLA-DRB mRNA was detected in non-induced MDA-MB-435, T-47D, MDA-MB-231, BT-20, MCF7, SK-BR-3 and BT-474.

All breast cancer cell lines up-regulated DRA and DRB mRNA following IFN-γ treatment.

3.3.1.2. HLA-DR Protein

MDA-MB-435, T-47D, MDA-MB-231 and BT-20 constitutively expressed cell surface HLA-DR as assessed by flow cytometry (Figure 3.8). Despite transcribing DRA and DRB mRNA, HLA-DR protein was not detected in MCF7.

All breast cancer cell lines up-regulated cell surface HLA-DR (Figure 3.8). The four cell lines which constitutively expressed DR protein expressed the highest levels of HLA-DR protein following IFN-γ treatment. There was a broad range of HLA-DR expression with MDA-MB-435 expressing the most DR (degree of expression = 345) and MDA-MB-468 (degree of expression = 6.0) expressing the least. Flow histograms depicting HLA-DR expression on select breast cancer cell lines are displayed in Figure 3.9.

Table 3.2. HLA-DRA and HLA-DRB transcription[a] in breast cancer cell lines as assessed using sequence specific primers and RT-PCR.

Breast Cancer Cell Lines	Constitutive mRNA		IFN-γ Induced mRNA	
	DRA	DRB	DRA	DRB
MDA-MB-435	+[b]	+	+	+
T-47D	+	+	+	+
MDA-MB-231	+	+	+	+
BT-20	+	+	+	+
MCF7	+	+	+	+
SK-BR-3	+	+	+	+
MDA-MB-436	+	-	+	+
Hs578T	-[c]	-	+	+
MDA-MB-157	-	-	+	+
BT-474	-	+	+	+
MDA-MB-468	-	-	+	+

[a] DRA transcription was assessed using cDNA synthesized from non-DNase treated RNA extracted from trypsinized cells. DRB transcription was assessed using cDNA synthesized from DNase treated RNA extracted from non-trypsinized cells.
[b] mRNA was transcribed.
[c] mRNA was not detected.

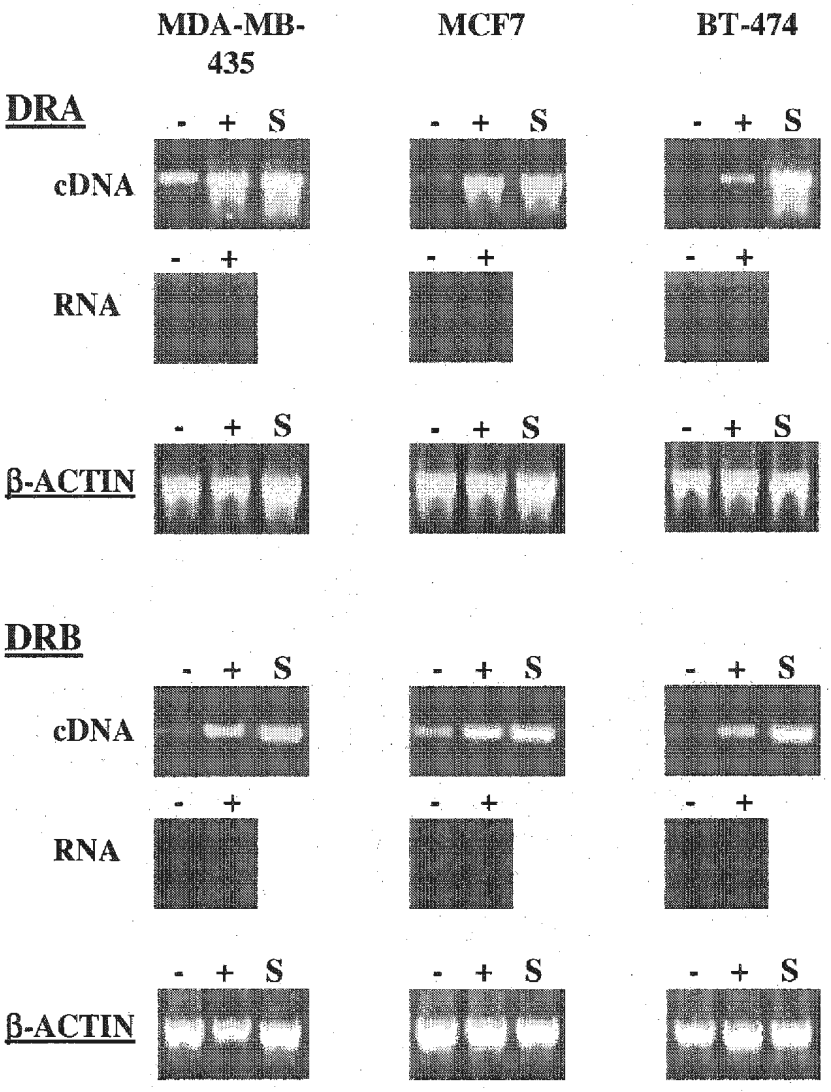


Figure 3.7. Representative gels depicting constitutive (-) and IFN-γ induced (+) DRA and DRB expression in breast cancer cell lines. DRA transcription was analyzed once by RT-PCR for 30 cycles while DRB transcription was analyzed twice by RT-PCR for 30 cycles. β-actin was amplified as a control for mRNA integrity. The B cell line SAVC (S) was the positive control. RNA was subjected to RT-PCR to ensure that it was not contaminated with DNA.

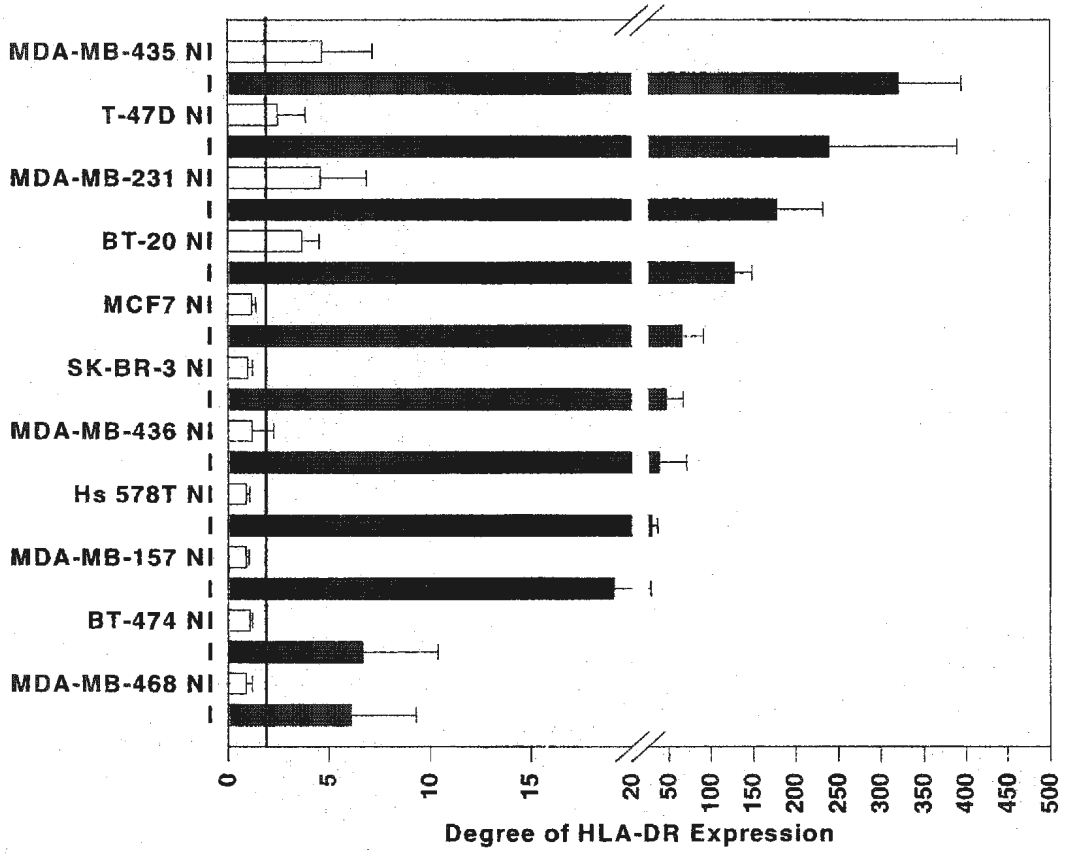


Figure 3.8. HLA-DR cell surface expression on breast cancer cell lines. HLA-DR expression was assessed using the antibody L243 and flow cytometry. Results are reported as degree of expression (see section 2.4.5) on non-induced (NI) cells (open bars) and on IFN-γ induced cells (filled bars). Standard deviations between at least three independent experiments are indicated. Values of 2.1 and above (to the right of the black line) are considered positive.

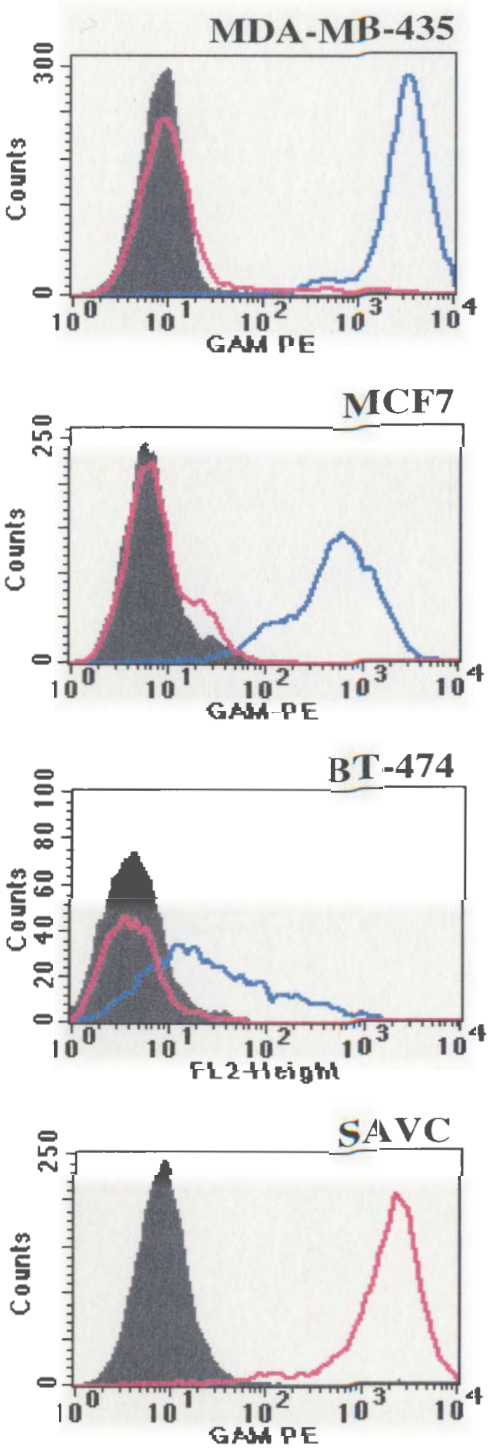


Figure 3.9. Representative flow histograms depicting constitutive (pink) and IFN-γ induced (blue) HLA-DR cell surface expression on breast cancer cell lines. Expression was assessed using the antibody L243 and flow cytometry. The negative control is shown in gray.

3.3.2. HLA-DP Expression

3.3.2.1. HLA-DPA and -DPB Transcription

Table 3.3 summarizes DPA and DPB transcription in breast cancer cell lines and representative gels depicting DPA and DPB transcription are shown in Figure 3.10. HLA-DPA was constitutively transcribed by MDA-MB-435, T-47D, MDA-MB-231, BT-20, MCF7, SK-BR-3, Hs578T, MDA-MB-157 and MDA-MB-468. DPB mRNA was detected in non-induced MDA-MB-435, MDA-MB-231, BT-20, MCF7 and MDA-MB-157. There is a band, larger than the predicted 376bp, present on HLA-DPB gels, particularly noticeable in non-induced samples. While this non-specific band is clearly visible in non-induced MDA-MB-435, MCF7 and BT-474, the specific DPB band is only visible in MDA-MB-435 and MCF7. Notably, more non-induced cells lines transcribed DPA mRNA than DPB mRNA.

All breast cancer cell lines transcribed DPA and DPB genes in response to IFN-γ.

3.3.2.2. HLA-DP Protein

While MDA-MB-435, MDA-MB-231, BT-20, MCF7 and MDA-MB-157 transcribed HLA-DPA and HLA-DPB genes, HLA-DP cell surface expression was only observed on MDA-MB-435 and MDA-MB-231 (Figure 3.11).

After IFN-γ treatment, cell surface HLA-DP was up-regulated on 10/11 breast cancer cell lines (Figure 3.11). BT-474 did not up-regulate cell surface HLA-DP despite transcribing DPA and DPB genes. The two cell lines which constitutively expressed DP protein, MDA-MB-435 and MDA-MB-231, had the highest DP expression following IFN-γ

Table 3.3. HLA-DPA and HLA-DPB transcription[a] in breast cancer cell lines as assessed using sequence specific primers and RT-PCR.

Breast Cancer Cell Lines	Constitutive mRNA		IFN-γ Induced mRNA	
	DPA[d]	DPB	DPA[d]	DPB
MDA-MB-435	+[b]	+	+	+
T-47D	+	-	+	+
MDA-MB-231	+	+	+	+
BT-20	+	+	+	+
MCF7	+	+	+	+
SK-BR-3	+	-	+	+
MDA-MB-436	-[c]	-	+	+
Hs578T	+	-	+	+
MDA-MB-157	+	+	+	+
BT-474	-	-	+	+
MDA-MB-468	+	-	+	+

[a] DPA and DPB transcription were determined using cDNA synthesized from non-DNase treated RNA extracted from trypsinized cells.
[b] mRNA was transcribed.
[c] mRNA was not detected.
[d] RT-PCR to determine HLA-DPA transcription was performed by Ingrid Pardoe.

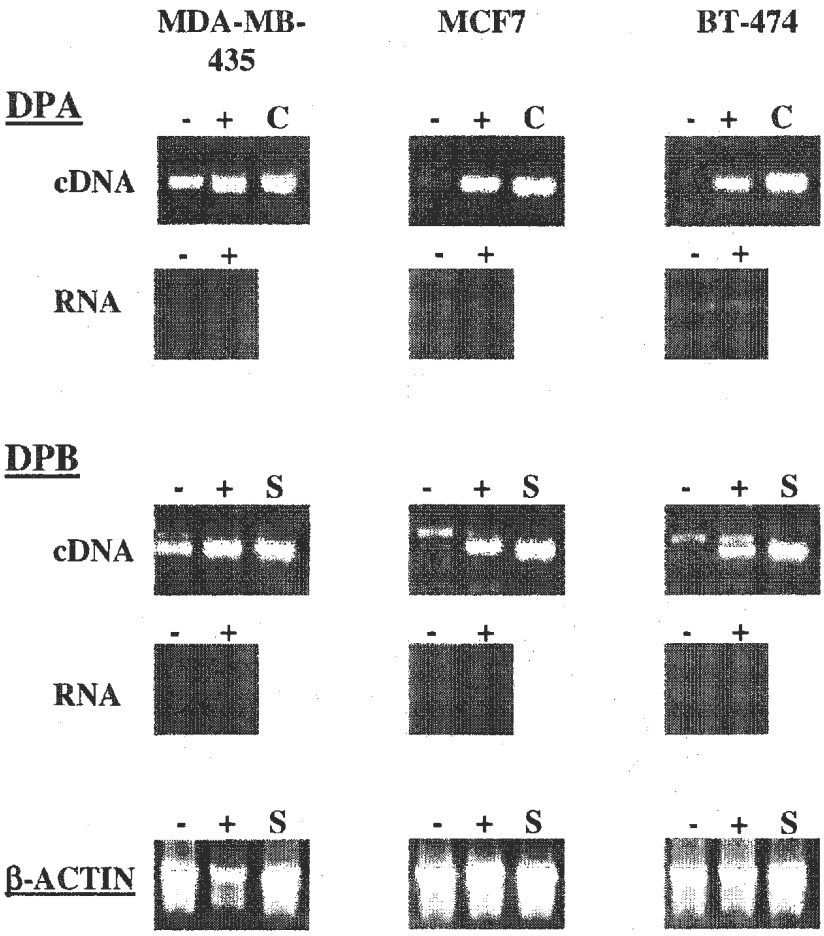


Figure 3.10. Representative gels depicting constitutive (-) and IFN-γ induced (+) DPA and DPB expression in breast cancer cell lines. DPA and DPB transcription were assessed once by RT-PCR for 30 cycles. β-actin was amplified as a control for mRNA integrity in DPB reactions but not in DPA reactions. The B cell lines COX (C) and SAVC (S) were positive controls. RNA was subjected to RT-PCR to ensure that it was not contaminated with DNA.

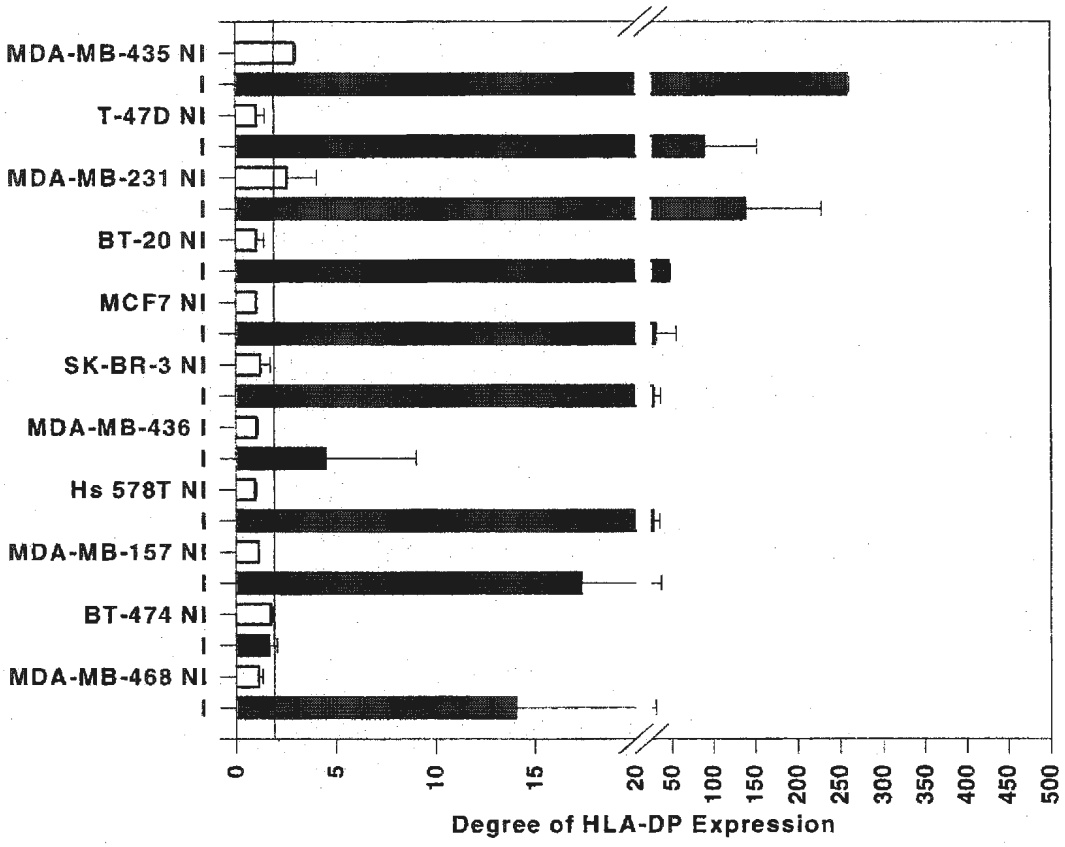


Figure 3.11. HLA-DP cell surface expression on breast cancer cell lines. HLA-DP expression was assessed using the antibody NFLD.M67 and flow cytometry. Results are reported as degree of expression (see section 2.4.5) on non-induced (NI) cells (open bars) and on IFN-γ induced cells (filled bars). Standard deviations between at least two independent experiments are indicated. Values of 2.1 and above (to the right of the black line) are considered positive.

treatment. There was a broad range of HLA-DP cell surface expression with MDA-MB-435 expressing the most DP (degree of expression = 259) and BT-474 expressing no DP (degree of expression 1.6). Representative flow cytometry plots depicting cell surface HLA-DP expression on breast cancer cell lines are displayed in Figure 3.12.

3.3.3. HLA-DQ Expression

3.3.3.1. HLA-DQA and -DQB Transcription

Breast cancer cell line DQA and DQB transcription is summarized in Table 3.4 and representative gels depicting DQA and DQB transcription are shown in Figure 3.13. In some instances, weak bands that are visible in gel photographs are not as apparent in Figure 3.13. HLA-DQA was constitutively transcribed by MDA-MB-435, T-47D, BT-20, MCF7 and SK-BR-3 while HLA-DQB mRNA was found in MDA-MB-435, T-47D, MDA-MB-231, BT-20, MCF7, SK-BR-3 and BT-474.

Following IFN-γ treatment, all breast cancer cell lines transcribed DQA and DQB mRNA.

3.3.3.2. HLA-DQ Protein

While five breast cancer cell lines, MDA-MB-435, T-47D, BT-20, MCF7 and SK-BR-3, transcribed DQA and DQB genes, no cell line expressed constitutive cell surface HLA-DQ protein (Figure 3.14).

MDA-MB-435, T-47D, BT-20, MCF7 and SK-BR-3 up-regulated cell surface HLA-DQ (Figure 3.14). Interestingly, these cell lines were the only ones to constitutively transcribe DQA and DQB. Despite transcribing DQA and DQB genes, all other breast

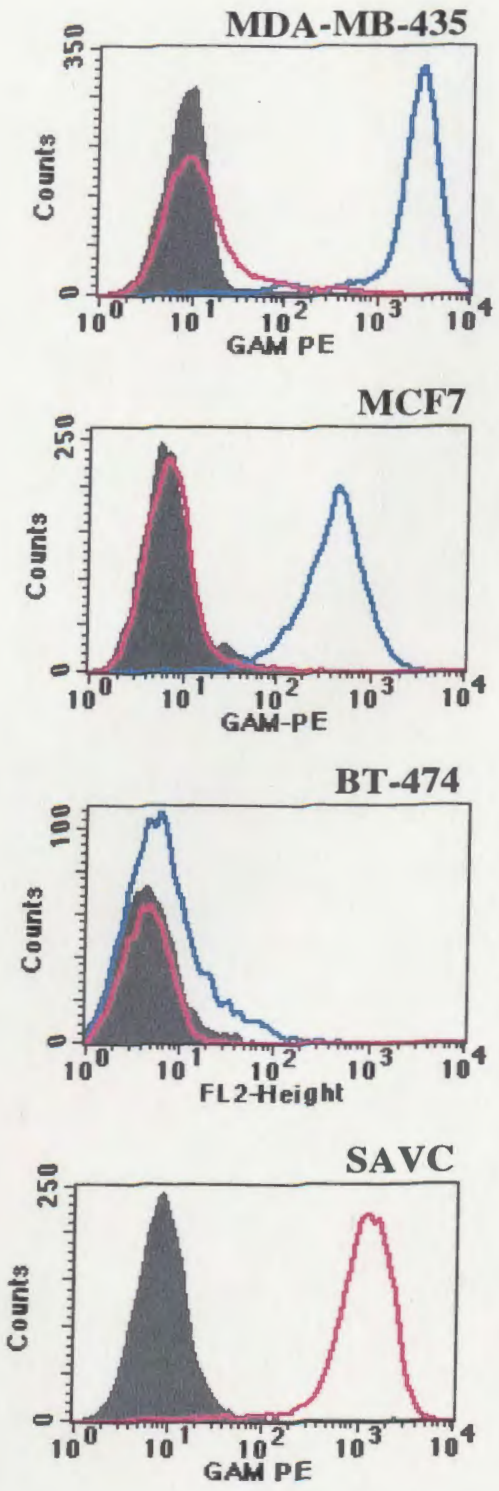


Figure 3.12. Representative flow histograms depicting constitutive (pink) and IFN-γ induced (blue) HLA-DP cell surface expression on breast cancer cell lines. Expression was assessed using the antibody NFLD.M67 and flow cytometry. The negative control is shown in gray.

Table 3.4. HLA-DQA and HLA-DQB transcription[a] in breast cancer cell lines as assessed using sequence specific primers and RT-PCR.

Breast Cancer Cell Lines	Constitutive mRNA		IFN-γ Induced mRNA	
	DQA	DQB	DQA	DQB
MDA-MB-435	+[b]	+	+	+
T-47D	+	+	+	+
MDA-MB-231	-[c]	+	+	+
BT-20	+	+	+	+
MCF7	+	+	+	+
SK-BR-3	+	+	+	+
MDA-MB-436	-	-	+	+
Hs578T	-	-	+	+
MDA-MB-157	-	-	+	+
BT-474	-	+	+	+
MDA-MB-468	-	-	+	+

[a] DQA and DQB transcription were determined using cDNA synthesized from DNase treated RNA extracted from non-trypsinized cells.
[b] mRNA was transcribed.
[c] mRNA was not detected.

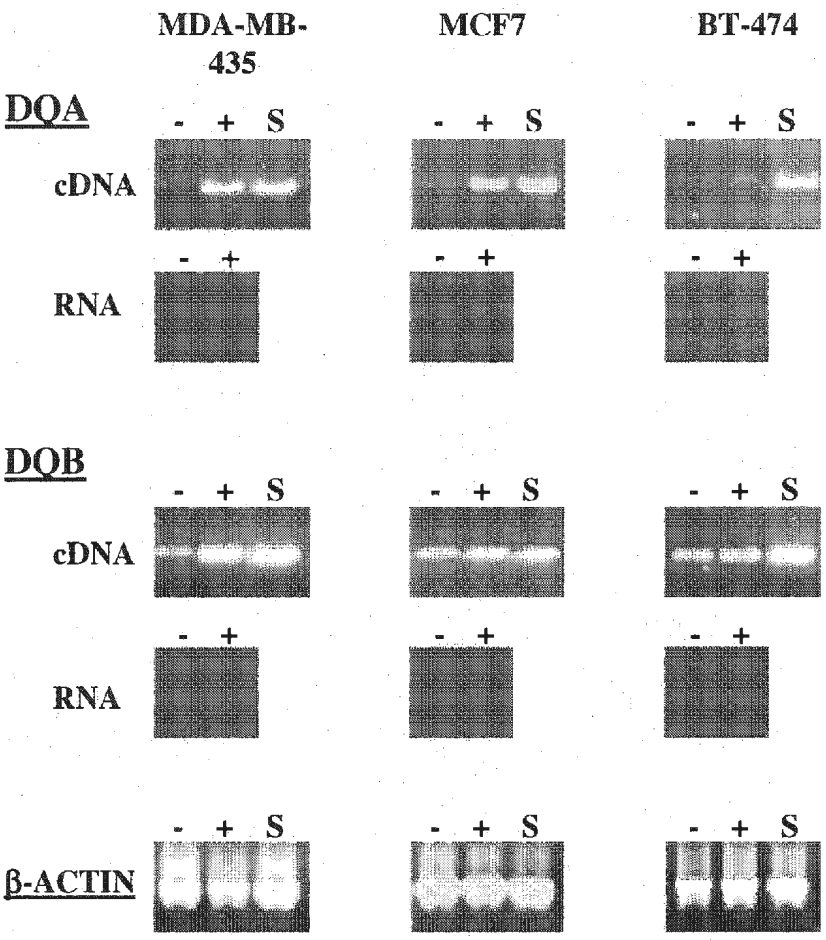


Figure 3.13. Representative gels depicting constitutive (-) and IFN-γ induced (+) DQA and DQB expression in breast cancer cell lines. DQA and DQB transcription were analyzed twice by RT-PCR for 30 cycles. β-actin was amplified as a control for mRNA integrity in both reactions. The B cell line SAVC (S) was the positive control. RNA was subjected to RT-PCR to ensure that it was not contaminated with DNA.

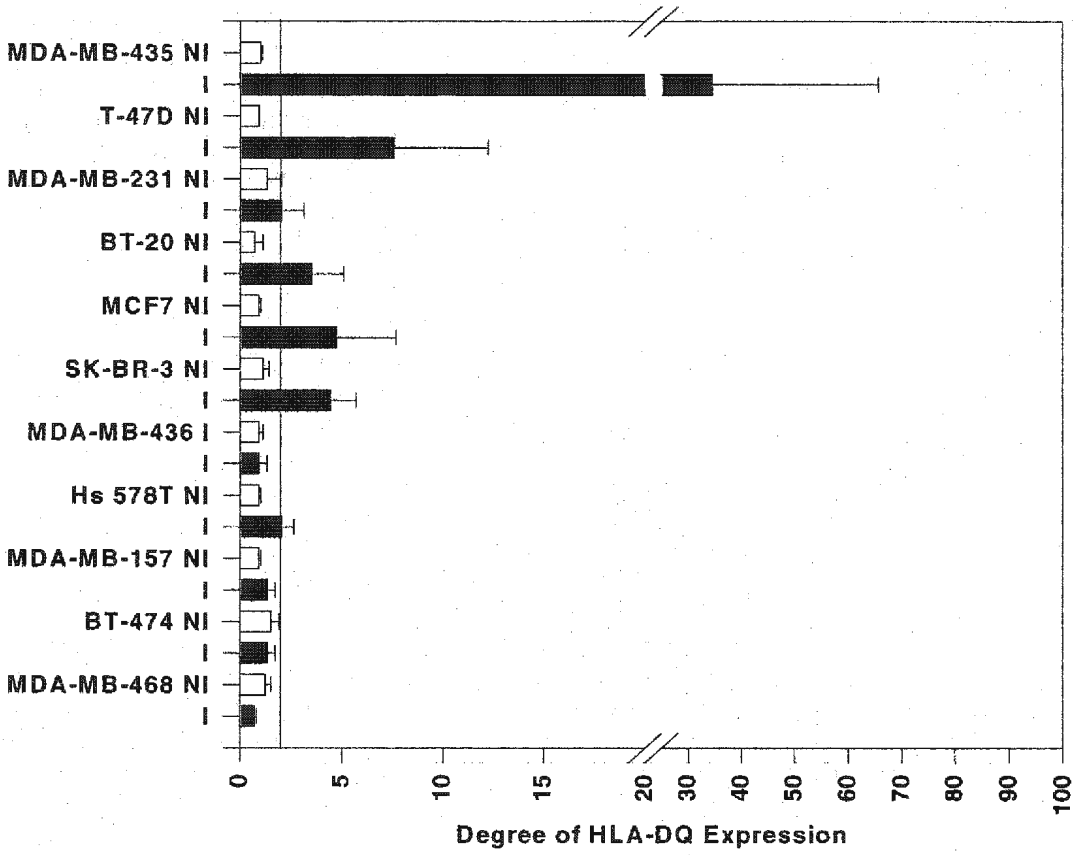


Figure 3.14. HLA-DQ cell surface expression on breast cancer cell lines. HLA-DQ expression was assessed using the antibody SPVL3 and flow cytometry. Results are reported as degree of expression (see section 2.4.5) on non-induced (NI) cells (open bars) and on IFN-γ induced cells (filled bars). Standard deviations between at least two independent experiments are indicated. Values of 2.1 and above (to the right of the black line) are considered positive.

cancer cell lines did not express cell surface HLA-DQ. Representative flow histograms depicting HLA-DQ cell surface expression in breast cancer cell lines are displayed in Figure 3.15.

3.4. HLA Class II Co-chaperone Expression in Breast Cancer Cell Lines

To further characterize breast cancer cell lines as potential HLA class II restricted APCs, constitutive and IFN-γ induced expression of class II co-chaperones, the invariant chain (Ii), HLA-DM and HLA-DO, were analyzed. Transcription of these genes was assessed using sequence specific primers and RT-PCR (see section 2.6). The B cell line SAVC or COX served as positive controls for all reactions.

Ii and HLA-DM protein expression were detected using mAbs and flow cytometry (see section 2.4). SAVC was used as a positive control in all assays. All flow cytometry assays were done at least twice with some exceptions. Ii expression in MCF7 and MDA-MB-468 and HLA-DM expression in T-47D and MDA-MB-468 were assessed only once. Co-chaperone protein expression was also examined using immunocytochemistry (IC) (see section 2.5). IC was performed on adherent breast cancer cells grown in chamber slides with the exception that IC on BT-474, MDA-MB-231 and the positive control, SAVC, was done using cytocentrifuge preparations. IC followed protocols described in section 2.5.5.2 with the exception that IC on BT-474 and MDA-MB-231 followed procedures in section 2.5.4.2.

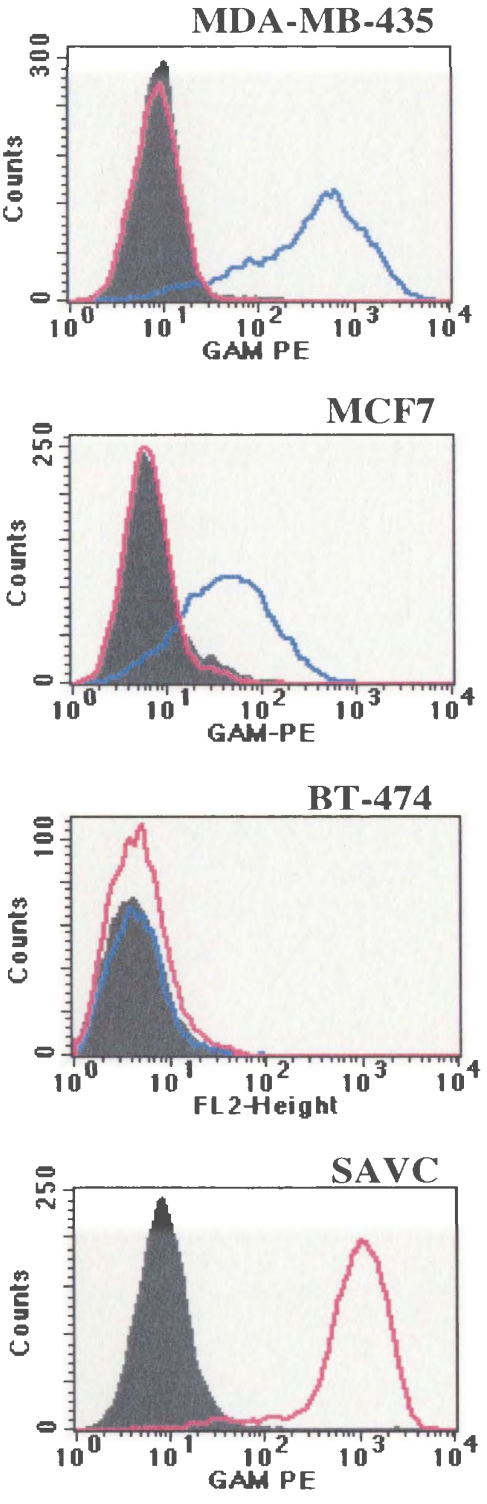


Figure 3.15. Representative flow histograms depicting constitutive (pink) and IFN-γ induced (blue) HLA-DQ cell surface expression on breast cancer cell lines. Expression was assessed using the antibody SPVL3 and flow cytometry. The negative control is shown in gray.

3.4.1. Invariant Chain Expression

3.4.1.1. Invariant Chain Transcription

Constitutive Ii transcription was detected in all breast cancer cell lines except Hs578T (Table 3.5). Following IFN-γ treatment, all cell lines transcribed Ii mRNA. Representative gels depicting Ii transcription are shown in Figure 3.16.

3.4.1.2. Invariant Chain Protein

While constitutive Ii mRNA was observed in 10/11 cell lines, only four of these cells, MDA-MB-435, T-47D, MDA-MB-231 and MCF7, constitutively expressed Ii protein as detected using intracellular flow cytometry (Figure 3.17) and IC. Following IFN-γ treatment, all cell lines up-regulated this co-chaperone. Representative flow cytometry histograms and representative IC data depicting Ii protein expression in breast cancer cell lines are displayed in Figure 3.18.

3.4.2. HLA-DM Expression

3.4.2.1. HLA-DMA and -DMB Transcription

HLA-DMA mRNA was transcribed constitutively by all breast cancer cell lines while HLA-DMB mRNA was detected in all cell lines except BT-474 (Table 3.6). In response to IFN-γ, DMA and DMB genes were transcribed by all cell lines. Representative gels depicting DMA and DMB transcription are shown in Figure 3.19. In some instances, weak bands that are visible in gel photographs are not as apparent in Figure 3.19.

3.4.2.2. HLA-DM Protein

HLA-DM protein was not detected constitutively by any breast cancer cell line when

Table 3.5. Invariant chain (Ii) transcription[a] in breast cancer cell lines as assessed using sequence specific primers and RT-PCR.

Breast Cancer Cell Lines	Constitutive Ii mRNA	IFN-γ Induced Ii mRNA
MDA-MB-435	+[b]	+
T-47D	+	+
MDA-MB-231	+	+
BT-20	+	+
MCF7	+	+
SK-BR-3	+	+
MDA-MB-436	+	+
Hs578T	-[c]	+
MDA-MB-157	+	+
BT-474	+	+
MDA-MB-468	+	+

[a] Ii transcription was determined using cDNA synthesized from non-DNase treated RNA extracted from trypsinized cells. RT-PCR to detect Ii transcription was performed by Ingrid Pardoe.

[b] mRNA was transcribed.

[c] mRNA was not detected.

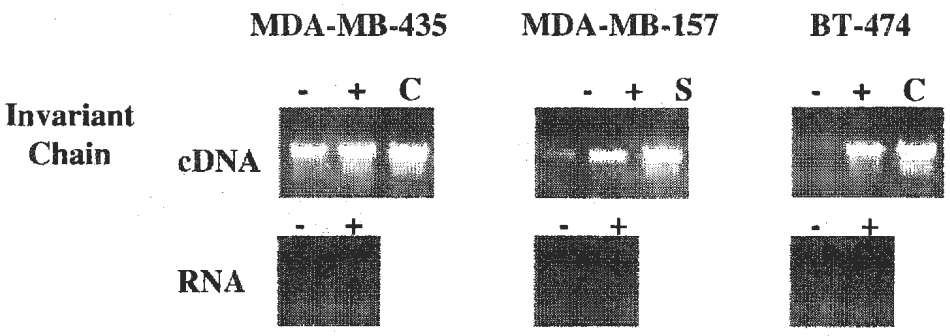


Figure 3.16. Representative gels depicting constitutive (-) and IFN-γ induced (+) invariant chain (Ii) transcription in breast cancer cell lines as assessed by RT-PCR. Ii transcription was assessed once by RT-PCR for 30 cycles. The B cell lines COX (C) and SAVC (S) were positive controls. RNA was subjected to RT-PCR to ensure that it was not contaminated with DNA.

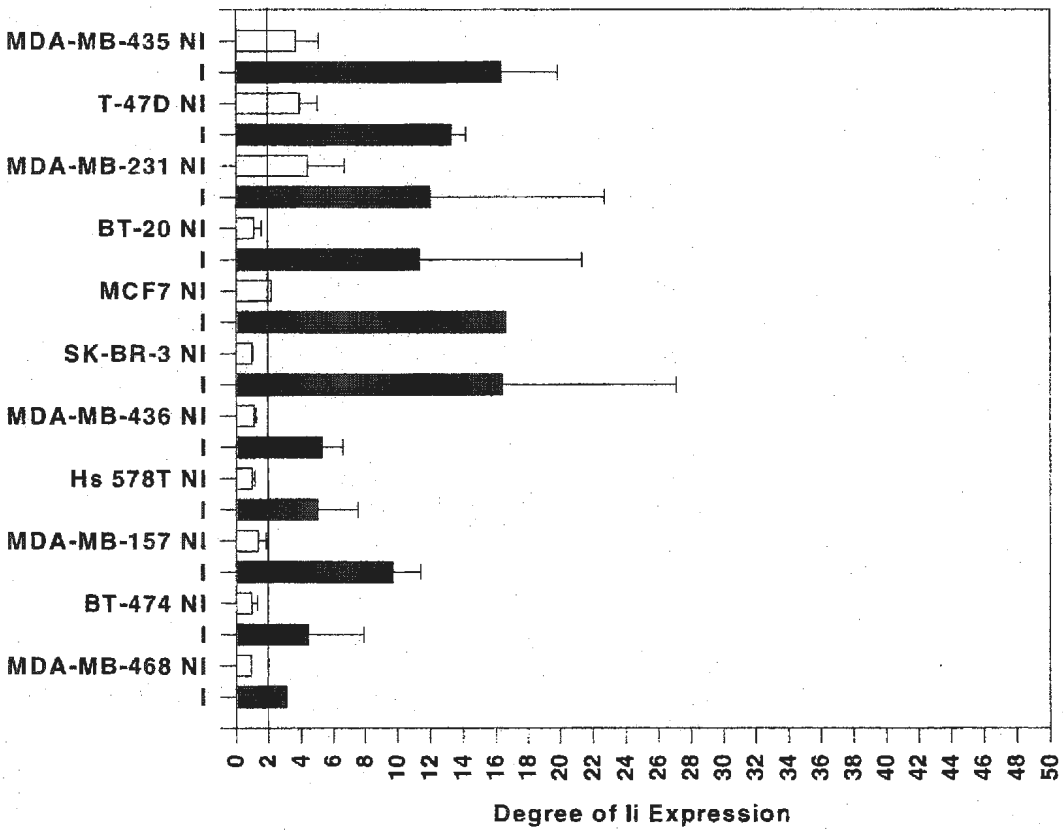


Figure 3.17. Intracellular invariant chain (Ii) protein expression in breast cancer cell lines. Ii expression was assessed using the antibody LN2 and flow cytometry. Results are reported as degree of expression (see section 2.4.5) on non-induced (NI) cells (open bars) and on IFN-γ induced cells (filled bars). Standard deviations between experiments are shown when more than one experiment was performed. Values of 2.1 and above (to the right of the black line) are considered positive.

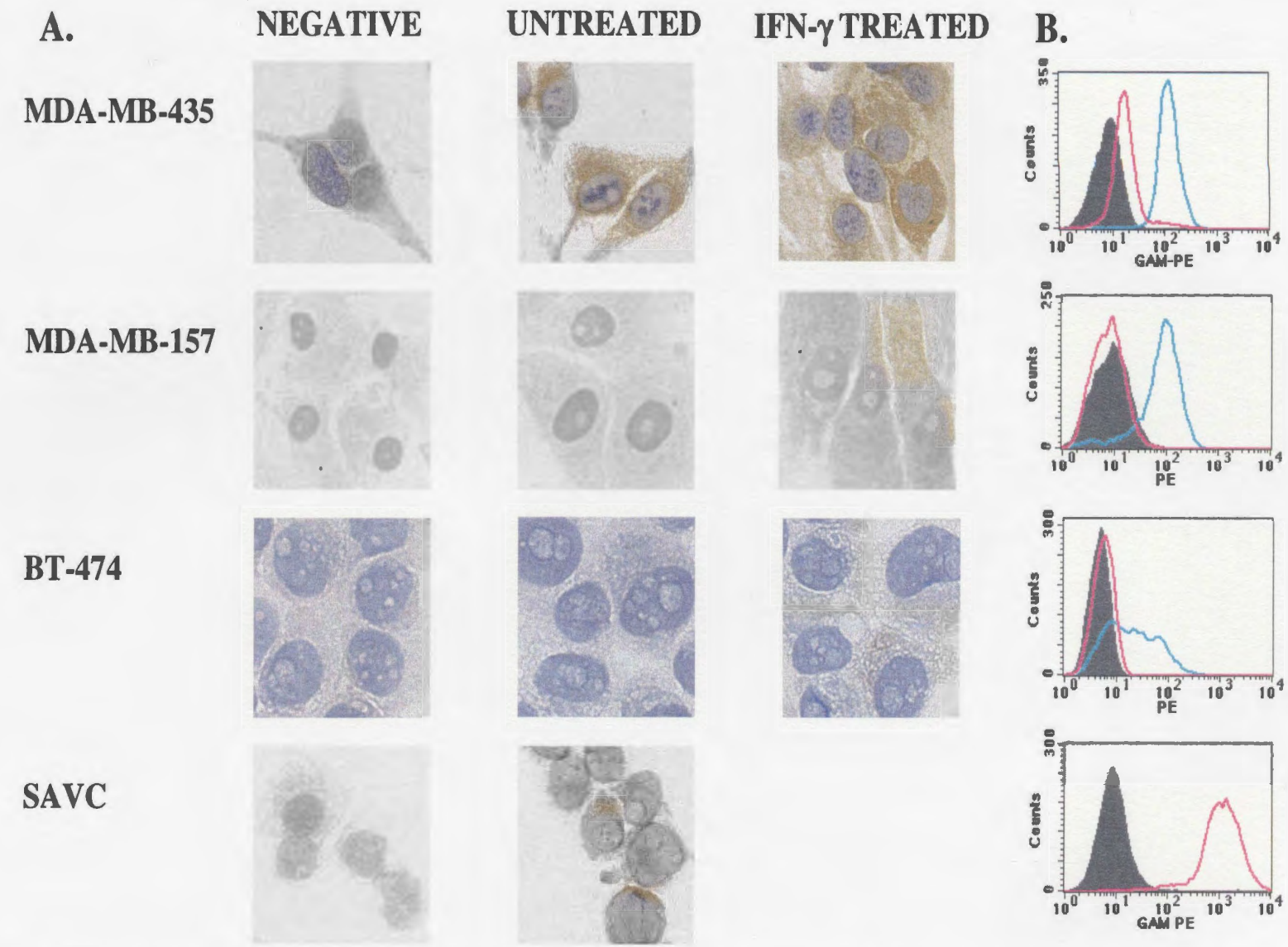


Figure 3.18. Invariant chain (Ii) protein expression by breast cancer cell lines. A) Immunocytochemical analysis of Ii expression in untreated and IFN-γ treated breast cancer cell lines. The negative control is shown alongside. B) Flow cytometry histograms depicting intracellular Ii expression in untreated (pink) and IFN-γ treated (blue) breast cancer cell lines. The negative control is shown in gray. SAVC is the positive control for Ii expression in both assays.

Table 3.6. HLA-DMA and HLA-DMB transcription[a] in breast cancer cell lines as assessed using sequence specific primers and RT-PCR.

Breast Cancer Cell Lines	Constitutive mRNA		IFN-γ Induced mRNA	
	DMA	DMB	DMA	DMB
MDA-MB-435	+[b]	+	+	+
T-47D	+	+	+	+
MDA-MB-231	+	+	+	+
BT-20	+	+	+	+
MCF7	+	+	+	+
SK-BR-3	+	+	+	+
MDA-MB-436	+	+	+	+
Hs578T	+	+	+	+
MDA-MB-157	+	+	+	+
BT-474	+	-[c]	+	+
MDA-MB-468	+	+	+	+

[a] DMA and DMB transcription were determined using cDNA synthesized from non-DNase treated RNA extracted from trypsinized treated cells. RT-PCR to assess HLA-DMA and HLA-DMB transcription was performed by Ingrid Pardoe.
[b] mRNA was transcribed.
[c] mRNA was not detected.

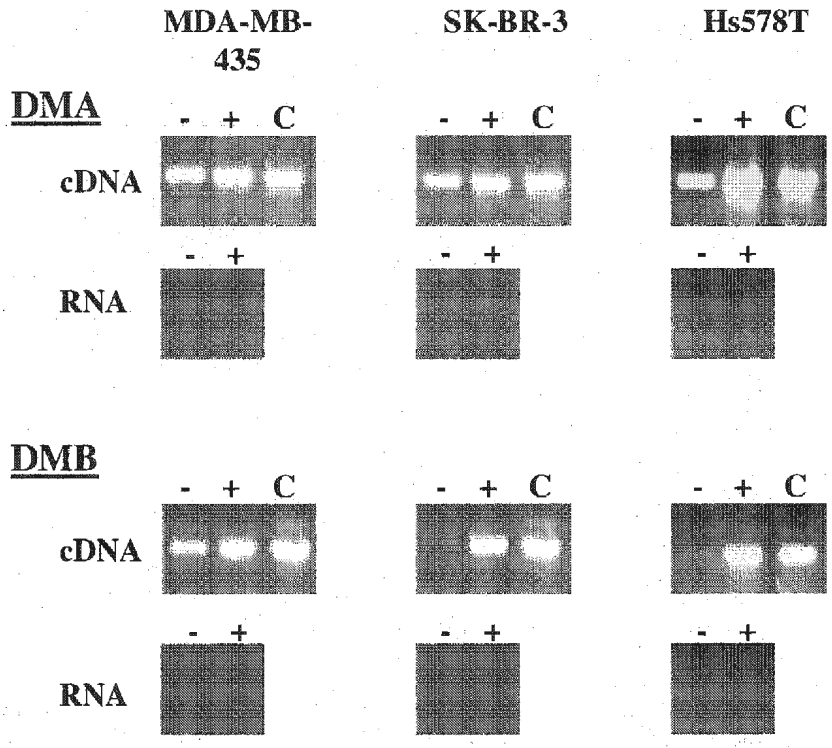


Figure 3.19. Representative gels depicting constitutive (-) and IFN-γ induced (+) DMA and DMB expression in breast cancer cell lines. DMA and DMB transcription were assessed once by RT-PCR for 30 cycles. The B cell line COX (C) was the positive control. RNA was subjected to RT-PCR to ensure that it was not contaminated with DNA.

assessed using intracellular flow cytometry (Figure 3.20). However, using IC, DM protein was detected in non-induced MDA-MB-435, MDA-MB-231 and BT-20 (Figure 3.21). IC was analyzed independently by two individuals to ensure accuracy of results. When cells were treated with IFN-γ, DM expression was up-regulated by MDA-MB-435, T-47D, MDA-MB-231, BT-20, MCF7 and MDA-MB-468 as shown by flow cytometry (Figure 3.20) but using IC, DM was also detected in four additional cell lines, SK-BR-3, MDA-MB-436, Hs578T and MDA-MB-157 (Figure 3.22).

It was serendipitously discovered that cell surface HLA-DM was expressed on certain cell lines. This was determined using extracellular flow cytometry *i.e.* cells were not permeabilized by saponin. MDA-MB-231 and BT-20 constitutively expressed surface DM while MDA-MB-435, MDA-MB-231, BT-20 and SK-BR-3 up-regulated surface DM in response to IFN-γ. Extracellular DM was not expressed on MDA-MB-436, Hs578T, MDA-MB-157 and BT-474. T-47D, MCF7 and MDA-MB-468 were not assayed for extracellular DM. Thus, it is probable that immunocytochemical detection of DM in non-induced MDA-MB-231 and BT-20 and IFN-γ induced SK-BR-3 reflected cell surface expression of this co-chaperone. It is not known why intracellular flow cytometry did not detect extracellular DM on breast cancer cell lines. However, it is possible that the fixation or the detergent required for intracellular flow cytometry destroyed the DM epitope.

As stated above, discrepancies between IC and flow cytometry were observed. Specifically, IC revealed constitutive DM expression in MDA-MB-435 and IFN-γ induced DM expression in MDA-MB-436, Hs578T and MDA-MB-157 but neither cell surface nor

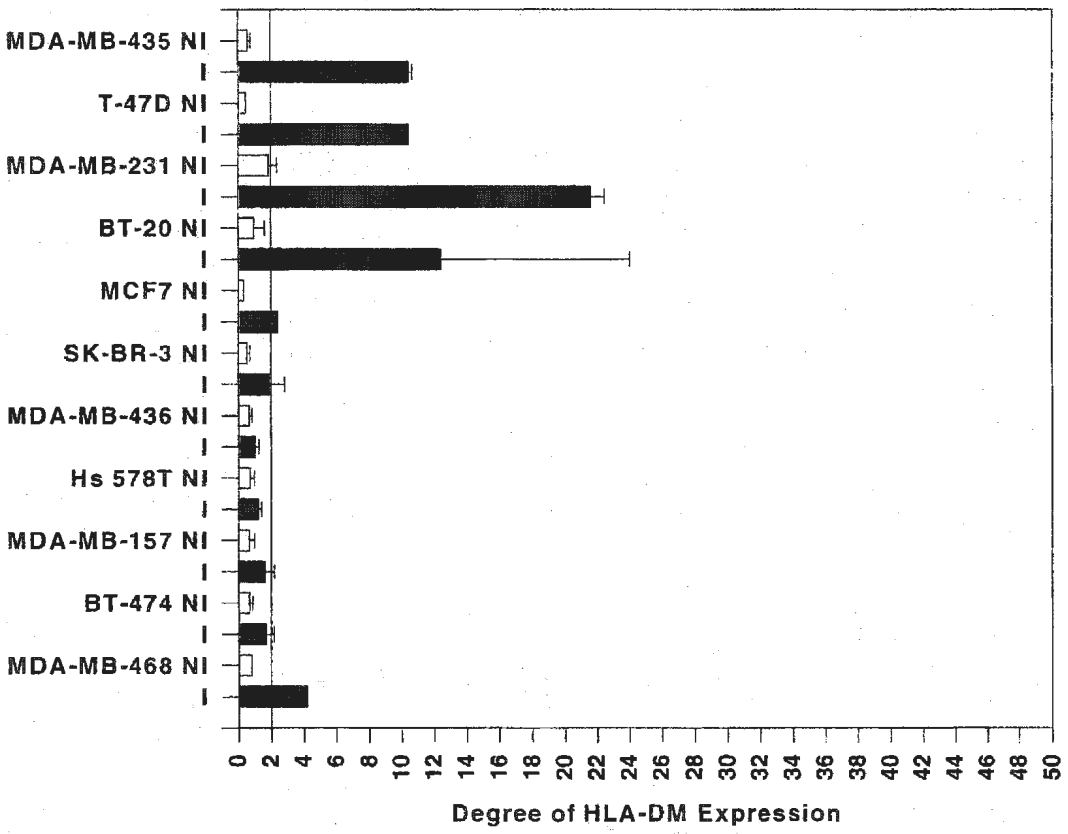


Figure 3.20. Intracellular HLA-DM protein expression in breast cancer cell lines. HLA-DM expression was assessed using the antibody MaP.DM1 and flow cytometry. Results are reported as degree of expression (see section 2.4.5) on non-induced (NI) cells (open bars) and on IFN-γ induced cells (filled bars). Standard deviations are shown when more than one experiment was performed. Values of 2.1 and above (to the right of the black line) are considered positive.

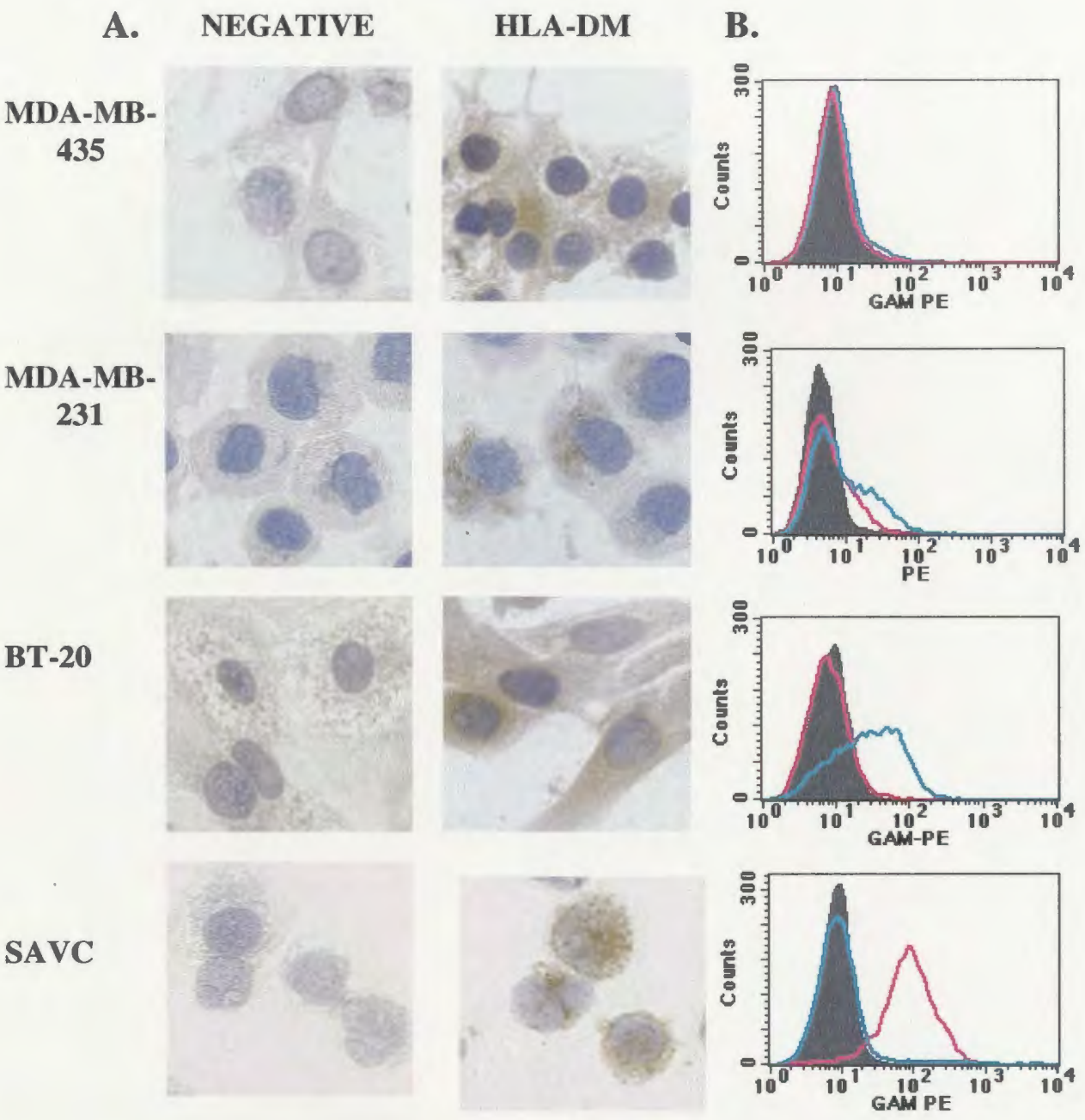


Figure 3.21. Constitutive HLA-DM expression in breast cancer cell lines. A) Immunocytochemical analysis of HLA-DM expression in breast cancer cell lines. The negative control is shown alongside. B) Flow cytometry histograms depicting cell surface (pink) and intracellular (blue) HLA-DM expression in breast cancer cell lines. The negative control is gray. SAVC is the positive control for HLA-DM expression in both assays. Note: Cell surface DM is not detected on SAVC.

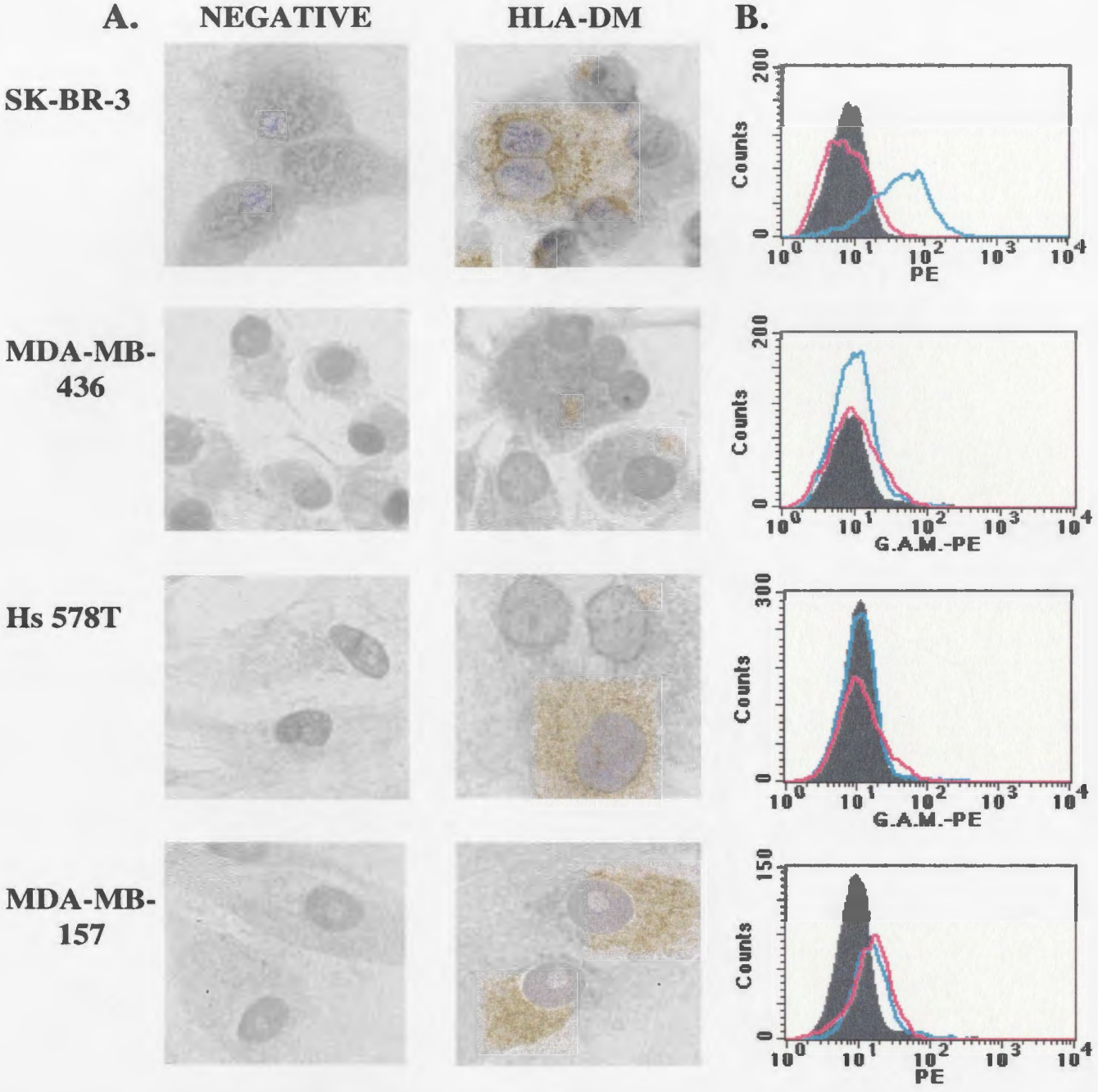


Figure 3.22. IFN-γ induced HLA-DM expression in breast cancer cell lines. A) Immunocytochemical analysis of HLA-DM expression in breast cancer cell lines. The negative control is shown alongside. B) Flow cytometry histograms depicting cell surface (pink) and intracellular (blue) HLA-DM expression in breast cancer cell lines. The negative control is gray. SAVC, the positive control in both assays, is shown in Figure 3.21.

intracellular DM were detected in these cells by flow cytometry (Figure 3.21, 3.22). Therefore, we decided to repeat flow cytometry on MDA-MB-435, MDA-MB-436 and Hs578T and, at the same time, prepare cytocentrifuge preparations. Thus, DM expression was assessed by flow cytometry and by IC simultaneously. Interestingly, DM was not observed in any of these cells using either methodology (data not shown). These discrepant findings may reflect differences in IC and flow cytometry protocols (see sections 2.4, 2.5) or differences in assay sensitivities.

In summary, while ten breast cancer cell lines constitutively transcribed DMA and DMB genes, DM protein was only consistently detected in MDA-MB-231 and BT-20. Additionally, while all cell lines transcribed DMA and DMB genes in response to IFN-γ, 7/11 cell lines consistently up-regulated DM protein.

3.4.3. HLA-DO Expression

3.4.3.1. HLA-DOA and -DOB Transcription

HLA-DOA was not transcribed constitutively by any breast cancer cell line. However, HLA-DOB mRNA was detected in all non-induced cell lines (Table 3.7). HLA-DO protein expression was not assessed but would not be expected given that DOA was not transcribed in any cell line.

Following IFN-γ treatment, HLA-DOA mRNA was detected in MDA-MB-435, T-47D, MDA-MB-231, BT-20, MCF7 and Hs578T (Table 3.7). HLA-DOB was transcribed by all cell lines. Representative RT-PCR results are displayed in Figure 3.23.

Table 3.7. HLA-DOA and HLA-DOB transcription[a] in breast cancer cell lines as assessed using sequence specific primers and RT-PCR.

Breast Cancer Cell Lines	Constitutive mRNA		IFN-γ Induced mRNA	
	DOA	DOB	DOA	DOB
MDA-MB-435	-[b]	+[c]	+	+
T-47D	-	+	+	+
MDA-MB-231	-	+	+	+
BT-20	-	+	+	+
MCF7	-	+	+	+
SK-BR-3	-	+	-	+
MDA-MB-436	-	+	-	+
Hs578T	-	+	+	+
MDA-MB-157	-	+	-	+
BT-474	-	+	-	+
MDA-MB-468	-	+	-	+

[a] DOA and DOB transcription were determined using cDNA synthesized from non-DNase treated RNA from trypsinized treated cells. RT-PCR to assess HLA-DOA and HLA-DOB transcription was performed by Ingrid Pardoe.
[b] mRNA was transcribed.
[c] mRNA was not detected.

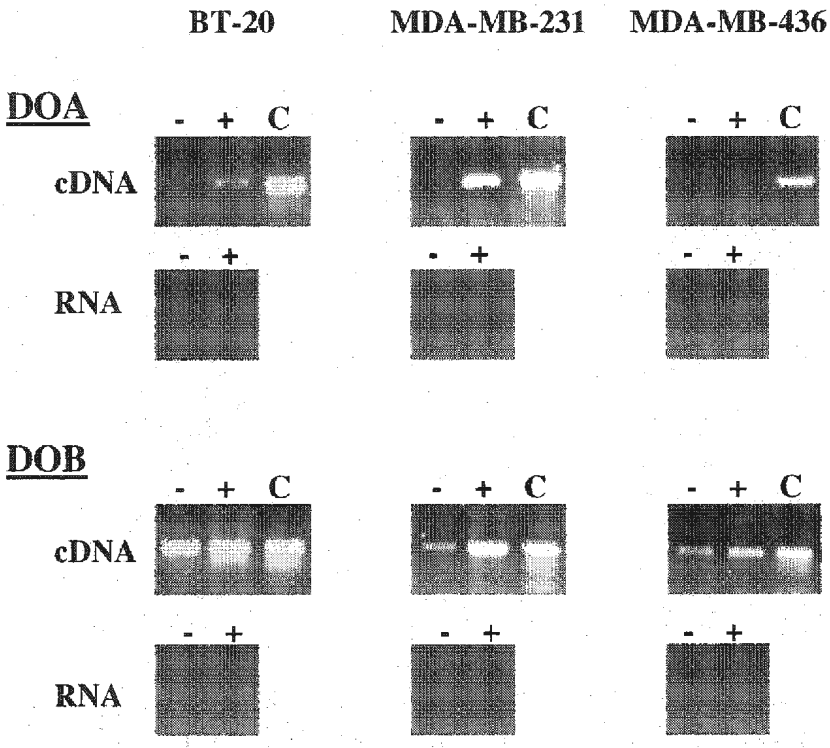


Figure 3.23. Representative gels depicting constitutive (-) and IFN-γ induced (+) DOA and DOB expression in breast cancer cell lines. DOA and DOB transcription were assessed once by RT-PCR for 30 cycles. The B cell line COX (C) was the positive control. RNA was subjected to RT-PCR to ensure that it was not contaminated with DNA.

Up-regulation of DO protein was not determined but IFN-γ treated MDA-MB-435, T-47D, MDA-MB-231, BT-20, MCF7 and Hs578T had the potential to express DO protein since they transcribed DOA and DOB genes.

3.5. Co-stimulatory Molecule Expression by Breast Cancer Cell Lines

Activation of T cell expansion and differentiation requires interactions between the T cell and the APC *i.e.* breast cancer cell. HLA/peptide interaction with a specific T cell receptor constitutes signal one. Signal two involves co-stimulatory molecule interaction with a corresponding ligand on T cells. CD80 and CD86 were believed to be the most important inducers of T cell co-stimulation. However, other molecules including CD40 may also act in this capacity (Croft & Dubey, 1997).

Cell surface expression of CD80, CD86 and CD40 on breast cancer cell lines was assessed using mAbs and flow cytometry (see section 2.4). The B cell line SAVC was used as a positive control in each assay. Co-stimulatory molecule expression was also examined using IC (see section 2.5). IC was performed on adherent breast cancer cells grown in chamber slides with the exception that IC on BT-474 and the positive control, SAVC, was done using cytocentrifuge preparations. IC followed protocols described in section 2.5.5.2 with the exception that IC on BT-474 followed procedures in section 2.5.4.2.

3.5.1. CD80 and CD86

Constitutive and IFN-γ induced CD80 and CD86 protein expression were not detected on any breast cancer cell line by flow cytometry. These results were confirmed by

IC. Representative data showing the lack of CD80 and CD86 expression on a breast cancer cell line as assessed by flow cytometry and IC is depicted in Figure 3.24.

3.5.2. CD40

CD40 was constitutively expressed on the cell surface of five breast cancer cell lines as assessed using flow cytometry (Figure 3.25). Strong CD40 expression was detected on MDA-MB-435, MDA-MB-231 and BT-20. Moderate CD40 expression was found on SK-BR-3 while weak expression was detected on T-47D. Unexpectedly, constitutive CD40 expression was not detected on any breast cancer cell line using IC. This was most surprising for MDA-MB-435, BT-20 and MDA-MB-231 which were found to express high levels of CD40 by flow cytometry.

CD40 was up-regulated by MDA-MB-435, T-47D, MDA-MB-231, BT-20, SK-BR-3, MDA-MB-436 and Hs578T but was not expressed by MCF7, MDA-MB-157, BT-474 and MDA-MB-468. These findings were confirmed by IC with one exception. Weak CD40 up-regulation observed on Hs578T by flow cytometry was not detected immunocytochemically. Representative IC results and flow cytometry histograms are presented in Figure 3.26.

3.6. Expression of HLA-DR Alleles in Breast Cancer Cell Lines

HLA class I alleles may be selectively down-regulated in tumors (reviewed in Marcincola et al., 2000). It is not known whether class II alleles are also selectively expressed. This study examined HLA-DR allele expression in breast cancer cell lines. HLA-DR was chosen, as opposed to HLA-DP or HLA-DQ, for several reasons. As described in

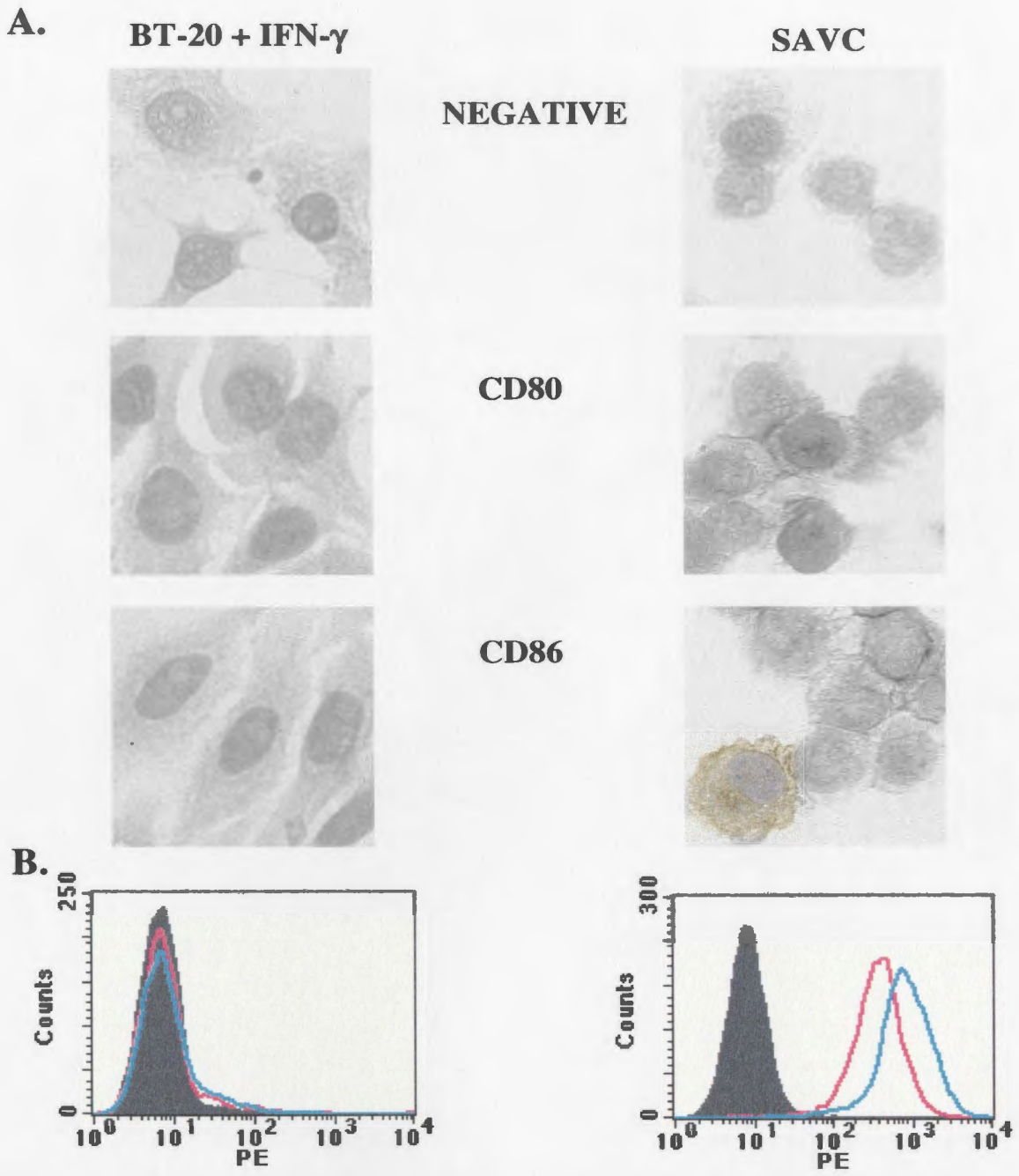


Figure 3.24. CD80 and CD86 expression in BT-20. A) Immunocytochemical analysis of CD80 and CD86 expression in IFN-γ treated BT-20 and the positive control, SAVC. The negative control is shown alongside. B) Flow cytometry histograms depicting CD80 (pink) and CD86 (blue) cell surface expression on IFN-γ treated BT-20 and on SAVC. The negative control is shown in gray.

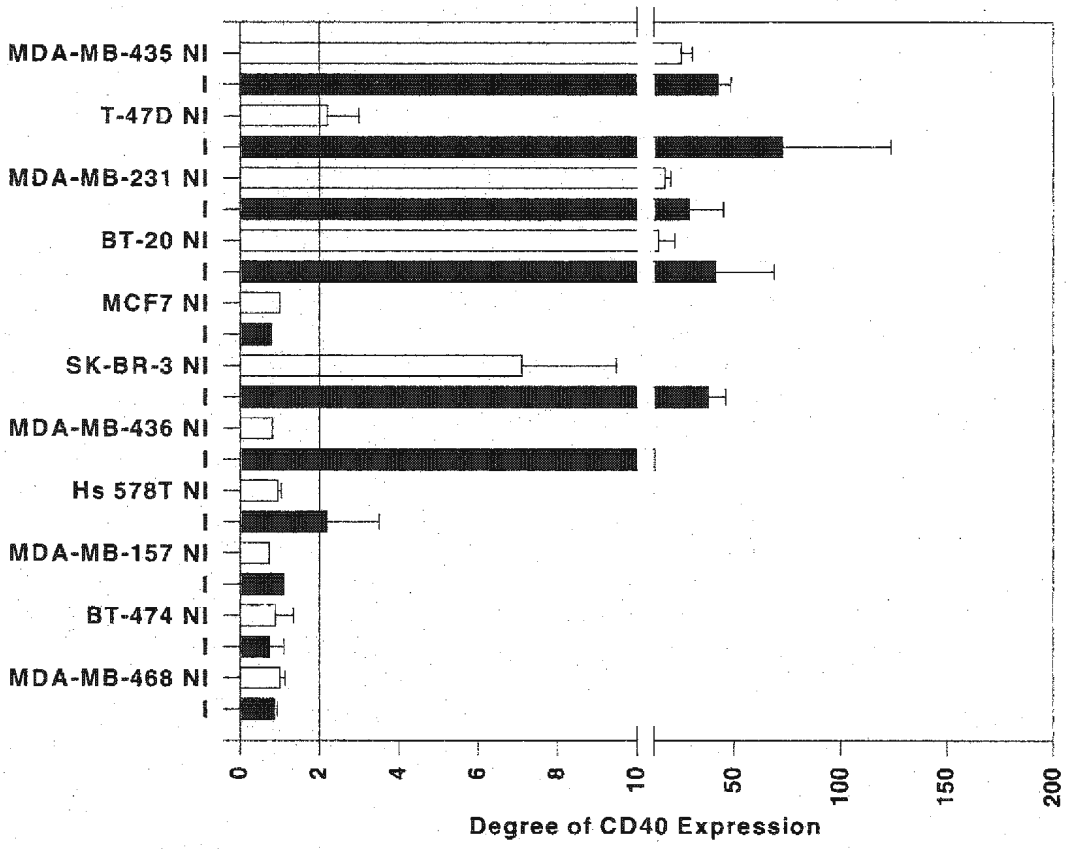


Figure 3.25. CD40 cell surface expression on breast cancer cell lines. CD40 expression was assessed using the antibody 5C3 and flow cytometry. Results are reported as degree of expression (see section 2.4.5) on non-induced (NI) cells (open bars) and IFN-γ induced cells (filled bars). Standard deviations are reported when more than one experiment was performed. Values of 2.1 and above (to the right of the black line) are considered positive.

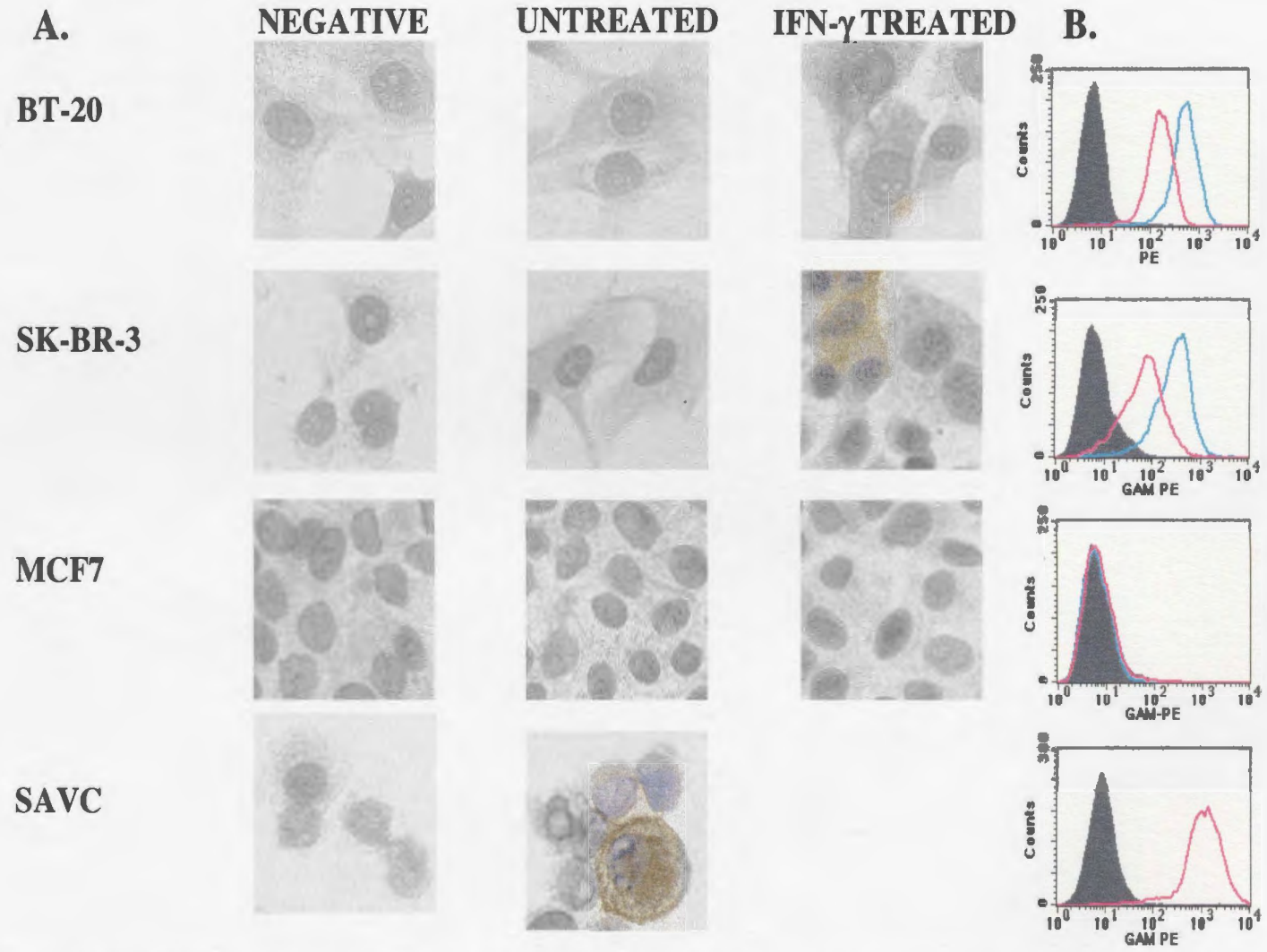


Figure 3.26. CD40 protein expression in breast cancer cell lines. A) Immunocytochemical analysis of CD40 expression in untreated and IFN-γ treated breast cancer cell lines. The negative control is shown alongside. B) Flow cytometry histograms depicting cell surface CD40 expression in untreated (pink) and IFN-γ treated (blue) breast cancer cell lines. The negative control is shown in gray. SAVC is the positive control for CD40 expression in both assays.

section 3.3, cell surface HLA-DR was up-regulated by all cell lines. In most cells (10/11), HLA-DR was expressed at higher levels than other class II isotypes. Importantly, monoclonal antibodies capable of recognizing one HLA-DR type or a combination of HLA-DR types were available. Even though HLA-DP was up-regulated on 10/11 cell lines, HLA-DP typing is not routinely performed so HLA-DP typing kits are not readily available. HLA-DQ was not studied given its comparatively low cell surface expression and its up-regulation on only 5/11 cell lines.

3.6.1. HLA Class II DNA Typing of Breast Cancer Cell Lines

To investigate selective up-regulation of HLA-DR allelic products, it was first necessary to identify the HLA-DRB alleles in each breast cancer cell line. This was done by DNA typing using commercially available HLA class II DNA typing and subtyping kits (see section 2.3.2). The HLA-DRB and HLA-DQB1 alleles in each breast cancer cell line are listed in Table 3.8. The HLA-DRB3 subtyping was performed by Dianne Codner.

3.6.2. Specificity of HLA-DR Specific Monoclonal Antibodies

To determine expression of HLA-DR allelic products in breast cancer cell lines, HLA-DR specific mAbs were utilized. The specificity of these antibodies has been extensively studied and published (see Table 2.4). However, to ensure that the antibodies bound a particular HLA-DR molecule, their binding to B cell lines expressing the appropriate HLA-DR type was assayed using flow cytometry (Table 3.9). Since all HLA-DR specific mAbs bound HLA-DR protein, it is reasonably certain that when HLA-DR allelic products were not detected in breast carcinoma cell lines, it was because these HLA-DR types were not up-

Table 3.8. HLA-DRB and HLA-DQB1 genes in breast cancer cell lines. DR and DQ types were determined by DNA typing using commercially available kits.

Breast Cancer Cell Line	HLA-DR Type	HLA-DQ Type
MDA-MB-435	DRB1*04051, DRB4, DRB1*1320, DRB3*0202	DQB1*08, DQB1*06
T-47D	DRB1*0102	DQB1*05
MDA-MB-231	DRB1*07, DRB4, DRB1*13, DRB3*0202	DQB1*02, DQB1*03
BT-20	DRB1*0404, DRB4, DRB1*1301, DRB3*01	DQB1*03, DQB1*06
MCF7	‡DRB1*03, DRB3*0202, DRB1*15, DRB5	‡DQB1*02, DQB1*06
SK-BR-3	DRB1*07, DRB4, DRB1*1302, DRB3*0302	DQB1*02, DQB1*06
MDA-MB-436	DRB1*03, DRB3*01	DQB1*02
Hs578T	‡DRB1*0101, DRB1*15, DRB5	‡DQB1*05, DQB1*06
MDA-MB-157	‡DRB1*0401, DRB4, DRB1*15, DRB5	‡DQB1*03, DQB1*06
BT-474	DRB1*0401, DRB4, DRB1*15, DRB5	DQB1*03, DQB1*06
MDA-MB-468	DRB1*0102, DRB1*07, DRB4	DQB1*05, DQB1*02

‡HLA-DRB and HLA-DQB1 types, excluding subtypings, reported previously by Tibbo, 1998.

Table 3.9. Binding[a] of HLA-DR allele specific monoclonal antibodies to cell surface HLA-DR on B cell lines.

Antibodies	B CELL LINES									
	JEST-HOM [b]B1*0101	**MGAR** B1*1501 B5*0101	**COX** B1*0301 B3*0101	**SAVC** B1*0401 B4*0101	**MT14B** B1*0404 B4*0101	**HAS15** B1*0405 B4*0101	**OMW** B1*1301 B3*0101	**CB6B** B1*1301 B3*0202	**HO301** B1*1302 B3*0301	**PLH** B1*0701 B4*0101
L243 [c]All DR	2806.3[d] (304.4)[e]	1563.6 (170.2)	3102.2 (343.8)	2271.0 (264.6)	[f]2627.0 (238.9)	5453.5 (444.9)	1232.3 (174.2)	[f]590.0 (74.8)	[f]1495.0 (250.2)	3082.0 (233.3)
JS-1 DR1	3544.5 (421.9)	-	-	-	-	-	-	-	-	-
NFLD.D10 DR1, DR4, DR15	2374.8 (259.3)	1207.5 (131.5)	-	2209.3 (243.0)	[f]1665.0 (152.4)	-	-	-	-	-
NFLD.D2 DR1, DR4	1733.0 (204.8)	-	-	[f]1368.0 (153.0)	[f]1609.0 (147.3)	-	-	-	-	-
NFLD.D1 DR4	-	-	-	486.0 (55.2)	[f]800.0 (73.7)	2847.5 (243.2)	-	-	-	-
NFLD.M1 DR4, DR52	-	-	456.5 (54.4)	544.5 (73.0)	[f]880.0 (81.0)	-	255.5 (31.2)	-	-	-
NFLD.D7 DR4,DR15, DR52	-	3140.0 (332.8)	3262.6 (366.4)	-	-	-	1717.5 (212.5)	[f]524.0 (66.5)	[f]141.0 (24.5)	-

Table 3.9. Continued.

Antibodies	B CELL LINES									
	JEST-HOM B1*0101	**MGAR** B1*1501 B5*0101	**COX** B1*0301 B3*0101	**SAVC** B1*0401 B4*0101	**MT14B** B1*0404 B4*0101	**HAS15** B1*0405 B4*0101	**OMW** B1*1301 B3*0101	**CB6B** B1*1301 B3*0202	**HO301** B1*1302 B3*0301	**PLH** B1*0701 B4*0101
NDS13 DR52	-	-	4476.8 (502.9)	-	-	-	1881.5 (210.6)	[f]979.0 (123.4)	[f]1964.0 (328.3)	-
7.3.19.1 DR3, DR52	-	-	7307.5 (852.8)	-	-	-	4478.3 (615.1)	[f]1811.0 (227.4)	[f]5083.0 (848.2)	-
UK8.1 DR3, DR13	-	-	4307.7 (481.9)	-	-	-	1687.5 (254.3)	-	-	-
SFR16 DR7	-	-	-	-	-	-	-	-	-	1336.5 (94.5)
PL3 DR7, DR53	-	-	-	987.3 (108.6)	-	1833.0 (157.3)	-	-	-	4073.0 (503.6)

[a] Antibody binding was determined using flow cytometry.

[b] B cell line HLA-DR types.

[c] HLA-DR types recognized by the antibodies. Note: Antibodies may recognize more HLA-DR types than listed (see Table 2.4). Presented here are the antibody specificities important for detection of HLA-DR on breast cancer cell lines.

[d] Mean fluorescence intensity with the background subtracted.

[e] Degree of HLA-DR expression (see section 2.4.5).

[f] Antibody binding was detected only once. All other data are an average of at least two separate assays.

regulated and not because mAbs were non-reactive.

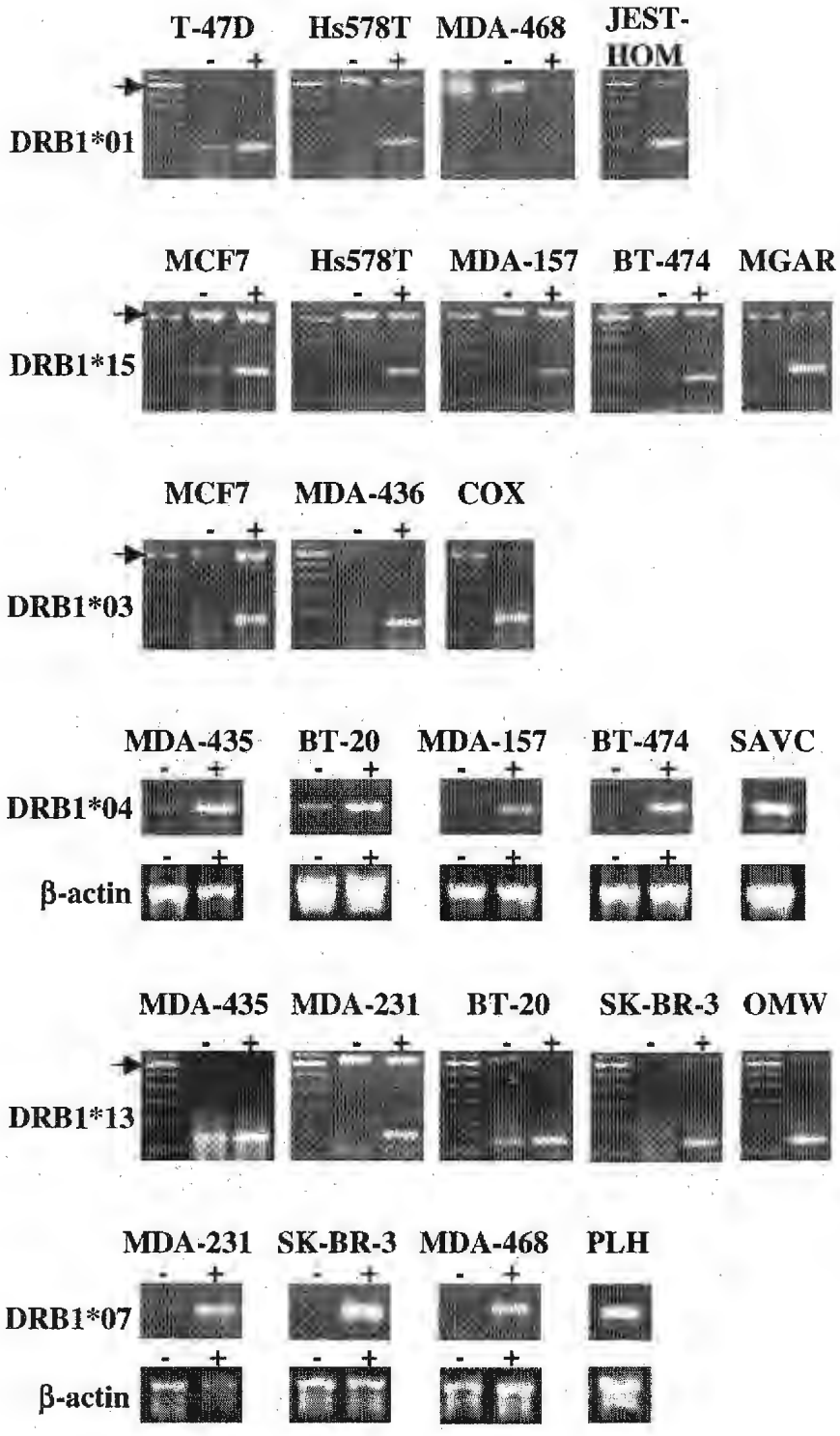
3.6.3. Expression of HLA-DR Allelic Products by Breast Cancer Cell Lines

HLA-DRB gene transcription was analyzed using sequence specific primers and RT-PCR (see section 2.6). Cell surface expression of HLA-DR allelic products was assessed using HLA-DR specific mAbs and flow cytometry (see section 2.4). When HLA-DR allotypes were not up-regulated on the cell surface, IC was performed to determine whether HLA-DR protein was made but retained intracellularly.

3.6.3.1. Transcription of HLA-DR Alleles

Constitutive and IFN-γ induced HLA-DRB allele transcription in breast cancer cell lines is depicted in Figure 3.27 and summarized in Table 3.10. In some instances, weak bands that are visible in gel photographs are not as apparent in Figure 3.27. HLA-DRB allele mRNA was constitutively transcribed by 9/11 breast cancer cell lines (Table 3.10). These findings correlated with those found using generic HLA-DRB primers with two notable exceptions. Non-induced MDA-MB-157 did not transcribe DRB as assessed using generic DRB primers but did weakly transcribe DRB4 mRNA. Similarly, non-induced MDA-MB-468 did not transcribe DRB as assessed using generic DRB primers but did weakly transcribe DRB1*01 mRNA.

We were unable to differentiate between all HLA-DR allelic products in breast cancer cell lines. For example, DR53 protein expression could not be determined in DR7 expressing cell lines, MDA-MB-231 and SK-BR-3, since the antibody PL3 recognizes both DR7 and DR53. Similarly, a DR51 antibody was not available for this study. However, transcription


T-47D
Hs578T
MDA-468
JEST-HOM
DRB1*01
MCF7
Hs578T
MDA-157
BT-474
MGAR
DRB1*15
MCF7
MDA-436
COX
DRB1*03
MDA-435
BT-20
MDA-157
BT-474
SAVC
DRB1*04
β-actin
MDA-435
MDA-231
BT-20
SK-BR-3
OMW
DRB1*13
MDA-231
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MDA-468
PLH
DRB1*07
β-actin

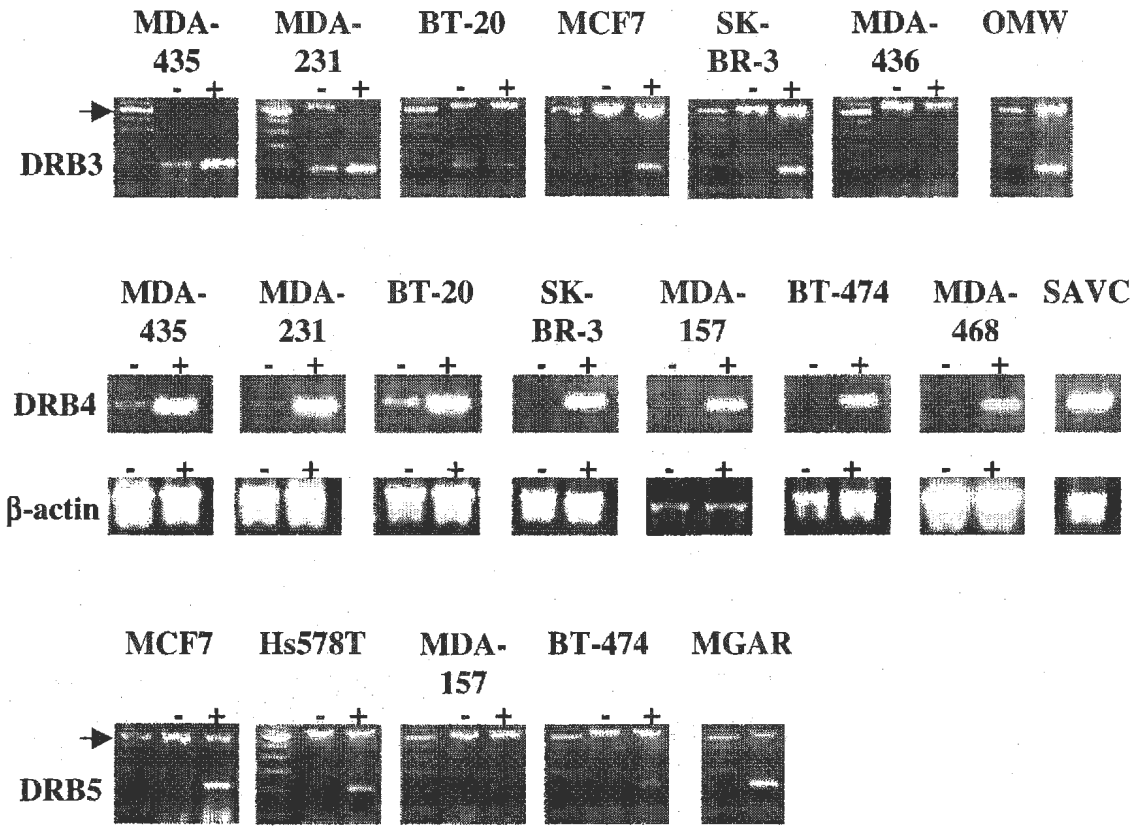


Figure 3.27. HLA-DRB transcription in breast cancer cell lines. Expression in non-induced cells (-) and IFN-γ induced cells (+) was assessed using sequence specific primers and RT-PCR (see section 2.6). β-actin amplification was a control for DRB1*04, DRB1*07 and DRB4 reactions. An internal control, a non allelic sequence in exon 3 of the DRB gene, was included in all other reactions and is shown at ~600bp. The arrow indicates 600bp on the DNA ladder.

Table 3.10. HLA-DRB transcription[a] in breast cancer cell lines.

Cell Line	HLA-DR Gene								
	DRB1 *01	DRB1 *15	DRB1 *03	DRB1 *04	DRB1 *13	DRB1 *07	DRB3	DRB4	DRB5
MDA-MB-435	-	-	-	C[b]	C	-	C	C	-
T-47D	C	-	-	-	-	-	-	-	-
MDA-MB-231	-	-	-	-	C	C	C	C	-
BT-20	-	-	-	C	C	-	C	C	-
MCF7	-	C	I[c]	-	-	-	I	-	C
SK-BR-3	-	-	-	-	I	I	I	C	-
MDA-MB-436	-	-	I	-	-	-	I	-	-
Hs578T	I	I	-	-	-	-	-	-	I
MDA-MB-157	-	I	-	I	-	-	-	C	I
BT-474	-	C	-	I	-	-	-	I	I
MDA-MB-468	C	-	-	-	-	I	-	I	-

[a] Expression was assessed using sequence specific primers and RT-PCR.
[b] Constitutive transcription.
[c] IFN-γ induced transcription.

of these genes was assessed. MDA-MB-231 and SK-BR-3 were found to constitutively express DRB4 and up-regulate DRB4 (Table 3.10). DRB5 was constitutively transcribed by MCF7 and was up-regulated by MCF7, Hs578T, MDA-MB-157 and BT-474. In certain instances, for unknown reasons, amplification of β-actin, used as a control for mRNA integrity, was poor. This was particularly noticeable for DRB1*07 reactions and for DRB4 amplification in MDA-MB-157. However, these cDNA were suitable for use in RT-PCR since amplification of β-actin was achieved readily in other PCR reactions.

3.6.3.2. Expression of HLA-DR Allelic Products

Since 9/11 cell lines constitutively transcribed HLA-DRB alleles and all cell lines transcribed HLA-DRB alleles in response to IFN-γ, cell surface expression of HLA-DR allelic products was assessed. A description of HLA-DR allele expression in each breast cancer cell line will follow.

3.6.3.2A. MDA-MB-435

MDA-MB-435 constitutively expressed cell surface HLA-DR and strongly up-regulated this antigen as assessed by the antibody L243 and flow cytometry (Figure 3.28). HLA-DRB1*13, detected using UK8.1, was constitutively expressed and was further up-regulated by IFN-γ. Similarly, HLA-DRB3 (DR52), determined using 7.3.19.1 and NDS13, was constitutively expressed and was strongly up-regulated. Using NFLD.D1 and NFLD.D10, HLA-DRB1*04 was found to be strongly up-regulated and using PL3, HLA-DRB4 (DR53) expression was found to be strongly induced.

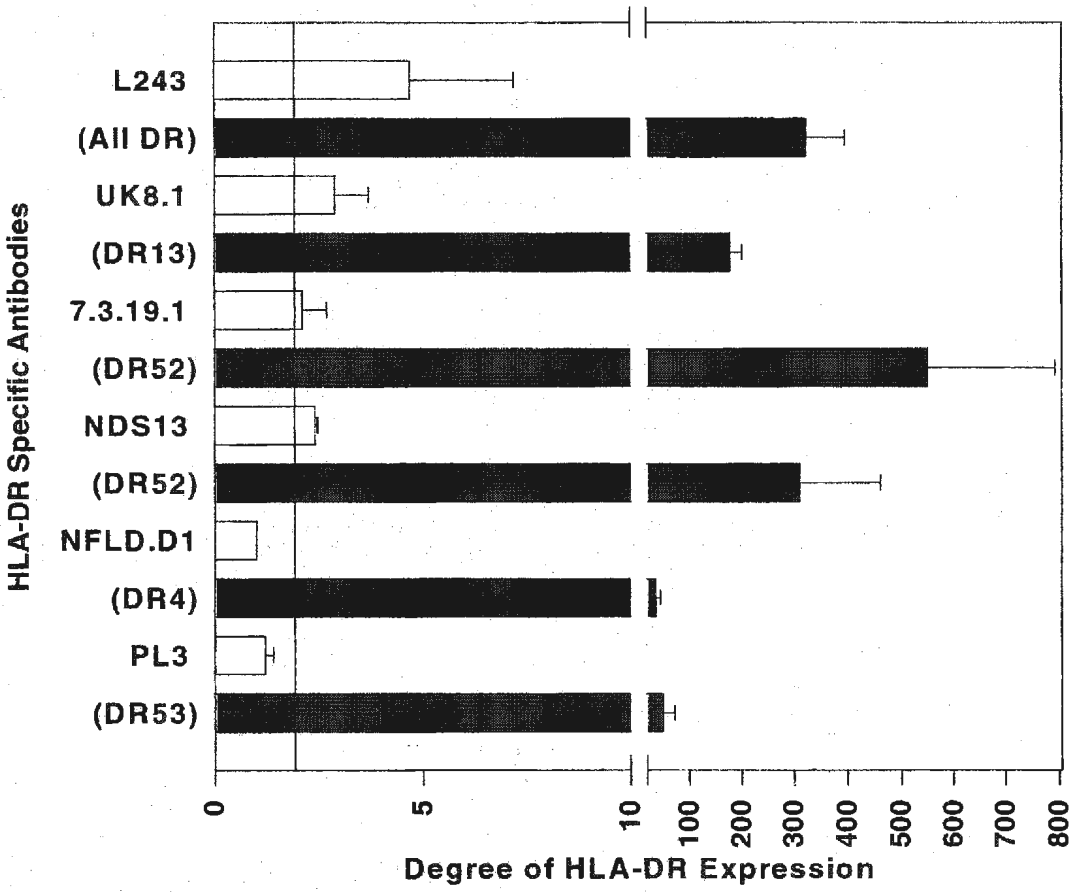


Figure 3.28. Cell surface expression of HLA-DR allelic products on MDA-MB-435. Non-induced (open bars) and IFN-γ induced (filled bars) expression were assessed using monoclonal antibodies that distinguished DR allotypes (shown in parenthesis) and flow cytometry. Results, reported as degree of HLA-DR expression (see section 2.4.5), are averages of at least two experiments. Standard deviations are indicated. Values of 2.1 and above (to the right of the black line) are considered positive.

3.6.3.2B. T-47D

T-47D constitutively expressed and strongly up-regulated cell surface HLA-DR as detected using the antibody L243 (Figure 3.29). This cell line had only one HLA-DR type, HLA-DRB1*0102. Interestingly, although constitutive HLA-DR expression was observed using L243, constitutive HLA-DR1 expression was not detected using NFLD.D10, JS-1 or NFLD.D2. Following IFN-γ treatment, HLA-DR1 was strongly up-regulated on the cell surface of T-47D (Figure 3.29).

3.6.3.2C. MDA-MB-231

Even though constitutive HLA-DR cell surface expression was observed on MDA-MB-231 using the pan anti-HLA-DR antibody L243, constitutive expression of HLA-DR allelic products was not detected using HLA-DR specific mAbs (Figure 3.30). Following IFN-γ treatment, cell surface HLA-DR was strongly up-regulated as assessed using L243. Similarly, HLA-DRB1*13 expression, detected using UK8.1, and HLA-DRB3 (DR52) expression, detected using NFLD.D7 and 7.3.19.1, were strongly induced. Using SFR16, it was determined that HLA-DR7 was moderately up-regulated. Unfortunately, HLA-DR7 expression was measured only once since we could not acquire more DR7-specific antibody. PL3, the antibody used to detect HLA-DRB4 (DR53) expression also measured HLA-DR7 expression. For this reason, it was not possible to determine whether DR53 protein was up-regulated by this cell line.

3.6.3.2D. BT-20

As described for T-47D and MDA-MB-231, constitutive HLA-DR expression was

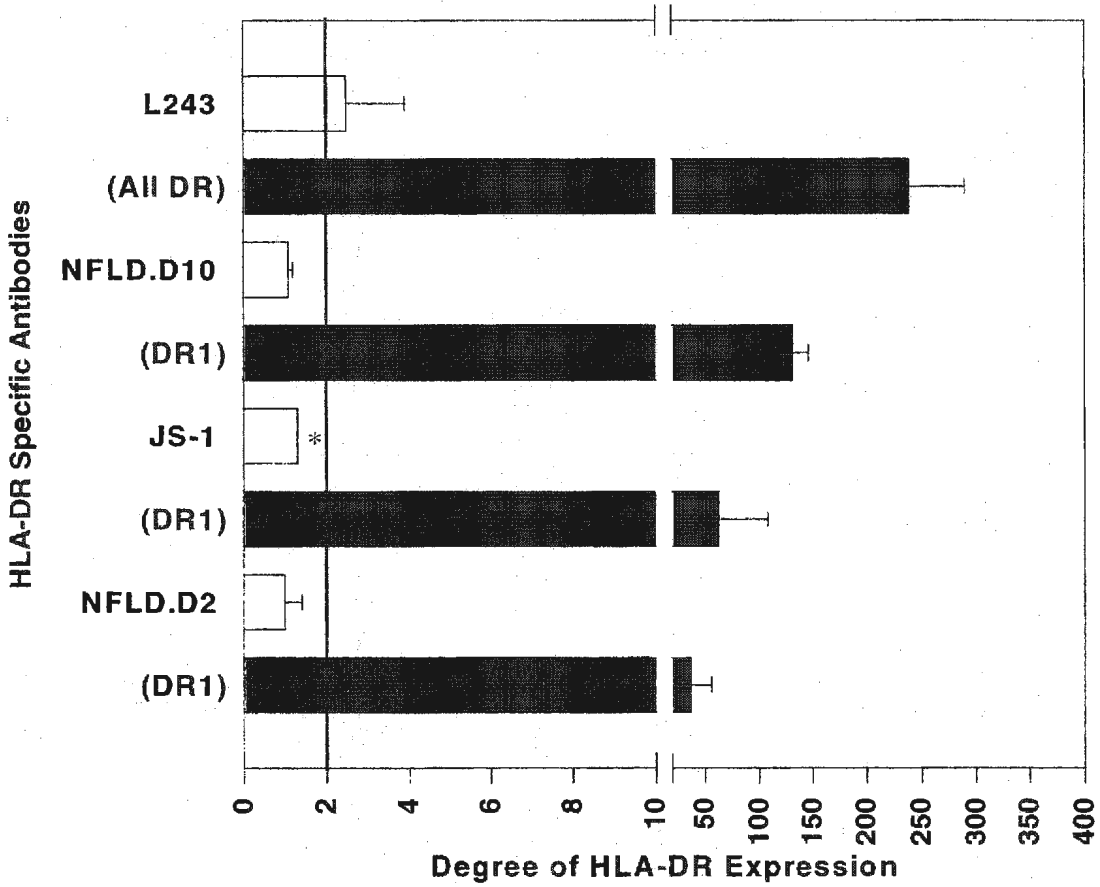


Figure 3.29. Cell surface expression of HLA-DR allelic products on T-47D. Non-induced (open bars) and IFN-γ induced (filled bars) expression were assessed using monoclonal antibodies that distinguished DR allotypes (shown in parenthesis) and flow cytometry. Results are reported as degree of HLA-DR expression (see section 2.4.5) and are averages of more than one experiment unless indicated by an asterisk. Standard deviations are indicated where appropriate. Values of 2.1 and above (to the right of the black line) are considered positive.

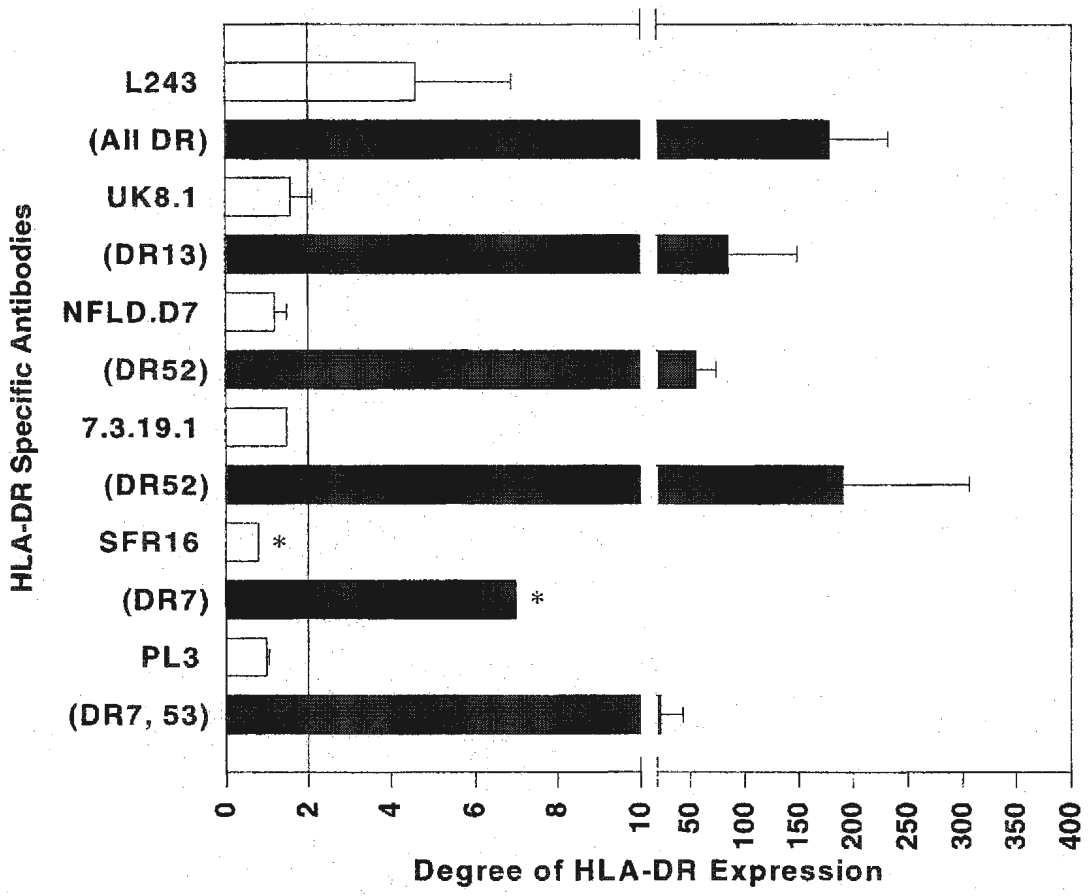


Figure 3.30. Cell surface expression of HLA-DR allelic products on MDA-MB-231. Non-induced (open bars) and IFN-γ induced (filled bars) expression were assessed using monoclonal antibodies that distinguished DR allotypes (shown in parenthesis) and flow cytometry. Results are reported as degree of HLA-DR expression (see section 2.4.5) and are averages of more than one experiment unless indicated by an asterisk. Standard deviations are shown where appropriate. Values of 2.1 and above (to the right of the black line) are considered positive.

detected on BT-20 using the antibody L243 but was not detected using HLA-DR specific mAbs (Figure 3.31). HLA-DR was strongly up-regulated in response to IFN-γ as assessed using L243 (Figure 3.31). Accordingly, cell surface HLA-DRB1*13 expression, ascertained using UK8.1 and HLA-DRB3 (DR52) expression, ascertained using 7.3.19.1, were strongly up-regulated. IFN-γ induced cell surface expression of HLA-DRB1*04 differed considerably depending on the antibody used to detect it (Figure 3.31). For example, when NFLD.D1 and NFLD.M1 were used, HLA-DR4 was found to be weakly up-regulated. However, when NFLD.D10 and NFLD.D2 were used, strong DR4 up-regulation was observed. This phenomenon was described previously by Tibbo (1998). HLA-DRB4 (DR53) expression, detected using PL3, was strongly induced.

Since NFLD.M1 also binds HLA-DR52 (Table 2.4), it would suggest that DR52 was weakly up-regulated by BT-20. However, using 7.3.19.1, DR52 was found to be strongly up-regulated.

Immunocytochemistry (IC), shown in Figure 3.32, was used to further characterize HLA-DR4 expression in BT-20. Moderate to strong HLA-DR expression was observed in ~75% of BT-20 using the antibody L243. Immunocytochemical detection of HLA-DR4 revealed that ~60% of cells were moderately to strongly positive as assessed using NFLD.D1; ~50% of cells were weakly to moderately positive as detected using NFLD.M1; ~70% of cells were moderately to strongly positive as determined using NFLD.D10 and ~35% of cells were weakly to moderately positive as assessed using NFLD.D2. These observations suggest that

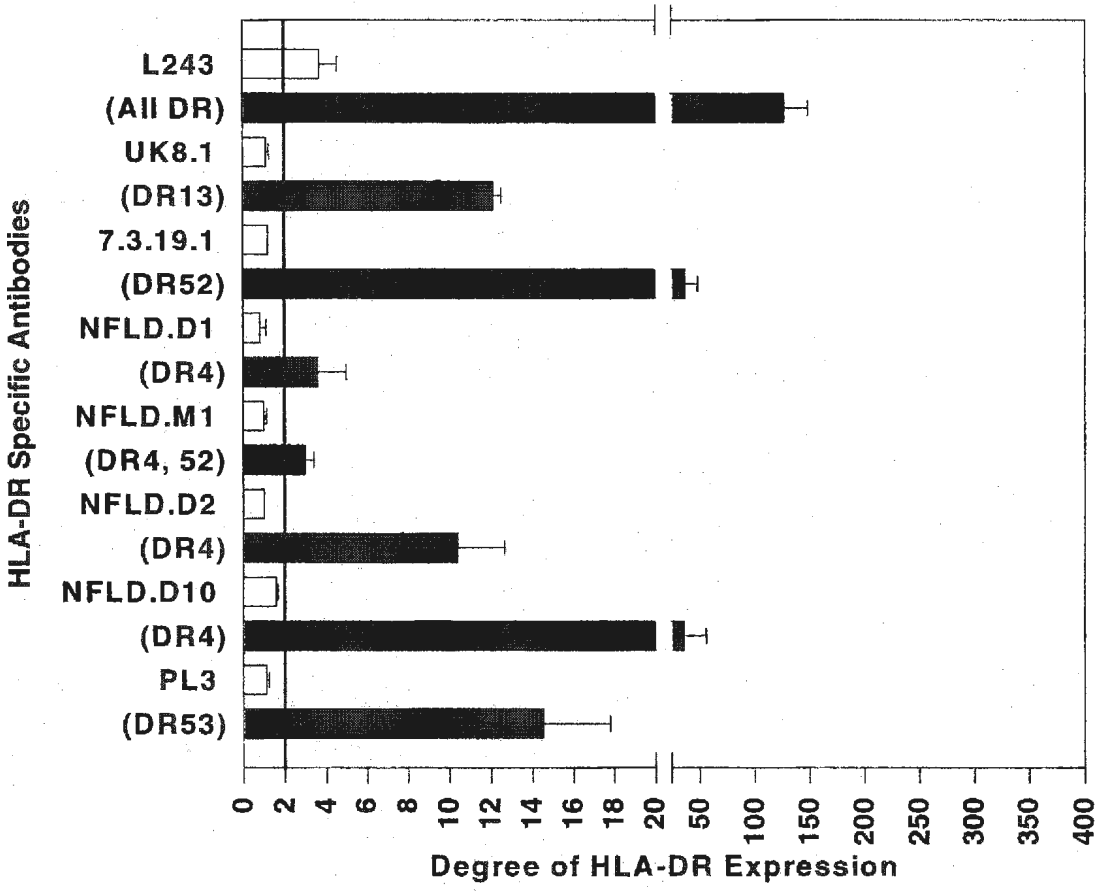
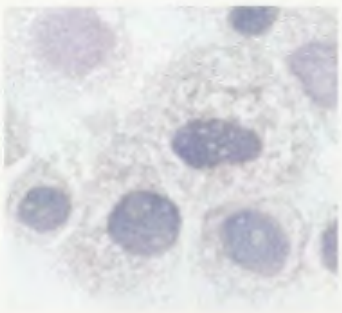
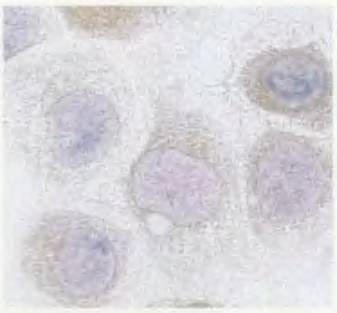


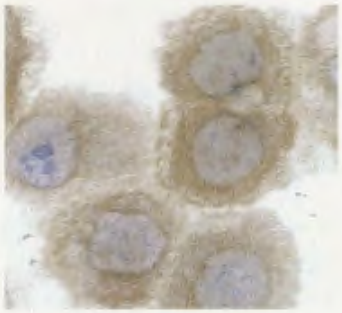
Figure 3.31. Cell surface expression of HLA-DR allelic products on BT-20. Non-induced (open bars) and IFN-γ induced (filled bars) expression were assessed using monoclonal antibodies that distinguished DR allotypes (shown in parenthesis) and flow cytometry. Results, reported as degree of HLA-DR expression (see section 2.4.5), are averages of at least two experiments. Standard deviations are indicated. Values of 2.1 and above (to the right of the black line) are considered positive.



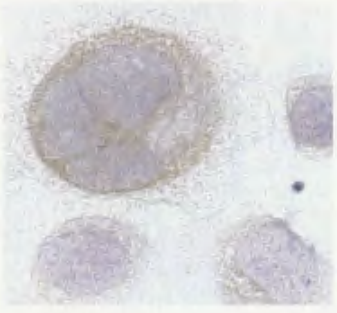
NEGATIVE



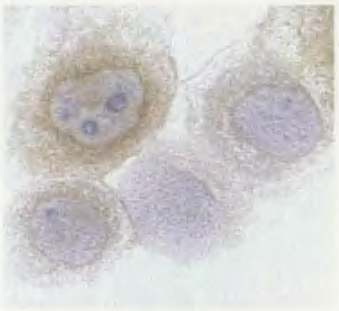
NFLD.M1 (DR4,DR52)



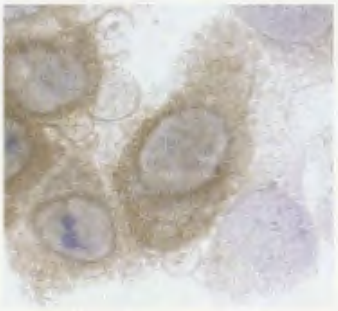
L243 (All DR)



NFLD.D2 (DR4)



NFLD.D1 (DR4)



NFLD.D10 (DR4)

Figure 3.32. Immunocytochemical analysis of HLA-DR4 up-regulation in IFN-γ treated BT-20. HLA-DR4 expression was assessed using NFLD.D1, NFLD.M1, NFLD.D2 and NFLD.D10. Expression of all DR types was analyzed using L243. The negative control is shown alongside.

NFLD.D1 and NFLD.M1 binding to cell surface HLA-DR4 was impeded in BT-20.

3.6.3.2E. MCF7

Cell surface HLA-DR was strongly up-regulated by MCF7 following IFN-γ treatment (Figure 3.33). HLA-DRB1*03 expression, determined by UK8.1, was strongly induced. Using NDS13, HLA-DRB3 (DR52) was found to be moderately up-regulated. Oddly, constitutive HLA-DR52 cell surface expression was also observed using NDS13 even though this gene was not constitutively transcribed (Figure 3.27) (see section 4.4.2 for further discussion). Constitutive HLA-DR expression, as assessed using L243, was also not detected. HLA-DRB1*15 expression, assessed using NFLD.D10, was strongly up-regulated by MCF7. HLA-DR15 expression was analyzed previously with similar findings (Tibbo, 1998). Unfortunately, HLA-DRB5 (DR51) protein expression was not assessed since a DR51 specific antibody was not available.

3.6.3.2F. SK-BR-3

HLA-DR was strongly up-regulated by SK-BR-3 in response to IFN-γ as assessed using the antibody L243 (Figure 3.34). HLA-DRB1*13 expression, determined using UK8.1, was moderately induced. HLA-DRB3 (DR52) was strongly up-regulated as assessed using 7.3.19.1. HLA-DRB1*07, detected using SFR16, was moderately up-regulated but was only measured once since the DR7 specific antibody was depleted. As previously stated, PL3, the antibody used to determine DRB4 (DR53) expression, also bound HLA-DR7. Thus, it was not possible to ascertain DR53 protein expression in this cell line.

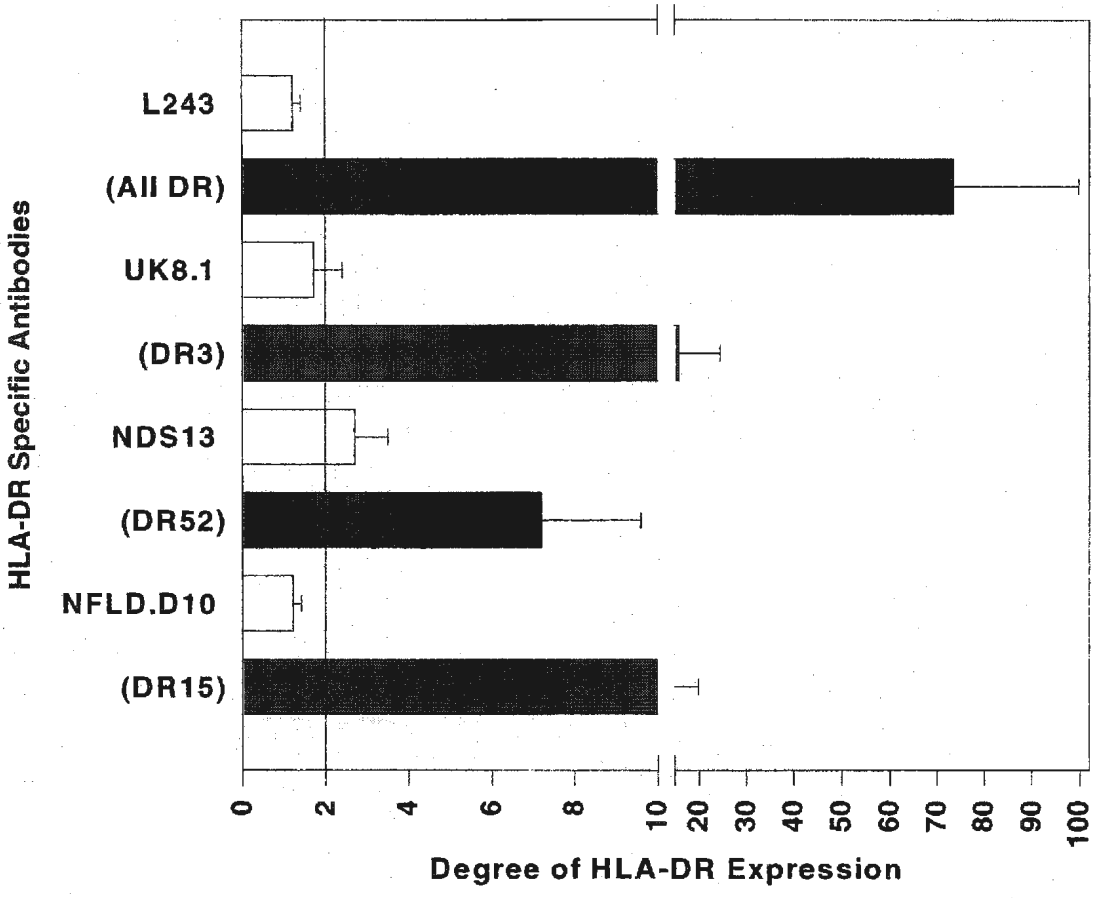


Figure 3.33. Cell surface expression of HLA-DR allelic products on MCF7. Non-induced (open bars) and IFN-γ induced (filled bars) expression were assessed using monoclonal antibodies that distinguished DR allotypes (shown in parenthesis) and flow cytometry. Results, reported as degree of HLA-DR expression (see section 2.4.5), are averages of at least two experiments. Standard deviations are indicated. Values of 2.1 and above (to the right of the black line) are considered positive.

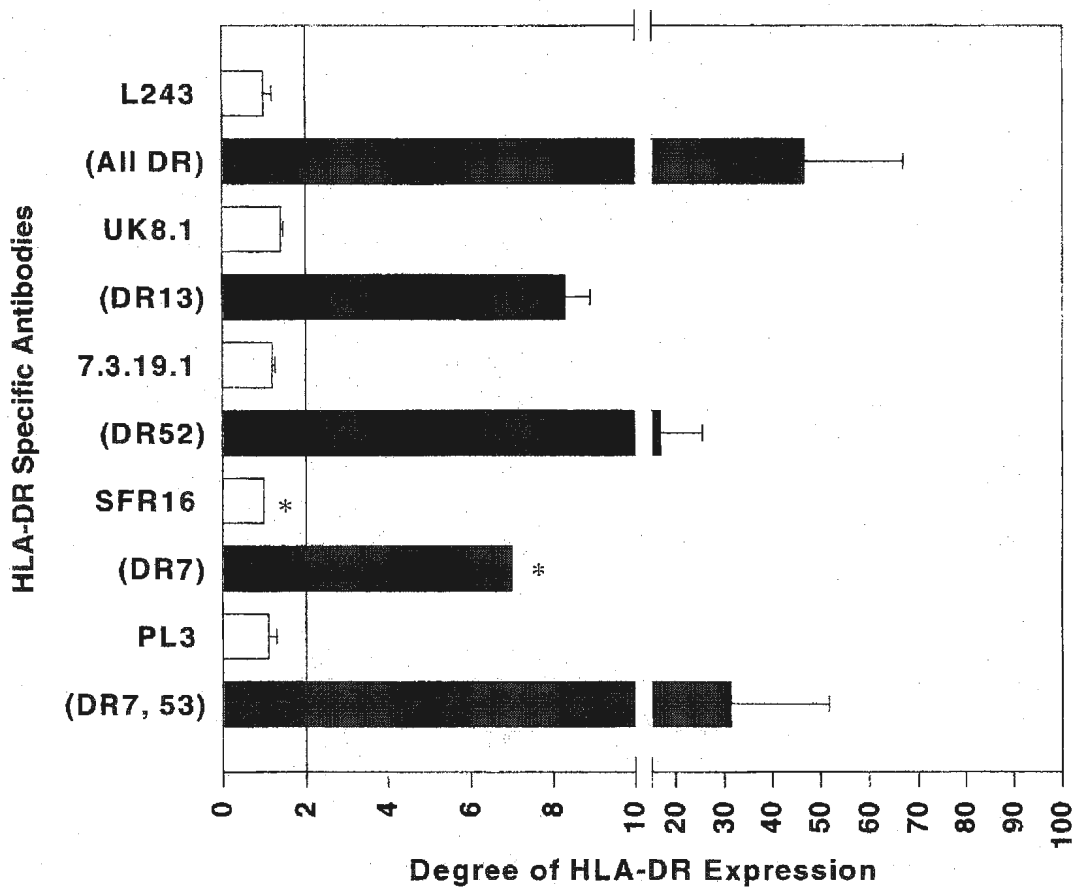


Figure 3.34. Cell surface expression of HLA-DR allelic products on SK-BR-3. Non-induced (open bars) and IFN-γ induced (filled bars) expression were assessed using monoclonal antibodies that distinguished DR allotypes (shown in parenthesis) and flow cytometry. Results are reported as degree of HLA-DR expression (see section 2.4.5) and are averages of more than one experiment unless indicated by an asterisk. Standard deviations are shown where appropriate. Values of 2.1 and above (to the right of the black line) are considered positive.

3.6.3.2G. MDA-MB-436

MDA-MB-436 strongly up-regulated cell surface HLA-DR as assessed using L243 (Figure 3.35). HLA-DRB1*03 expression, determined by UK8.1, was strongly induced. In comparison, HLA-DRB3 (DR52) expression, detected using NFLD.D7 and NDS13, was weakly up-regulated.

Immunocytochemically, weak to moderate HLA-DR52 expression was observed in ~14% of MDA-MB-436 using NFLD.D7 (Figure 3.36). This confirmed flow cytometry results which revealed weak DR52 up-regulation in this cell line. In contrast, no DR52 protein was detected using the antibody NDS13. NFLD.D7 was effective for use in IC as observed by its moderate to strong binding to ~90% of the B cell line control COX. However, the epitope recognized by NDS13 may have been altered by acetone fixation or by detergent since NDS13 weakly to moderately bound ~77% of COX. (Figure 3.36).

3.6.3.2H. Hs578T

Cell surface HLA-DR was strongly up-regulated by Hs578T as determined using the antibody L243 (Figure 3.37). HLA-DRB1*01, assessed by JS-1, was not up-regulated. HLA-DRB1*15 expression, determined by NFLD.D7, was strongly induced following IFN-γ treatment. HLA-DRB5 (DR51) expression was not analyzed since we did not have a DR51 specific antibody.

Immunocytochemically, no DR1 protein was detected in Hs578T (Figure 3.38). This confirmed flow cytometry data. JS-1 was suitable for use in IC since weak to moderate staining of ~80% of cells was observed in the positive control JESTHOM.

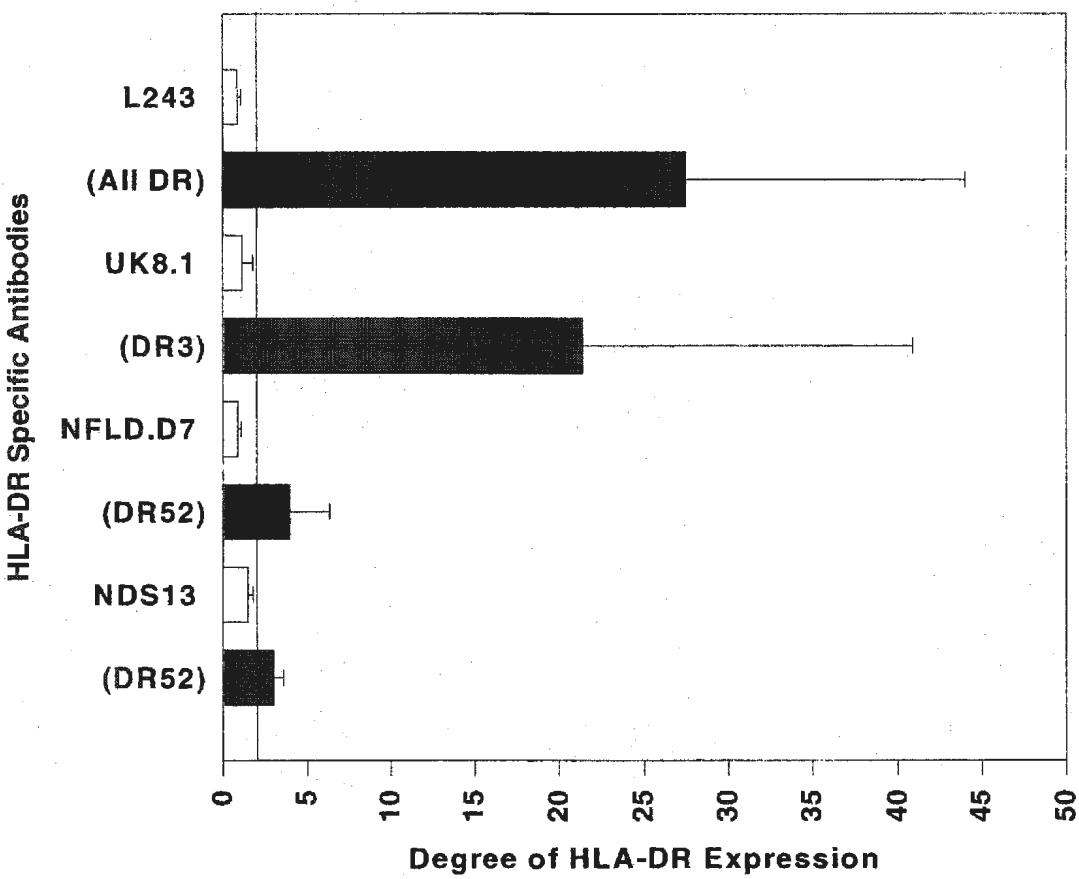


Figure 3.35. Cell surface expression of HLA-DR allelic products on MDA-MB-436. Non-induced (open bars) and IFN-γ induced (filled bars) expression were assessed using monoclonal antibodies that distinguished DR allotypes (shown in parenthesis) and flow cytometry. Results, reported as degree of HLA-DR expression (see section 2.4.5), are averages of at least two experiments. Standard deviations are indicated. Values of 2.1 and above (to the right of the black line) are considered positive.

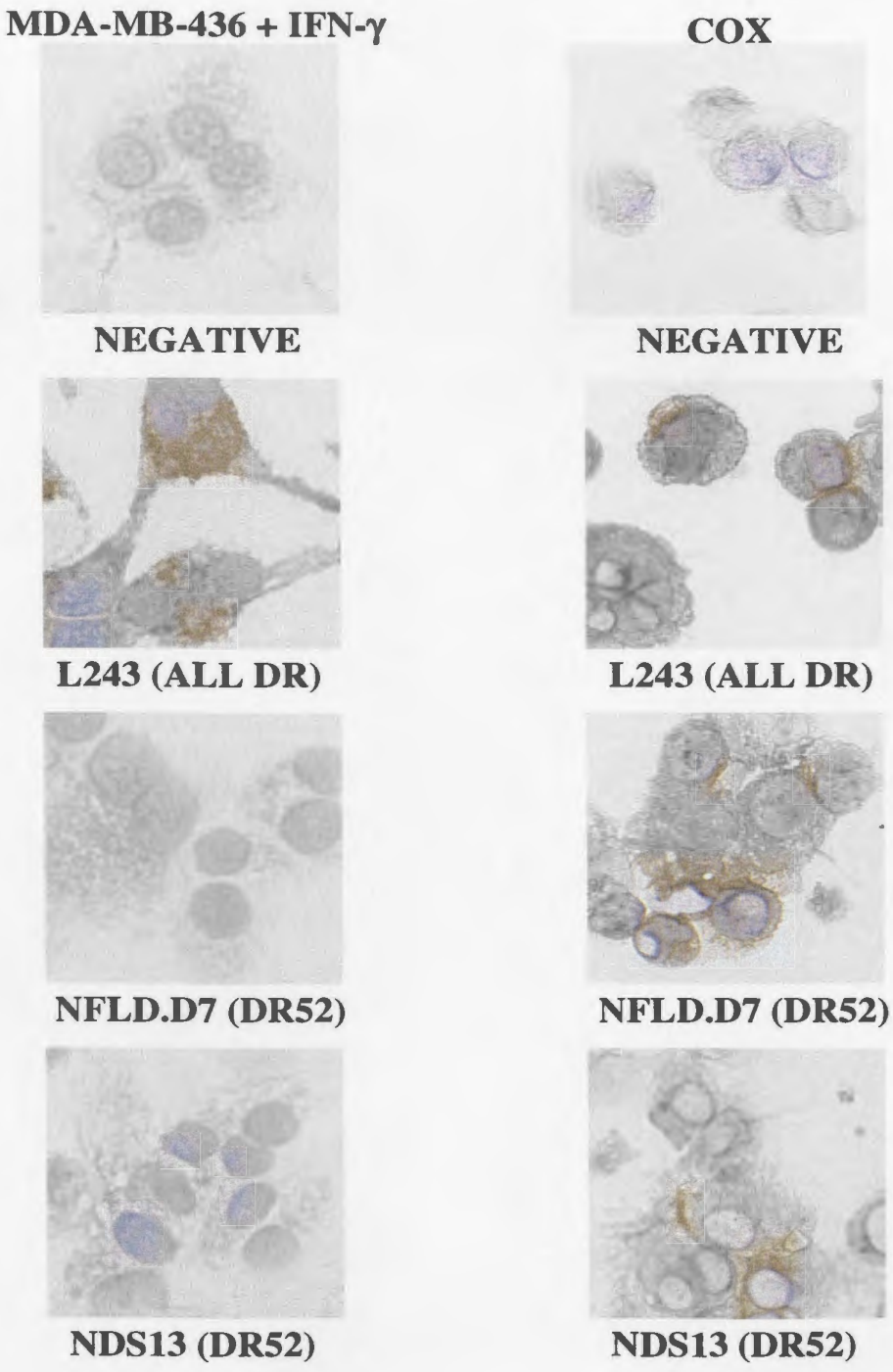


Figure 3.36. Immunocytochemical analysis of HLA-DR52 up-regulation in IFN-γ treated MDA-MB-436. HLA-DR52 expression was assessed using NFLD.D7 and NDS13. Expression of all DR types was analyzed using L243. The negative control is shown alongside. COX is the positive control for DR52 antibody binding.

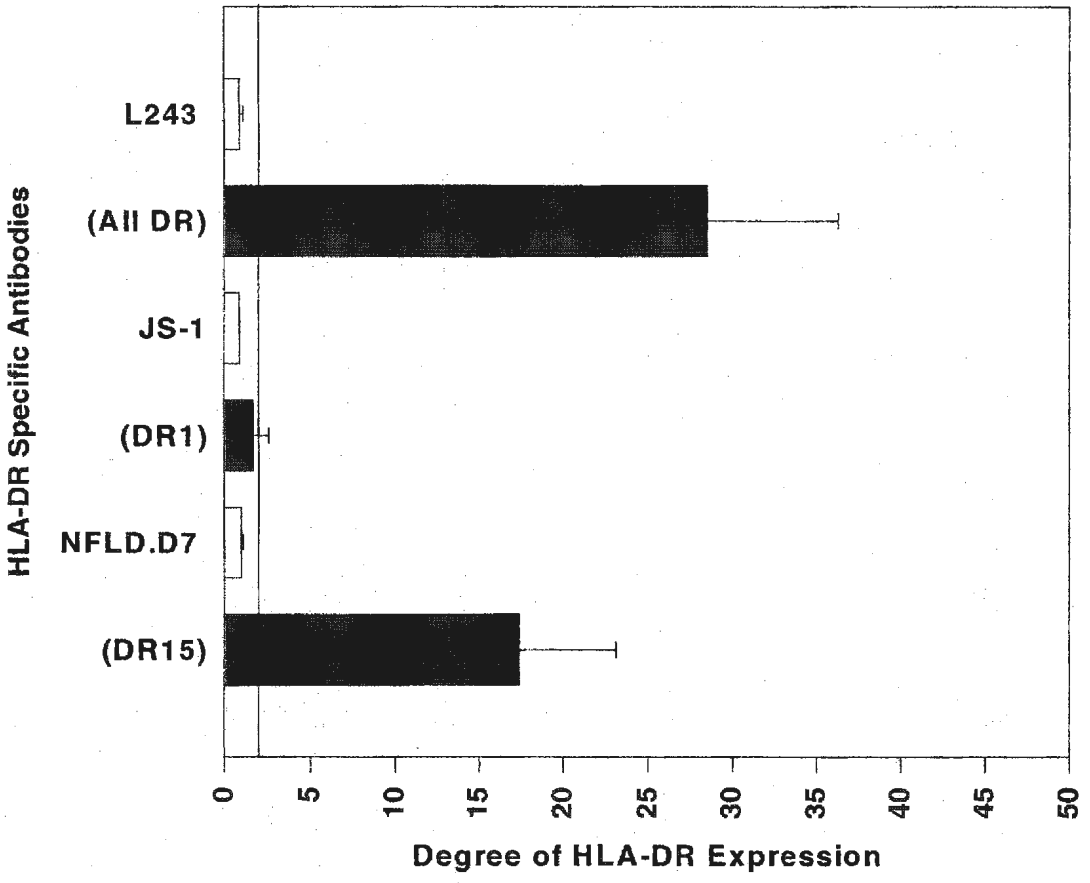


Figure 3.37. Cell surface expression of HLA-DR allelic products on Hs578T. Non-induced (open bars) and IFN-γ induced (filled bars) expression were assessed using monoclonal antibodies that distinguished DR allotypes (shown in parenthesis) and flow cytometry. Results, reported as degree of HLA-DR expression (see section 2.4.5), are averages of at least two experiments. Standard deviations are indicated. Values of 2.1 and above (to the right of the black line) are considered positive.

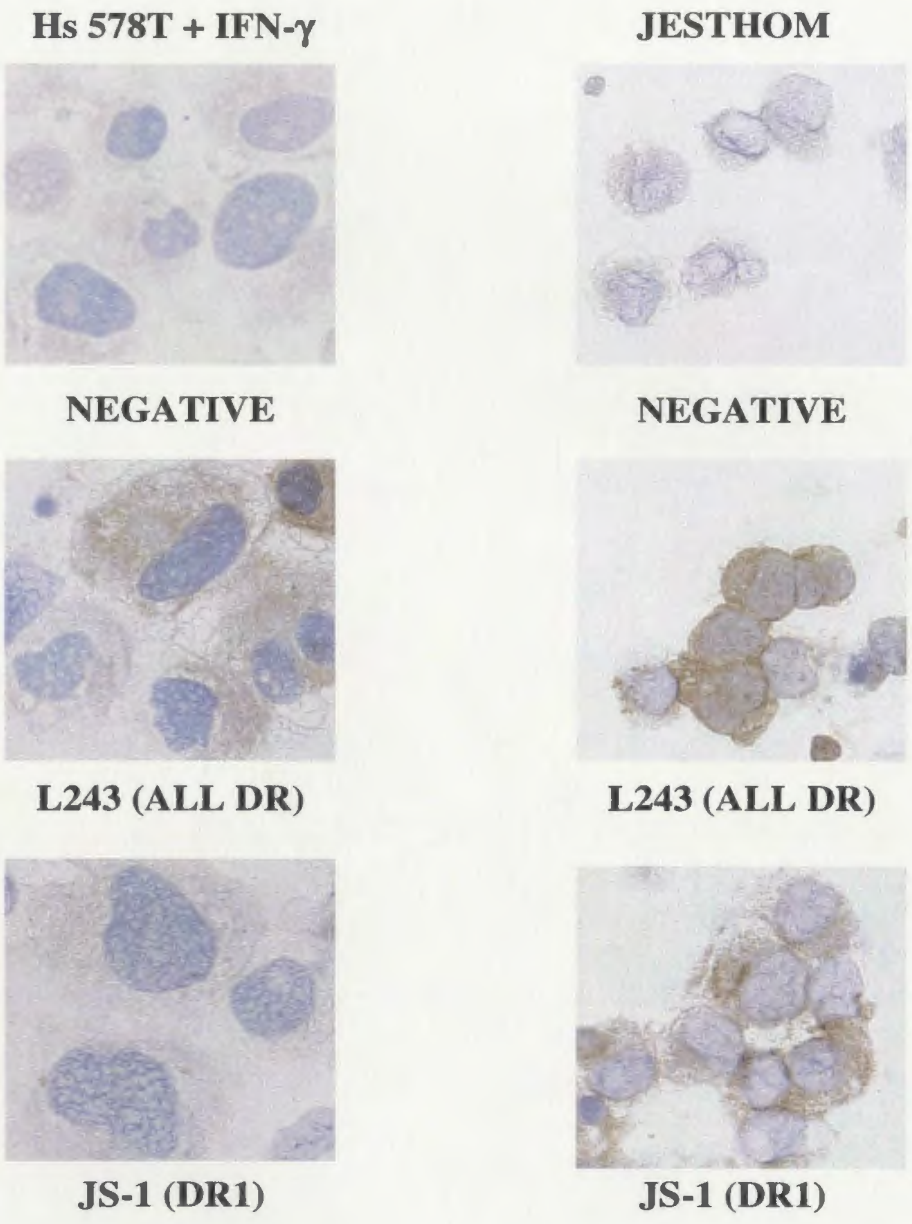


Figure 3.38. Immunocytochemical analysis of HLA-DR1 up-regulation in IFN-γ treated Hs578T. HLA-DR1 expression was assessed using JS-1. Expression of all DR types was analyzed using L243. The negative control is shown alongside. JESTHOM is the positive control for JS-1 binding.

3.6.3.2I. MDA-MB-157

Following IFN-γ treatment, MDA-MB-157 strongly up-regulated cell surface HLA-DR as assessed using L243 (Figure 3.39). However, HLA-DRB1*04 expression, analyzed using NFLD.D1 and NFLD.M1, and HLA-DRB4 (DR53) expression, assessed using PL3, were not induced. HLA-DRB1*15 cell surface expression, detected using NFLD.D10 and NFLD.D7, was moderately up-regulated. NFLD.D10 and NFLD.D7 recognize DR4 and DR15 but since DR4 was not up-regulated by MDA-MB-157, these antibodies were useful for DR15 detection. HLA-DRB5 (DR51) protein expression could not be assessed since a DR51 specific mAb was not available. These data confirmed findings by Tibbo (1998) regarding HLA-DR4 and DR15 protein expression in MDA-MB-157.

Immunocytochemically, weak to moderate HLA-DR4 expression, assessed by NFLD.D1 and NFLD.M1, was observed in ~15% of cells (Figure 3.40). NFLD.D1 and NFLD.M1 were suitable for use in IC as observed by their binding to positive control cell lines (Figure 3.38). Specifically, NFLD.D1 strongly bound ~95% of SAVC while NFLD.M1 moderately bound ~85% of MDA-MB-435. Additionally, IC analysis, using the antibody PL3, revealed that ~20% of cells were weakly positive for DR53 expression. Thus, DR53 protein was weakly up-regulated intracellularly in MDA-MB-157. PL3 was suitable for IC since this antibody strongly bound ~87% of MDA-MB-435 (Figure 3.40).

3.6.3.2J. BT-474

BT-474 moderately up-regulated cell surface HLA-DR following exposure to IFN-γ as assessed using L243 (Figure 3.41). HLA-DRB1*04 expression, assessed by NFLD.D1 and

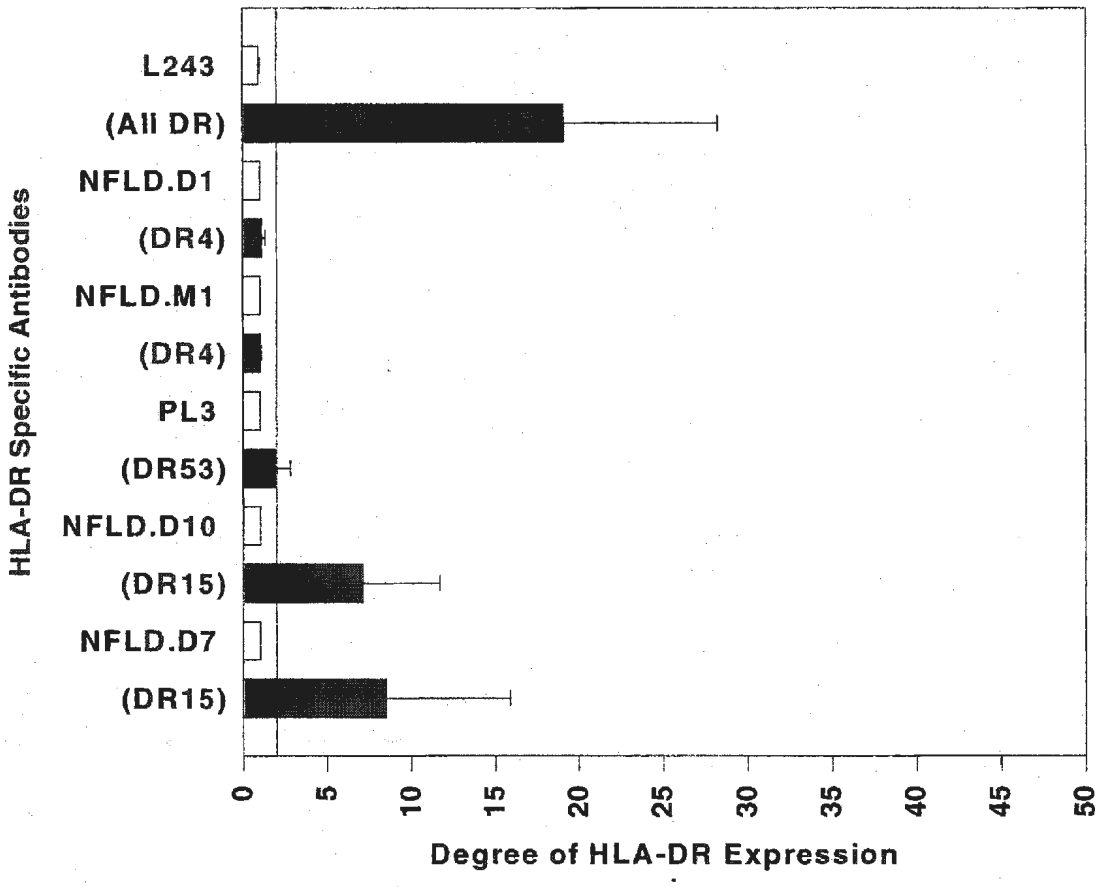


Figure 3.39. Cell surface expression of HLA-DR allelic products on MDA-MB-157. Non-induced (open bars) and IFN-γ induced (filled bars) expression were assessed using monoclonal antibodies that distinguished DR allotypes (shown in parenthesis) and flow cytometry. Results, reported as degree of HLA-DR expression (see section 2.4.5), are averages of at least two experiments. Standard deviations are indicated. Values of 2.1 and above (to the right of the black line) are considered positive.

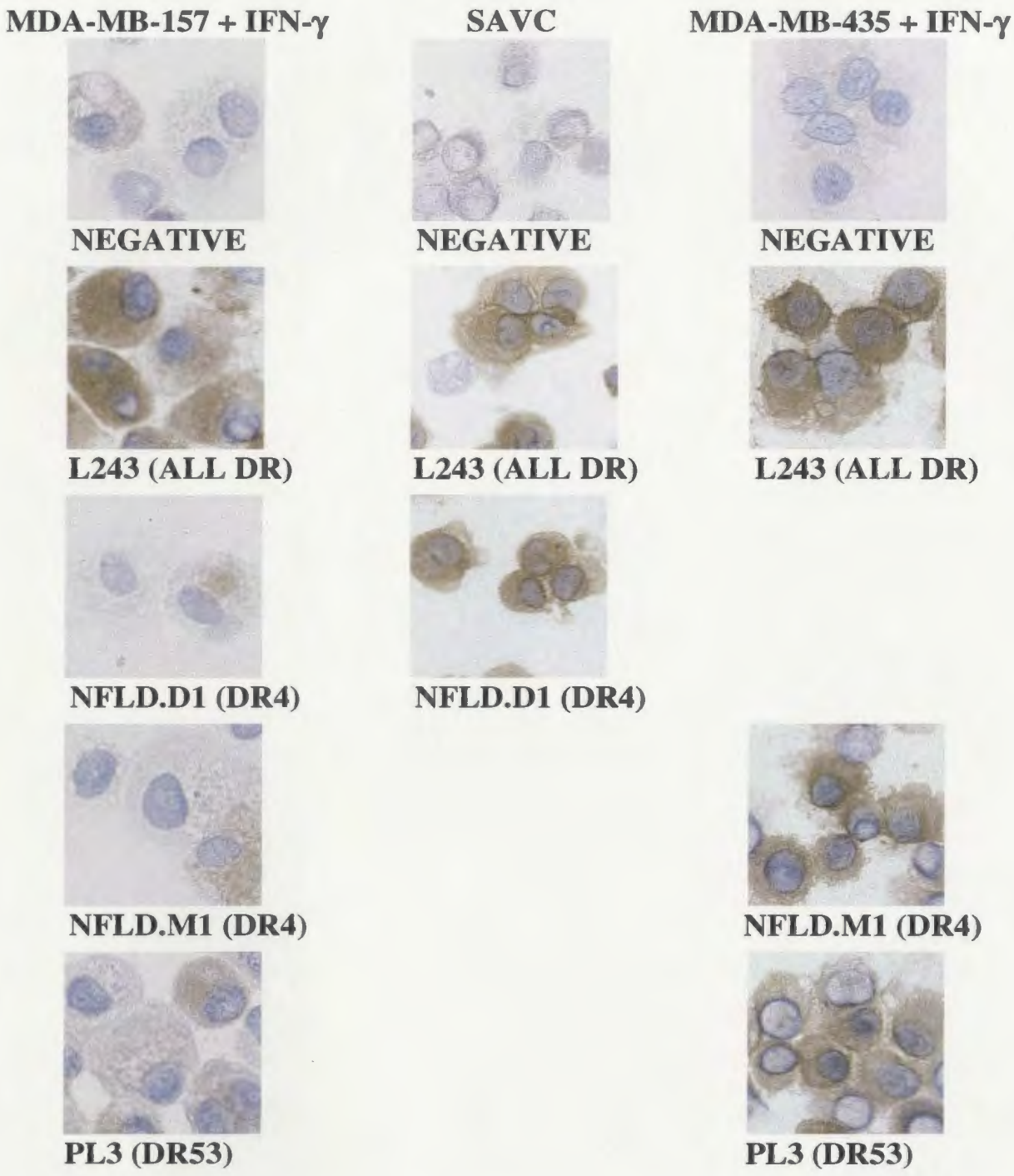


Figure 3.40. Immunocytochemical analysis of HLA-DR4 and HLA-DR53 expression in IFN-γ treated MDA-MB-157. DR4 expression was assessed using NFLD.D1 and NFLD.M1 while DR53 expression was assessed using PL3. Expression of all DR types was analyzed using L243. The negative control is shown alongside. SAVC and MDA-MB-435 are positive controls for DR4 and DR53 antibody binding. MDA-MB-435 IC was done by Sharon Oldford.

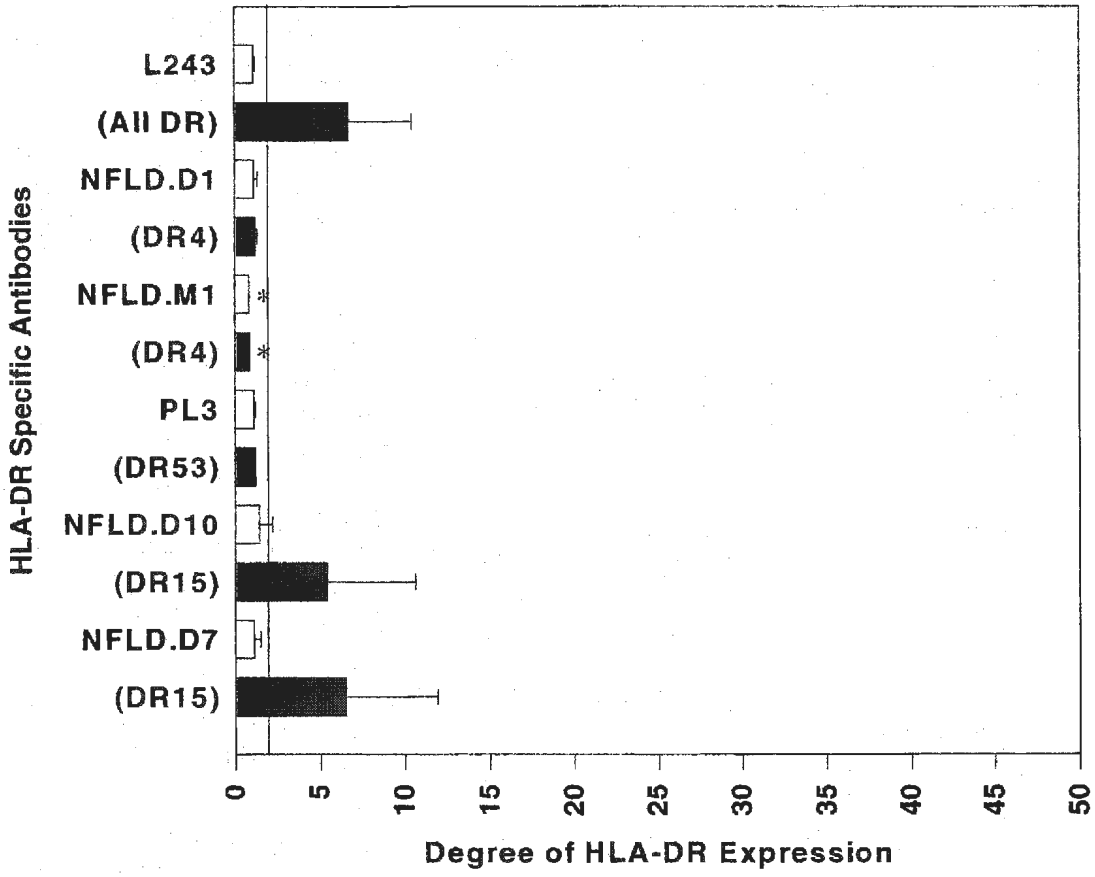


Figure 3.41. Cell surface expression of HLA-DR allelic products on BT-474. Non-induced (open bars) and IFN-γ induced (filled bars) expression were assessed using monoclonal antibodies that distinguished DR allotypes (shown in parenthesis) and flow cytometry. Results are reported as degree of HLA-DR expression (see section 2.4.5) and are averages of more than one experiment unless indicated by an asterisk. Standard deviations are indicated where appropriate. Values of 2.1 and above (to the right of the black line) are considered positive.

NFLD.M1, and HLA-DRB4 (DR53) expression, analyzed by PL3, were not observed. NFLD.M1 detection of DR4 expression was done only once. HLA-DRB1*15 expression, determined by NFLD.D10 and NFLD.D7, was moderately induced. Again, HLA-DRB5 (DR51) expression could not be analyzed.

Immunocytochemically, ~14% of cells were weakly or strongly positive for HLA-DR4 as assessed using NFLD.D1 and ~6% of cells were strongly positive for DR4 expression as determined using NFLD.M1 (Figure 3.42). Additionally, <5% of cells were weakly to moderately positive for HLA-DR53 expression as detected using PL3. These observations confirmed flow cytometry results. NFLD.D1, NFLD.M1 and PL3 were effective for use in IC given that they strongly bound positive control cell lines (see section 3.7.3.2I.).

3.6.3.2K. MDA-MB-468

Following IFN-γ treatment, MDA-MB-468 moderately up-regulated cell surface HLA-DR as assessed using L243. This cell line had the lowest HLA-DR up-regulation of all breast cancer cell lines (Figure 3.8). HLA-DRB1*01 expression, assessed by NFLD.D10, was not detected (Figure 3.43). Similarly, HLA-DRB1*07 expression, analyzed using SFR16, was not up-regulated. Since DR7 expression was not induced by MDA-MB-468, PL3 was suitable for assessing DR53 expression. However, using PL3, cell surface DR53 up-regulation was not observed.

IC confirmed flow cytometry data (Figure 3.44). Using NFLD.D10, ~9% of cells were weakly to moderately positive for DR1. IC using SFR16 was not done because this antibody was depleted. Immunocytochemically, <5% of cells were weakly to moderately

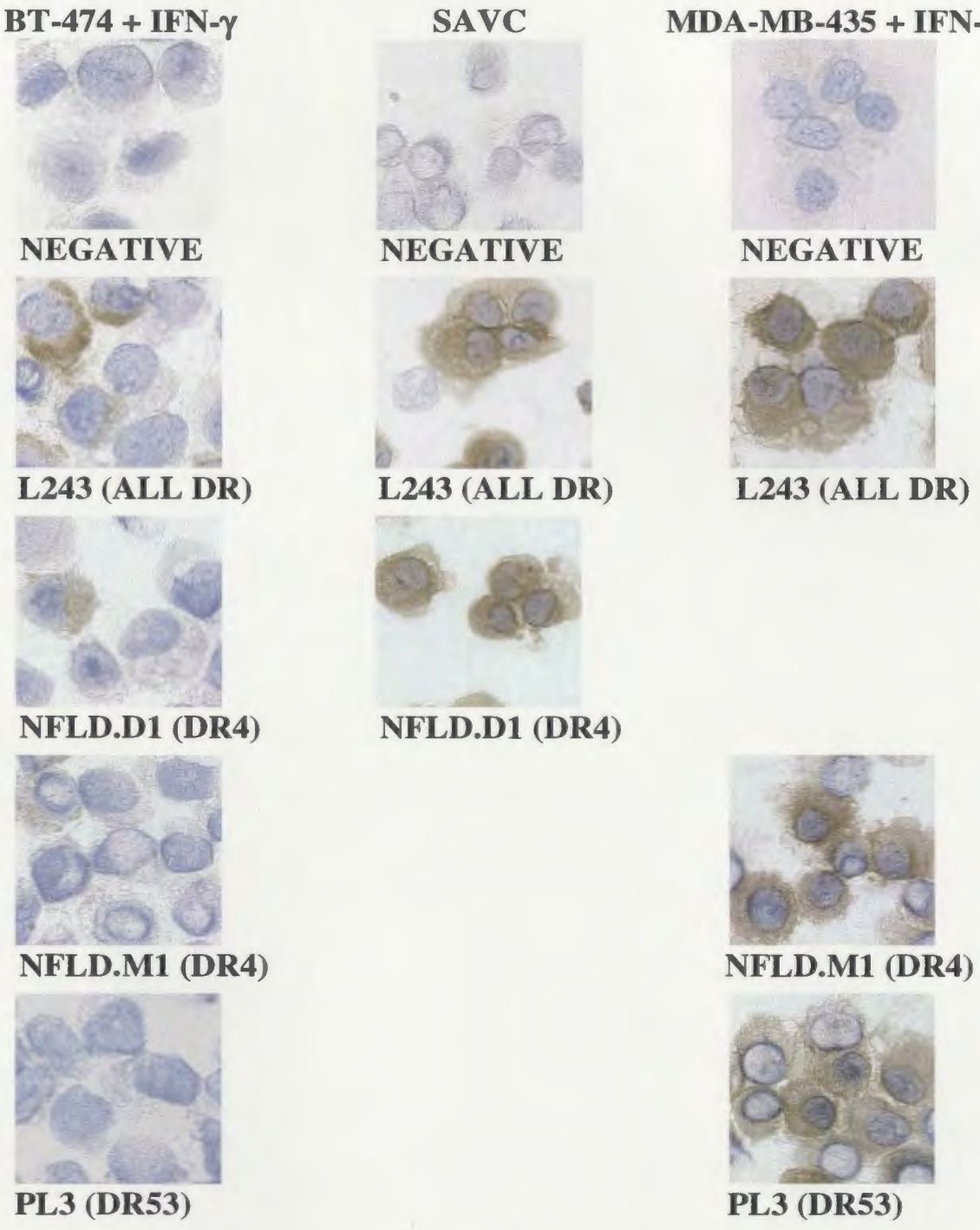


Figure 3.42. Immunocytochemical analysis of HLA-DR4 and HLA-DR53 expression in IFN-γ treated BT-474. DR4 expression was assessed using NFLD.D1 and NFLD.M1 while DR53 expression was assessed using PL3. Expression of all DR types was analyzed using L243. The negative control is shown alongside. SAVC and MDA-MB-435 are positive controls for DR4 and DR53 antibody binding. MDA-MB-435 IC was done by Sharon Oldford.

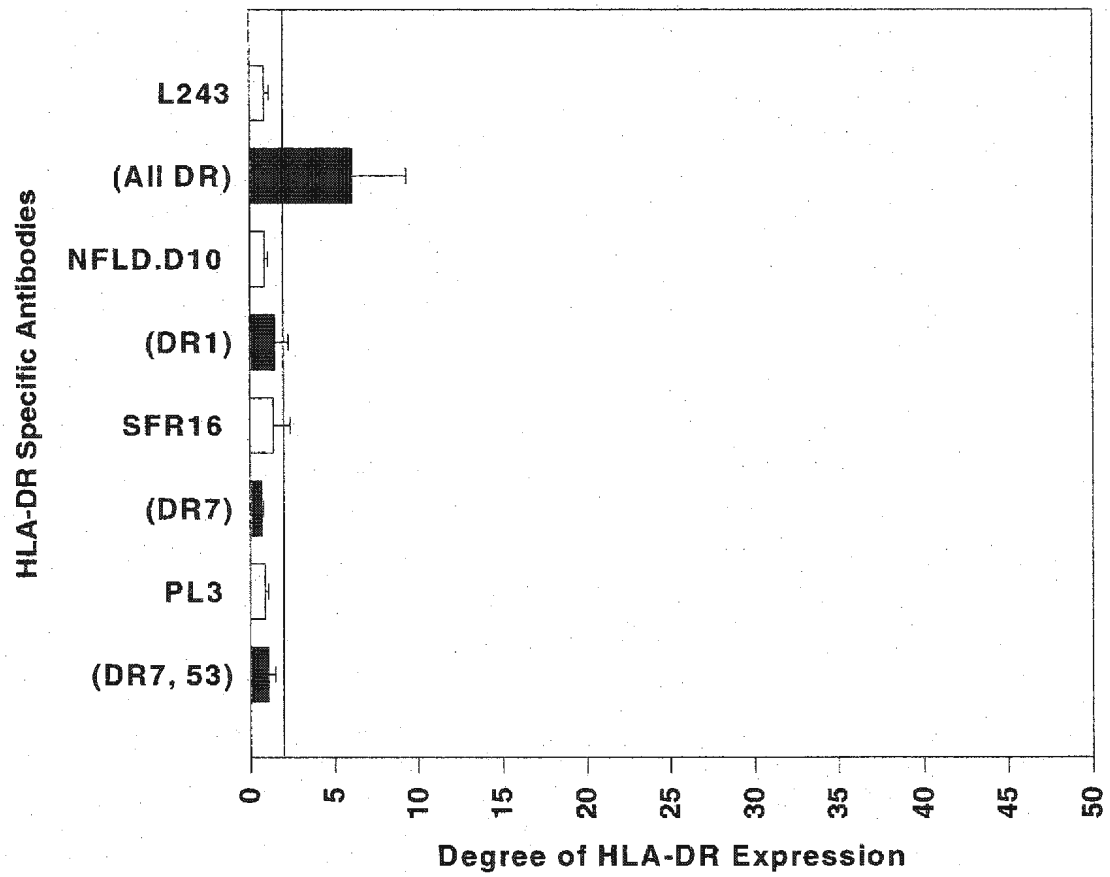


Figure 3.43. Cell surface expression of HLA-DR allelic products on MDA-MB-468. Non-induced (open bars) and IFN-γ induced (filled bars) expression were assessed using monoclonal antibodies that distinguished DR allotypes (shown in parenthesis) and flow cytometry. Results, reported as degree of HLA-DR expression (see section 2.4.5), are averages of at least two experiments. Standard deviations are indicated. Values of 2.1 and above (to the right of the black line) are considered positive.

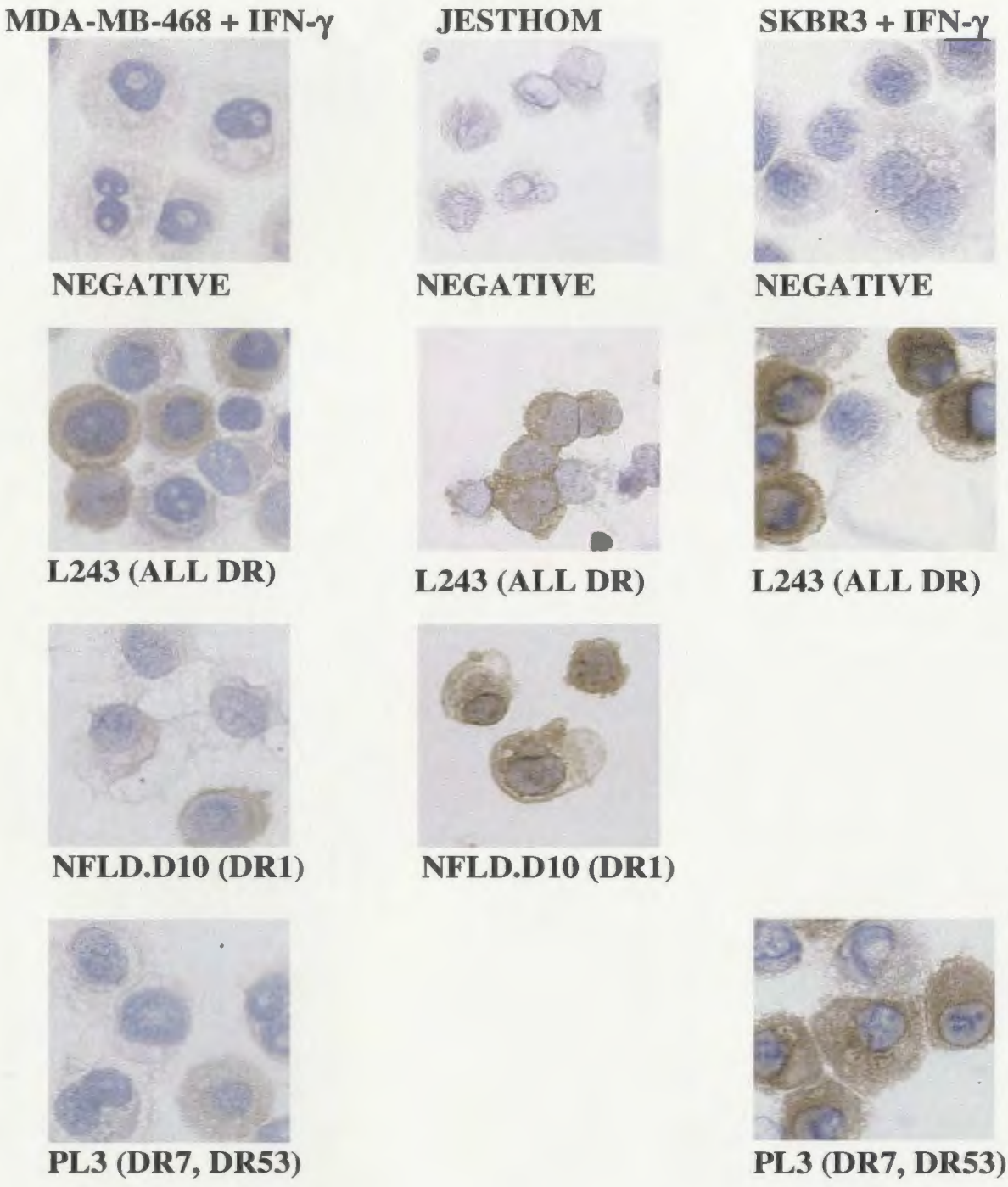


Figure 3.44. Immunocytochemical analysis of HLA-DR allele expression in IFN-γ treated MDA-MB-468. DR1 expression was assessed using NFLD.D1. DR7 and DR53 expression were assessed using PL3. Expression of all DR types was analyzed using L243. The negative control is shown alongside. JESTHOM and SK-BR-3 are positive controls for NFLD.D10 and PL3 binding.

positive when analyzed using PL3. NFLD.D10 and PL3 were suitable for use in IC since they moderately to strongly bound positive control cell lines (Figure 3.44). For example, NFLD.D10 bound ~90% of JESTHOM while PL3 bound ~65% of SK-BR-3.

CHAPTER 4. DISCUSSION

The CD4+ T cell tumor antigen-specific immune response may have a critical role in the eradication of breast tumors (Ostrand-Rosenberg, 1994, Baskar, 1996, Armstrong et al., 1998c). Since this immune response may be significantly altered by HLA class II expressing carcinomas, an important goal of our research was to characterize breast cancer cells as potential APC.

4.1. Study Concerns

4.1.1. Cell Lines

Breast carcinoma cell lines utilized in our investigation were kindly provided by several laboratories (see section 2.1). When working with cultured cell lines, there is always the possibility of cell line mix-up or contamination (Kaplan et al., 1998, MacLeod et al., 1999, Masters et al., 2001). However, the identity of each breast cancer cell line used in our study has been confirmed via comparison with short tandem repeat (STR) sequences published by the ATCC (data not shown).

One cell line, MDA-MB-435, classified as a breast carcinoma by the ATCC, has been found to express several genes associated with melanoma (Ross et al., 2000). While this gene expression may have been indicative of neuroendocrine features of some breast tumors, more likely these results indicate that MDA-MB-435 originated from a melanoma (Ross et al., 2000). Thus, even though this thesis reports MDA-MB-435 findings, it is cautioned that this cell line may not be a breast carcinoma.

Of course, it is not known whether data obtained using our *in vitro* model of breast

cancer parallel the situation *in vivo*. Current work in our laboratory is addressing this issue by examining the selective expression of HLA-DR allelic products *in situ*.

4.1.2. Trypsin

The idea that weak class II expression on certain breast carcinoma cells was due to trypsin induced class II shedding was addressed. Trypsin degrades the extracellular matrix of proteins involved in cell adhesion thereby facilitating the removal of adherent cells (Vogel, 1978, Corver et al., 1995). This enzyme may also strip glycopeptides, glycosaminoglycans and proteins from the cell surface (Anghileri & Dermietzel, 1976, Vogel, 1978). Despite these trypsin induced cell alterations, Corver et al. (1995) observed limited loss of cell surface antigens on ovarian cancer cell lines following trypsin treatment. In agreement, our study found that class II cell surface expression on the B cell line MT14B was not significantly altered by trypsin (Figure 3.3). Furthermore, trypsin was not responsible for low levels of class II cell surface antigens on IFN-γ treated BT-474 and MDA-MB-468 since these cells had correspondingly weak up-regulation of intracellular class II (data not shown).

4.2. CIITA Independent HLA Class II and Class II Co-chaperone Regulation in Breast Cancer Cell Lines

4.2.1. Primer Concerns

CIITA transcription in breast carcinoma cells was analyzed by RT-PCR using two primer sets which amplified different regions of the CIITA transcript. Notably, constitutive

CIITA expression was detected in more breast cancer cell lines using primer set #2 than primer set #1 (Table 3.1). While both primer sets amplified CIITA mRNA in all IFN-γ treated breast cancer cell lines, primer set #2 consistently amplified more product (Figure 3.6). These data suggest that primer pair #2 had a higher affinity for the CIITA transcript than primer pair #1 and indeed primer pair #2 has a higher melting temperature than primer pair #1. Thus, all conclusions are based on data obtained using primer set #2. Future work could obtain additional primers to confirm these results.

4.2.2. Findings

To our knowledge, this was the first investigation of CIITA expression in breast carcinoma cells. CIITA expression was assessed in terms of gene transcription, but since transcription does not always parallel translation it is important that future work analyze CIITA protein expression in these cells.

As expected, all cell lines transcribed CIITA following IFN-γ stimulation. These data support the dogma that CIITA mediates IFN-γ induction of class II and class II co-chaperone genes (Steimle et al., 1994).

Constitutive CIITA transcription was observed in MDA-MB-435, MDA-MB-231, BT-20, MCF7, BT-474 and MDA-MB-468 but was not detected in T-47D, SK-BR-3, Hs578T, MDA-MB-436 and MDA-MB-157. Interestingly, all CIITA negative cell lines constitutively transcribed one or more class II and class II co-chaperone genes suggesting CIITA independent regulation of these genes. One cell line, T-47D, even constitutively expressed HLA-DR and Ii protein. Notably, a CIITA transcript was amplified in T-47D but

its size was smaller than the predicted size. Whether this indicates a mutation or deletion in CIITA is not known and requires further investigation.

This is not the first study to provide evidence for CIITA independent class II and class II co-chaperone regulation. Chang et al. (1996) described class II protein expression in the thymus of CIITA knockout mice. Furthermore, Williams et al. (1998), using different CIITA deficient mice, demonstrated class II protein in thymic cortex cells, germinal center B cells and lymph node dendritic cells. Chang et al. (1996) emphasized that low levels of class II transcripts detected in APCs from CIITA deficient mice may not be sufficient for translation (Chang et al., 1996). Future research could quantify class II transcripts in cell lines not expressing CIITA to assess whether levels are suitable for translation. This would also be informative for CIITA positive cell lines constitutively transcribing class II or class II co-chaperones, but not expressing protein.

Douhan et al. (1997) described a CIITA negative B cell line, clone 13, which expressed HLA-DQ protein but no HLA-DR nor HLA-DP protein. This suggests differential regulation of class II isotypes by CIITA. This phenomenon was not observed in our study as no cell line expressed HLA-DQ protein without concomitant HLA-DR and HLA-DP expression. However, our results do suggest that CIITA may preferentially regulate class II β-chains. For example, HLA-DQB mRNA was detected in only 2/5 CIITA negative cell lines but was detected in 5/6 CIITA positive cell lines. HLA-DQA mRNA was found in 2/5 CIITA negative cell lines and similarly in 3/6 CIITA positive cell lines. A similar phenomenon was observed for transcription of HLA-DR and HLA-DP α- and β-chains.

CIITA independent regulation of class II co-chaperones is also supported by our findings. Specifically, DMA and DMB were constitutively transcribed by all CIITA negative breast cancer cell lines and Ii was transcribed in 4/5 of these cell lines. Similarly, Chang et al. (1996) showed that HLA-2M and Ii genes were transcribed in splenocytes from CIITA deficient mice, but at reduced levels. Williams et al. (1998) described Ii protein in the thymus, spleen and lymph nodes of CIITA deficient mice. Sartoris et al. (1998) also reported constitutive Ii, DMA and DMB transcription in the absence of constitutive CIITA transcription in a pancreatic carcinoma cell line, CFPAC-1. Tai et al. (1999) have even described the molecular basis for CIITA independent Ii transcription. It is not surprising that factors other than CIITA may activate Ii and DM transcription since their promoters contain extra regulatory regions in addition to the characteristic W/S, X1, X2 and Y box class II promoter elements (Westerheide et al., 1997, Moore et al., 1998, Tai et al., 1999).

Intriguingly, constitutive HLA-DOA transcription was not detected in any cell line, regardless of CIITA expression. Following IFN-γ treatment, DOA mRNA was detected in 6/11 cell lines. This suggests that another factor, not present constitutively in breast carcinoma cells but inducible by IFN-γ in some cells, may act independently or in concert with CIITA to regulate HLA-DOA expression. HLA-DOB transcription is not regulated by CIITA (Taxman et al., 2000).

Our investigation and the others described above support the notion that CIITA is not a master regulator of class II and class II co-chaperone expression in all cells. However, CIITA may be required for optimal class II and class II co-chaperone transcription and

translation. There is evidence to suggest that other factors may activate class II transcription in the absence of CIITA. For example, CIITA deficient RJ2.2.5 cells expressed cell surface HLA-DR and HLA-DQ following transfection with activated *ras* genes (Hume et al., 1987).

4.3. Breast Carcinoma Cell Lines as Potential HLA Class II Restricted APCs

There is evidence suggesting that cancer cells may act as APCs (Ostrand-Rosenberg, 1994, Armstrong et al., 1998b, Armstrong et al., 1998c). Thus, our study examined whether breast cancer cells express components of the class II antigen presentation pathway and co-stimulatory molecules.

4.3.1. IFN-γ as an Inducer of Class II and Class II Co-chaperone Expression

IFN-γ was used since this agent is the most potent inducer of HLA class II and class II co-chaperones (Jabrane-Ferrat et al., 1990). As well, this factor may be released into the tumor microenvironment by tumor infiltrating lymphocytes such as CD4+ and CD8+ T cells and natural killer cells; thus it has a physiological role (Blanck, 1999). Based on previous work in our laboratory and preliminary research, we decided that cells would be stimulated with 500 units/ml IFN-γ for 96 hours. In fact, successful class II induction has been achieved using a broad range of IFN-γ concentrations from 10 units/ml to 1000 units/ml (Schwartz et al., 1985, Jabrane-Ferrat et al., 1990, Lu et al., 1994, Muczynski et al., 1998, Kooy et al., 1999, Walter et al., 2000). The 96 hour incubation period is longer than that utilized in most studies in which 48-72 hours are standard (Jabrane-Ferrat et al., 1990, Sedlak et al., 1992, Lu et al., 1994) but as shown in our study (section 3.1.1.2), the optimal time is also cell line

dependent.

4.3.2. HLA Class II and Class II Co-chaperone Expression in Breast Cancer Cell Lines

Our findings, summarized in Table 4.1, show that after treatment with IFN-γ, most breast cancer cell lines up-regulate the molecules involved in class II antigen presentation. These data both confirm and contradict the limited published data describing class II and class II co-chaperone expression in breast carcinoma cell lines (Table 4.2). Contradictory findings are not surprising considering that culture conditions and/or methodologies used in other studies often differed from those in our study (Table 4.2). As well, cultured cell lines may simply mutate over time. Thus, cell line passage number may influence the cell's ability to express different molecules.

4.3.2.1. HLA-DR

Our study found constitutive HLA-DR protein in MDA-MB-435, T-47D, MDA-MB-231 and BT-20 (Figure 3.8). Even though constitutive DRA and DRB transcription were detected in MCF7 and SK-BR-3, constitutive DR protein expression was not. Gene transcription in the absence of protein expression was frequently observed in our study. There are several potential explanations for this phenomenon including mRNA instability which could hinder accumulation of protein product or low levels of mRNA which could prevent efficient gene translation or gene mutations which could prevent successful gene translation or proper protein folding. Also, the assays used to assess gene transcription and protein expression had differences in their detection sensitivity *i.e.* PCR is very sensitive while flow cytometry is not as sensitive.

Table 4.1. Summary of HLA class II, class II co-chaperone and co-stimulatory molecule protein expression[a] on breast cancer cell lines.

Cell Line	Protein						
	HLA-DR	HLA-DP	HLA-DQ	Ii	HLA-DM	CD80/ CD86	CD40
MDA-MB-435	C[b]	C	I[c]	C	I	-[d]	C
T-47D	C	I	I	C	I	-	C
MDA-MB-231	C	C	-	C	C	-	C
BT-20	C	I	I	I	C	-	C
MCF7	I	I	I	C	I	-	-
SK-BR-3	I	I	I	I	I	-	C
MDA-MB-436	I	I	-	I	-	-	I
Hs578T	I	I	-	I	-	-	I
MDA-MB-157	I	I	-	I	-	-	-
BT-474	I	-	-	I	-	-	-
MDA-MB-468	I	I	-	I	I	-	-

[a] Data is based on flow cytometry findings

b Constitutive and IFN-γ induced expression observed.

c IFN-γ induced expression observed.

d No expression detected.

Table 4.2. Comparison of other studies analyzing HLA class II and class II co-chaperone expression in breast cancer cell lines.

Study	Cell Lines Analyzed	Culture Conditions	Methodologies	Results
Armstrong et al., 1998a	MDA-MB-435 MDA-MB-231 MCF7 SK-BR-3	• IMDM medium • Culture time not reported	• Immunofluorescence and flow cytometry using L243 (anti-HLA-DR), PIN.1 (anti-Ii) and polyclonal rabbit anti-DM	• MDA-435 48% DR positive; MDA-231 40% DR positive; MCF7 38% DR positive; SK-BR-3 17% positive • MDA-435 55% Ii positive; MDA-231 25% Ii positive; MCF7 33% positive; SK-BR-3 39% positive • MDA-435 49% DM positive; MDA-231 9% DM positive, MCF7 28% DM positive; SK-BR-3 22% DM positive
Sedlak et al., 1992	BT-20 MDA-MB-468 MCF7	• 500 U/ml IFN-γ • 72 hours • MEM medium	• Immunofluorescence and flow cytometry using Bra30 (anti-HLA-DR)	• All cell lines up-regulated HLA-DR
Nouri et al., 1992	T-47D MCF7	• 100 U/ml IFN-γ • 48 hours	• Radioimmunoassay using L243 (anti- HLA-DR)	• No constitutive HLA-DR protein • All cell lines up-regulated HLA-DR
Jabrane-Ferrat et al., 1990	T-47D MDA-MB-231 MCF7	• 1000 U/ml IFN-γ • 48 hours • DMEM medium	• Immunofluorescence and fluorescence microscopy using D1-12 (anti-HLA-DR), L2 and TU22 (anti- HLA-DQ) & B7-21 (anti-HLA-DP) • Northern blotting for mRNA • 2-D gel electrophoresis using VIC-Y1 (anti-Ii)	• No constitutive mRNA or protein • All cells up-regulated DR & DP • T-47D up-regulated DQ; MDA-MB-231 & MCF7 did not • DQA & DQB not transcribed in MDA-MB-231 and MCF7 • Ii up-regulated but not constitutive

Table 4.2. Continued.

Study	Cell Lines Analyzed	Culture Conditions	Methodologies	Results
Boyer et al., 1989	BT-20 MCF7 SK-BR-3	• 1000 U/ml IFN-γ • 48-72 hours • RPMI 1640 (BT-20, SKBR3) ; DMEM (MCF7)	• Radioimmunoassay using L243 (anti-HLA-DR)	• No constitutive HLA-DR • All cell lines up-regulated HLA-DR
Gastl et al., 1985	BT-20 MCF7 Hs578T	• 500 units/ml IFN-γ • 72 hours • MEM medium	• Immunofluorescence and fluorescence microscopy using VID-1 (anti-HLA-DR) and anti-DR from Becton Dickinson	• No constitutive HLA-DR • BT-20 90-100% HLA-DR positive; MCF7 & Hs578T <5% HLA-DR positive

Armstrong et al. (1998a) also detected constitutive HLA-DR on MDA-MB-435 and MDA-MB-231 but in contrast to our findings, they found constitutive expression on MCF7 and SK-BR-3 as well. However, other investigations have not reported constitutive DR expression by breast cancer cell lines (Table 4.2). Specifically, Gastl et al. (1985) and Boyer et al. (1989) did not detect constitutive DR protein in BT-20. HLA-DR protein nor mRNA were detected in T-47D, MDA-MB-231 and MCF7 (Jabrane-Ferrat et al., 1990). Constitutive DR protein was also not detected in T-47D by Nouri et al. (1992). In agreement with other studies (Jabrane-Ferrat et al., 1990, Sedlak et al., 1992, Nouri et al., 1992), we found strong HLA-DR up-regulation on T-47D, MDA-MB-231, BT-20, MCF7 and SK-BR-3. However, our findings disagreed with those of Gastl et al. (1985) who described minimal HLA-DR up-regulation in MCF7 and Hs578T. While MDA-MB-468 up-regulated HLA-DR the least in our study, Sedlak et al. (1992) reported higher levels of HLA-DR in MDA-MB-468 than MCF7. We also showed that MDA-MB-435, MDA-MB-436, MDA-MB-157 and BT-474 up-regulated HLA-DR (Figure 3.8) but no other studies reported such data.

4.3.2.2. HLA-DP

We observed constitutive HLA-DP cell surface expression only on MDA-MB-435 and MDA-MB-231 even though constitutive DPA and DPB transcription were also detected in BT-20, MCF7 and MDA-MB-157 (Figure 3.11, Table 3.3). Jabrane-Ferrat et al. (1990) is the only other group to describe HLA-DP expression in several of the cell lines examined in our study. Specifically, Jabrane-Ferrat et al. (1990) also reported constitutive HLA-DPB transcription in T-47D but unlike our study, this group did not detect constitutive HLA-DPB

mRNA in MDA-MB-231 or MCF7 nor HLA-DP protein in MDA-MB-231.

Following IFN-γ stimulation, we found that 10/11 breast cancer cell lines expressed cell surface HLA-DP (Figure 3.11). BT-474 did not express this class II antigen but transcribed DPA and DPB genes (Table 3.3). Coinciding with our data, Jabrane-Ferrat et al. (1990) also found that T-47D, MDA-MB-231 and MCF7 up-regulated HLA-DP protein.

4.3.2.3. HLA-DQ

Constitutive HLA-DQ cell surface expression was not detected on any breast cancer cell line even though MDA-MB-435, T-47D, BT-20, MCF7 and SK-BR-3 constitutively transcribed DQA and DQB genes (Figure 3.14, Table 3.4). Again, the study by Jabrane-Ferrat et al. (1990) was the only other describing HLA-DQ expression in cell lines used in our study. This group also reported the absence of constitutive HLA-DQ expression in T-47D, MDA-MB-231 and MCF7, but contrasting with our findings, they did not detect constitutive DQA and DQB transcription in T-47D and MCF7.

Following IFN-γ treatment, all cell lines transcribed DQA and DQB genes but only MDA-MB-435, T-47D, BT-20, MCF7 and SK-BR-3 expressed cell surface HLA-DQ (Table 3.4, Figure 3.14). Our data, therefore, confirm those of Jabrane-Ferrat et al. (1990) in that HLA-DQ protein was detected in T-47D and not in MDA-MB-231 but contradicting our findings, Jabrane-Ferrat et al. (1990) reported that MCF7 did not up-regulate HLA-DQ and that DQA and DQB were not transcribed by MDA-MB-231 and MCF7.

4.3.2.4. Invariant Chain

Even though we detected constitutive Ii transcription in all breast cancer cell lines

except Hs578T, intracellular protein expression was observed in only four cell lines, MDA-MB-435, T-47D, MDA-MB-231 and MCF7 using flow cytometry or IC (Table 3.5 and Figure 3.17). These data confirm those of Armstrong et al. (1998a) in that MDA-MB-435, MDA-MB-231 and MCF7 constitutively expressed Ii but unlike our study, Armstrong et al. (1998a) also detected constitutive Ii expression in SK-BR-3. In contrast to these findings, Jabrane-Ferrat et al. (1990) did not detect constitutive Ii protein in T-47D, MDA-MB-231 and MCF7.

Our data confirmed those of Jabrane-Ferrat et al. (1990) in that Ii was up-regulated by T-47D, MDA-MB-231 and MCF7. Our study also found that nine other breast carcinoma cell lines up-regulated Ii (Figure 3.17); findings that had not been previously published.

4.3.2.5. HLA-DM

While no cell line constitutively expressed intracellular HLA-DM, as assessed by flow cytometry, DM was detected in MDA-MB-435, MDA-MB-231 and BT-20 by IC (Figures 3.20, 3.21). Armstrong et al. (1998a) also detected constitutive DM in MDA-MB-435 but in contrast to our findings, they found constitutive DM in MCF7 and SK-BR-3 but not in MDA-MB-231.

Since Armstrong et al. (1998a) only reported constitutive DM expression in breast cancer cell lines, our study was the first to examine IFN-γ induced DM expression. While all breast cancer cell lines transcribed HLA-DMA and HLA-DMB genes in response to IFN-γ (Table 3.6), intracellular DM protein, as assessed by flow cytometry, was up-regulated by MDA-MB-435, T-47D, MDA-MB-231, BT-20, MCF7 and MDA-MB-468 (Figure 3.20).

However, using IC, DM was detected in all cell lines except BT-474 (Figure 3.22).

Notably, our study is the first to describe cell surface HLA-DM expression by breast carcinoma cells (see section 3.4.2.2). These novel findings may partially resolve discrepancies between intracellular flow cytometry and IC data (described above). Since surface DM was constitutively expressed by MDA-MB-231 and BT-20 and was up-regulated by SK-BR-3, it is speculated that IC revealed surface DM that was not detected by intracellular flow cytometry. Theoretically, intracellular flow should have detected surface DM but it is possible that the fixative or the detergent used in IC altered the DM epitope.

Other discrepancies between intracellular flow cytometry and IC are more difficult to interpret. For example, constitutive DM detected in MDA-MB-435 using IC was not detected by intracellular nor extracellular flow cytometry. When IC and intracellular flow cytometry were repeated simultaneously, HLA-DM expression was not detected by either method (data not shown). Similar observations were made for MDA-MB-436, Hs578T and MDA-MB-157 (see section 3.4.2.2). Notably, when DM expression was detected in these cells immunocytochemically, cells were grown in chamber slides. In other experiments analyzing DM expression (*i.e.* flow cytometry and IC using cytocentrifuge preparations), cells were trypsinized prior to the assay. This suggests that trypsin may have destroyed the DM epitope in certain cell lines. Future work necessitates repeating IC using chamber slides. Alternatively, discrepant findings may be clarified by an experiment assessing constitutive intracellular HLA-DM expression in MDA-MB-435 at 24-hour intervals for 120 hours. HLA-DM was detected at 24, 48, 72 and 120 hours but not at 96 hours (David Spurrell,

unpublished observations). This suggests that DM expression may be cyclic but further experiments are required to confirm this observation.

4.3.2.6. HLA-DO

HLA-DO expression in breast carcinoma cells had never been assessed prior to this study. While no cell line constitutively transcribed HLA-DOA, all cells except MCF7 transcribed HLA-DOB suggesting differential regulation of these genes. Indeed, it is known that DOA is regulated by CIITA while DOB is not (Taxman et al., 2000). Following IFN-γ stimulation, DOA transcription was detected in six cell lines while DOB was transcribed in all cell lines (Table 3.7). Notably, of all class II and class II co-chaperone genes, DOA was up-regulated the least suggesting differential regulation of DOA in breast carcinoma cells. HLA-DO protein expression was not determined but could be assessed using a commercially available anti-DO antibody and IC.

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

Interestingly, each breast cancer cell line expressed different levels of HLA class II and class II co-chaperones. Some cell lines constitutively expressed and strongly up-regulated class II *i.e.* MDA-MB-435, T-47D, MDA-MB-231 and BT-20 while others displayed comparatively weak class II up-regulation *i.e.* BT-474 and MDA-MB-468.

The above mentioned low expressors were susceptible to IFN-γ induction *i.e.* possessed IFN-γ receptors and components of the IFN-γ signaling pathway since they up-regulated class I (data not shown), class II and class II co-chaperone genes. However, IFN-γ

receptor expression could have drastically differed between cell lines thereby promoting variations in class II cell surface expression. This theory is supported by a study by Ucer et al. (1985) that demonstrated a relationship between the number of IFN-γ receptors on cancer cell lines and the concentration of IFN-γ required to up-regulate HLA-DR. Thus, cells with more IFN-γ receptors should up-regulate higher levels of HLA class II than cell lines with fewer receptors when cultured in 500 units/ml IFN-γ. Future studies could attempt to correlate class II up-regulation on breast cancer cell lines with the expression of IFN-γ receptors.

Prolactin has been implicated in inducing class II on non-neoplastic breast epithelium and on a breast cancer cell line, MCF7 (Klareskog et al., 1980, Bernard et al., 1986, Bartek et al., 1987). Ginsburg & Vonderhaar (1995) have reported that MCF7 and T-47Dco (clone of T-47D) secrete prolactin and possess prolactin receptors suggesting autocrine regulation of class II expression in these cells. Indeed, as discussed above, these cell lines constitutively transcribed DRA and DRB genes and T-47D constitutively expressed HLA-DR protein.

Another potential autocrine mediator of constitutive class II expression, epidermal growth factor (EGF), was shown to up-regulate *de novo* class II expression in breast cancer cell lines (Bernard et al.,1992). Martinez-Carpio et al. (1999) demonstrated that MDA-MB-231 secretes EGF and possesses EGF receptors and as stated above, this cell line constitutively expressed HLA-DR and HLA-DP. It is possible that autocrine mediated class II induction was more noticeable in our study given that our 96 hour incubation is longer than that used in most other studies (see section 4.3.1).

Oncogenes may also induce *de novo* class II expression. For example, when Albino et al. (1986) transferred viruses containing mutated (*i.e.* activated) *ras* genes into class II negative melanocytes, HLA-DR was up-regulated. Similarly, Hume et al. (1987) partially restored class II expression in a mutant Burkitt's lymphoma cell line, RJ2.2.5, upon infection of these cells with retroviruses containing activated *ras* genes. MDA-MB-231 has an activated *ras* gene (Kozma et al., 1987) but whether this cell line or others possess mutant *ras* genes capable of activating class II expression is not known.

Other factors secreted by breast cancer cell lines may have impeded efficient class II up-regulation. For example, TGF-β impairs class II expression by impairing CIITA mRNA accumulation (Lee et al., 1997, Dong et al., 2001). Several breast cancer cell lines including MDA-MB-468, MCF7, T-47D, MDA-MB-231, BT-20, SK-BR-3 and Hs578T secrete this factor (Zugmaier & Lippman, 1990, Arteaga et al., 1988). However, not all are TGF-β sensitive. Unfortunately, contradictory findings regarding TGF-β sensitivity have been reported (Arteaga et al., 1988, Reiss & Barcellos-Hoff, 1997, Pouliot & Labrie, 1999, Lynch et al, 2001) and suggest that TGF-β resistant clones are selected during routine cell culture (Lynch et al., 2001). Thus, while TGF-β is a candidate for class II down-regulation in breast cancer cell lines, future experiments would be required to elucidate whether these cells are TGF-β sensitive. Future studies could also characterize growth factors and cytokines secreted by breast cancer cell lines and deduce their involvement in class II regulation.

4.3.4. Differential Expression of HLA Class II Isotypes by Breast Cancer Cell Lines

In agreement with published data, HLA class II isotypes were differentially expressed on breast cancer cell lines. Most cell lines expressed higher levels of HLA-DR than HLA-DP. HLA-DQ was expressed at much lower levels or not at all. This pattern of differential class II expression has been described on breast carcinomas (Koretz et al., 1989, Moller et al., 1989, Jabrane-Ferrat et al., 1990) and other non-conventional APCs (Natali et al., 1986, Alcaide-Loridan et al., 1999, Redondo et al., 1999).

The above mentioned cases indicate that HLA class II isotype specific regulation exists and indeed several have been previously described. For example, Otten et al. (1998) demonstrated that low levels of CIITA induced class II cell surface expression in a non-coordinate manner in HeLa-derived HtTa cells *i.e.* HLA-DR and HLA-DP protein were strongly up-regulated while HLA-DQ was weakly expressed. This disparity disappeared when high levels of CIITA were present. Since HtTa cells with low levels of CIITA transcribed all class II genes, it was speculated that other factors regulated by CIITA were critical for inducing DQ cell surface expression (Otten et al., 1998, Alcaide-Loridan et al., 1999). Our study did not quantify mRNA but future research could attempt to associate levels of CIITA transcripts with DQ cell surface expression.

De Lerma Barbaro et al. (1994) suggested that a specific post-transcriptional mechanism controlled HLA-DQ expression but not HLA-DR or HLA-DP expression in interspecies somatic cell hybrids. These cells had stable DQA and DQB mRNA which were not translated or were not properly assembled given the lack of mature αβ heterodimers. Our

study did not examine post-transcriptional mechanisms regulating HLA-DQ in breast cancer cell lines but future studies could ascertain the presence of mature αβ heterodimers.

Several investigations have described distinct cell surface transport of HLA-DQ molecules (Alcaide-Loridan et al., 1999). For example, Maurer et al. (1987) examined the intracellular expression of class II isotypes in IFN-γ treated dermal fibroblasts. DR and DP were localized in discrete vesicles while DQ was dispersed throughout the cytoplasm suggesting differential intracellular transport of class II antigens (Maurer et al., 1987).

Muczynski et al. (1998) reported delayed cell surface expression of HLA-DP and HLA-DQ in an IFN-γ treated melanoma cell line, ThM. Cell surface HLA-DR was apparent after 48 hours of IFN-γ treatment while cell surface HLA-DP and HLA-DQ appeared after 1 to 2 weeks of culture. This delay was not due to impaired class II transcription since class II mRNA was detected following IFN-γ exposure for 48 hours. Similar observations were made in primary culture of kidney proximal tubular cells and a transformed kidney cell line (Muczynski et al., 1998). Similarly, Beaty et al. (1992) found that HLA-DQ requires a longer IFN-γ induction than HLA-DR for cell surface expression on primary skin fibroblasts.

Breast cancer cell lines may also exhibit delayed cell surface expression of HLA-DQ. For example, in one experiment, MDA-MB-231 was assayed for IFN-γ induced class II cell surface expression over time. While cell surface expression of HLA-DR appeared instantly (at time 0) and HLA-DP appeared at 24 hours, HLA-DQ expression was not apparent until 144 hours (data not shown). Future investigations could assess cell surface and intracellular HLA-DQ expression over time in other breast cancer cell lines to address whether these cells

exhibit delayed HLA-DQ intracellular transport.

One cell line, MDA-MB-468, expressed higher levels of HLA-DP than HLA-DR and no HLA-DQ. A similar pattern of class II expression was observed in a malignant B cell line, BALM-4 (Pesando et al., 1986). Although Pesando et al. (1986) did not elucidate the mechanism(s) responsible for this differential class II expression, different regulatory factors or processes were suggested.

4.3.5. Co-stimulatory Molecule Expression by Breast Carcinoma Cell Lines

4.3.5.1. CD80 and CD86

No breast cancer cell line constitutively expressed or up-regulated CD80 or CD86. This is not surprising given that most tumors of non-hematopoietic origin do not express these co-stimulatory molecules (Nieland et al., 1998). However, it was logical to assess CD80 and CD86 expression on breast carcinoma cells given their expression on gastrointestinal and hepatocellular carcinomas where they help induce anti-tumor immunity (Li et al., 1996, Tatsumi et al., 1997, Koyama et al., 1998).

4.3.5.2. CD40

As seen in Figure 3.25, CD40 expression was detected on MDA-MB-435, T-47D, MDA-MB-231, BT-20 and SK-BR-3 constitutively and was up-regulated on these cells plus two additional ones, MDA-MB-436 and Hs578T. These findings confirm those reported in other studies in that CD40 was constitutively expressed and up-regulated by T-47D, MDA-MB-231 and BT-20 and that no CD40 was detected on MCF7 (Wingett et al., 1998, Hirano et al., 1999, Alexandroff et al., 2000, Tong et al., 2001).

Oddly, some breast cancer cell lines were found to express CD40 by flow cytometry but not by IC (see section 3.5.2) suggesting that the CD40 antibody (clone 5C3) may not be suitable for IC. It is possible that acetone fixation or detergent used in IC altered the CD40 epitope. This speculation is supported by the observation that the positive control SAVC strongly expressed CD40 when assessed by flow cytometry but weakly expressed CD40 when assessed immunocytochemically (Figure 3.26). Especially disconcerting was that the CD40 antibody did not detect constitutive CD40 on breast carcinoma cells immunocytochemically. Perhaps trypsin treatment, required prior to flow cytometry, unmasked the CD40 epitope on non-induced breast cancer cells allowing its easy detection by flow cytometry.

4.3.6. Significance of Breast Carcinoma Cells as Potential HLA Class II Restricted APCs

Our findings show that breast carcinoma cells possess the antigen presentation molecules and the co-stimulatory molecule CD40 required to engage T cells and thus may potentially act as HLA class II restricted APCs. The next step is to determine whether these cells activate CD4+ T cells by measuring T cell proliferation in a mixed lymphocyte reaction.

Since we found that all breast carcinoma cell lines up-regulated Ii following IFN-γ treatment, their ability to present endogenous tumor antigens may be impaired. For example, mice injected with class II transfected SaI sarcoma successfully rejected this tumor. However, class II positive SaI transfected with Ii or CIITA were highly malignant (Clements et al., 1992, Armstrong et al., 1997). As well, Jiang et al. (1999) correlated high levels of Ii

with poorly differentiated colon carcinomas which suggested that Ii reduces tumor immunogenicity. Since one pivotal function of Ii is to inhibit peptide loading of the class II peptide binding site until class II localization to endosomes (Cresswell, 1996, Wubbolts & Neefjes, 1999), Ii may prevent loading and presentation of endogenous tumor antigens by tumor cells (Ostrand-Rosenberg, 1994, Armstrong et al., 1997, Xu et al., 2000). However, Loss et al. (1993) reported that saturating levels of Ii did not impair presentation of a class II restricted endogenously derived class I peptide in murine fibroblasts. Thus, whether Ii hinders class II mediated presentation of endogenous tumor antigens in breast tumors is not known but could be investigated further.

While this was the first study to identify cell surface DM on breast carcinoma cells, surface DM has been detected on B cells and immature dendritic cells. Here, it associates with empty class II molecules and facilitates loading of extracellular peptides (Santambrogio et al., 1999, Arndt et al., 2000). Extracellular DM may also facilitate peptide exchange on recycled class II molecules in early endosomal compartments (Arndt et al., 2000, Parhak et al., 2001). It is possible that cell surface DM on breast cancer cells has a role in extracellular antigen presentation or altering the peptide repertoire on recycling class II molecules. Further research could investigate these intriguing possibilities.

HLA-DO expression in breast carcinoma cells is a novel finding. The primary function of this co-chaperone in B cells, as described in section 1.6.3, is to modulate HLA-DM activity (Alfonso et al., 1999). Its role in cells other than B cells has not been elucidated. Interestingly, transfection of HLA-DO αβ dimers into a melanoma cell line, Mel JuSo,

drastically impaired HLA-DM mediated antigen presentation to T cell clones (van Ham et al., 1997). This suggests that DO may negatively regulate class II restricted antigen presentation in non-conventional APCs such as breast carcinoma cells. This intriguing possibility infers that tumor cells may be poor presenters of tumor antigen if they express HLA-DO.

Co-stimulatory molecules on APCs are required for T cell activation. In fact, antigen presentation in the absence of co-stimulation is thought to induce T cell anergy (Croft & Dubey, 1997). Our study found that CD40 was expressed on several breast carcinoma cell lines but that CD80 and CD86 were not. However, breast carcinoma cells not expressing CD80, CD86 and CD40 may still co-stimulate T cells given the existence of several other co-stimulatory molecules including CD70, OX40L, ICAM, 41BB-ligand and B7-h (Baskar, 1996, Watts & DeBenedette, 1999, Mueller, 2000). The requirement of co-stimulation for T cell activation has recently been challenged by Ochsenbein et al. (2001) and Zinkernagel (2001), who proposed that negative signals *i.e.* antigen presentation alone, do not regulate T cell activation. Instead, the dose of antigen, the time it is available and its localization within or outside lymphoid tissue are critical.

Class II restricted tumor antigen presentation by carcinoma cells may not necessarily activate CD4+ T cells. In fact, T cells may be anergized if class II presentation proceeds in the absence of co-stimulation (Croft & Dubey, 1997, Martin et al., 1999). The observation that class II expressing breast tumors tend to be associated with a favorable clinical outcome (Zuk & Walker, 1988, Brunner et al., 1991, Concha et al., 1991, Concha et al., 1995)

supports the idea that these tumors activate CD4+ T cells. Even when CD4+ T cells are not directly activated by tumor cells, they may be activated by conventional APCs (cross-presentation). These T cells may then engage class II expressing tumor cells and orchestrate their destruction (Armstrong et al., 1998a, Armstrong et al., 1998b, Hung et al., 1998, Pardoll & Topalian, 1998).

4.4. Selective Expression of HLA-DR by Breast Carcinoma Cell Lines

To our knowledge, this is the first study to report selective up-regulation of HLA-DR alleles by human cancer cell lines. These findings are summarized in Table 4.3. Even though selective down-regulation of HLA class I alleles is frequently observed in carcinomas (reviewed in Marincola et al., 2000), several factors have impeded the study of class II allele expression in cancers. The availability of class II allele specific antibodies was one factor. More importantly, the significance of CD4+ T cells in the anti-tumor immune response has only recently been acknowledged (Armstrong et al. 1998b, Hung et al., 1998, Topalian & Pardoll 1998).

4.4.1. Assay Limitations

Cell surface expression of HLA-DR allelic products was assessed using HLA-DR specific antibodies and flow cytometry. An HLA-DR type was deemed expressed when the test antibody mean fluorescence was more than two times that of the negative control. When assessing protein expression in this manner, several limitations must be considered. For example, primary antibodies bind antigens with different affinities. Thus, low expression of a particular HLA-DR type could have been due to poor antibody binding rather than poor

Table 4.3. Selective expression[a] of HLA-DR by breast cancer cell lines.

DR Type	Cells on which allotypes up-regulated	Cells on which allotypes weakly up-regulated or not up-regulated
DR1	T-47D	Hs578T, MDA-MB-468
DR15	MCF7, Hs578T, MDA-MB-157, BT-474	-
DR3	MCF7, MDA-MB-436	-
DR4	MDA-MB-435, BT-20	MDA-MB-157, BT-474
DR13	MDA-MB-436, MDA-MB-231, BT-20, SK-BR-3	-
DR7	MDA-MB-231, SK-BR-3	MDA-MB-468
DR51	ND[b]	ND
DR52	MDA-MB-435, MDA-MB-231, BT-20, MCF7, SK-BR-3	MDA-MB-436
DR53[b]	MDA-MB-435, BT-20	MDA-MB-157, BT-474, MDA-MB-468

[a]Cell surface expression was determined using HLA-DR allele specific antibodies and flow cytometry.
[b]Not determined since we did not have a DR51 specific antibody.
[c]DR53 expression could not be assessed in DR7 expressing cell lines since PL3, the antibody used to detect DR53, also recognized DR7.

expression of a HLA-DR type. However, binding of mAbs to positive control B cell lines was a control for this possibility and mAbs were used at saturating concentrations. Similarly, the secondary antibody may bind primary antibodies with differing affinities depending on the primary antibody isotype. One way to control for this problem is to directly label primary antibodies with fluorescent tags. Unfortunately, this procedure often destroys antigen recognition by primary antibodies (Drover, personal communication).

Further, even though HLA-DR specific antibodies bound positive control B cell lines, this does not absolutely mean that they will bind DR molecules expressed by breast cancer cells. HLA-DR molecules in different cell types may have different patterns of glycosylation or may bind different peptides so antibodies may bind certain HLA-DR conformations better than others (Drover et al., 1994a). It is important to note, however, that the mAbs used in this study were selected because they bound to HLA-DR molecules on many cell types. Additionally, each mAb bound to one or more breast cancer cell line indicating their suitability for binding HLA-DR on breast carcinoma cells.

Thus, despite possible limitations, our method of analyzing expression of HLA-DR allelic products on breast cancer cells is effective. Future research could use quantum beads to quantitatively determine the actual number of HLA-DR molecules on a cell's surface. The expression of a particular HLA-DR allele could then be assessed based on the number of HLA-DR molecules required for successful T cell engagement.

4.4.2. Findings

HLA class II typing revealed a total of nine different HLA-DR genes in the eleven breast carcinoma cell lines. HLA-DR specific antibodies, used to assess DR protein expression, were shown to strongly bind HLA-DR allelic products using the appropriate DR expressing B cell line controls (Table 3.9). Thus, failure to detect a particular HLA-DR type was most probably due to its lack of expression rather than weakly reactive antibodies. However, DR specific mAbs may not have detected expression of a particular DR type if expression levels were markedly reduced.

Constitutive cell surface expression of HLA-DR allelic products using HLA-DR specific mAbs was observed in only one cell line, MDA-MB-435. However, constitutive HLA-DR expression was detected in three additional cell lines, T-47D, MDA-MB-231 and BT-20, using the pan anti-DR antibody L243. T-47D typed positive for only one HLA-DR allele, DRB1*0102, suggesting that L243 has a higher affinity for HLA-DR1 than the HLA-DR1 specific mAbs, NFLD.D10, JS-1 and NFLD.D2.

Alternatively, this cell line could have constitutively expressed another HLA-DR type not detected by the HLA class II DNA typing kit. Since MDA-MB-231 and BT-20 had four HLA-DR alleles it is possible that low levels of HLA-DR allelic products were undetectable using HLA-DR allele specific antibodies but were detectable using L243 since this antibody recognizes all DR types collectively. Another possibility, although unlikely, is that L243 was recognizing something other than HLA-DR on these cells.

Several intriguing findings were made regarding up-regulation of HLA-DR allelic products by breast carcinoma cell lines. Some HLA-DR alleles were consistently up-regulated regardless of the cell line on which they were expressed. For example, MCF7 and MDA-MB-436 (2/2 cell lines) which typed positive for HLA-DRB1*03, up-regulated DR3. MDA-MB-435, BT-20, MDA-MB-231 and SK-BR-3 (4/4 cell lines) which typed positive for HLA-DRB1*13, up-regulated cell surface DR13. Similarly, MCF7, Hs578T, MDA-MB-157 and BT-474 (4/4 cell lines), which typed positive for HLA-DRB1*15, up-regulated this HLA-DR type. In contrast, other HLA-DR types were not up-regulated or were weakly up-regulated by certain breast cancer cell lines (discussed below). Biologically, failure to up-regulate a particular HLA or even its weak up-regulation may inhibit a tumor specific T cell response (Costello et al., 1999).

HLA-DRB1*01 protein was not up-regulated by Hs578T nor by MDA-MB-468 (2/3 cell lines). Notably, MDA-MB-468 failed to up-regulate any HLA-DR allelic product (*i.e.* DRB1*01, DRB1*07 or DRB4) as assessed using HLA-DR specific mAbs even though DR up-regulation was observed using L243. This suggests that levels of individual DR allotypes were too low for detection by DR specific mAbs but that L243 bound all three DR types collectively.

T-47D strongly up-regulated DRB1*01. The antibodies used to assess DR1 expression on T-47D seemed to bind DR1 with differing affinities with NFLD.D10 showing the highest binding and NFLD.D2 showing the lowest (Figure 3.29). The relatively low DR1

binding by NFLD.D2 may be explained by the fact that this antibody is peptide dependent (Drover et al., 1994b).

Of the four cell lines that typed positive for HLA-DRB1*04, only one cell line, MDA-MB-435, strongly up-regulated cell surface HLA-DR4. Interestingly, DR4 expression on BT-20 varied considerably depending on the antibody used to assess it. Weak DR4 up-regulation was detected using NFLD.D1 and NFLD.M1 while strong DR4 up-regulation was assessed using NFLD.D2 and NFLD.D10 (Figure 3.31). Since NFLD.D10 may weakly bind DR53 (Drover, personal communication), its assessment of DR4 expression may have been an overestimate. On the other hand, NFLD.D2 assessment of DR4 expression may have been an underestimate since the NFLD.D2 epitope is peptide dependent (Drover et al., 1994b). Notably, NFLD.D1 and NFLD.M1 binding to DR4 on the B cell line control MT14B was two-fold less than NFLD.D2 and NFLD.D10 binding suggesting that NFLD.D1 and NFLD.M1 affinity for DR4 was less than that of NFLD.D2 and NFLD.D10. However, since NFLD.D1 and NFLD.M1 binding to DR4 on BT-20 was three times less than NFLD.D2 binding and eleven times less than NFLD.D10 binding, antibody affinity cannot be the sole explanation for differences in mAb binding to DR4 on BT-20.

A closer look at the epitopes recognized by the DR4 specific antibodies is informative. NFLD.D1 and NFLD.M1 bind epitopes in the DRβ2 domain *i.e.* the membrane proximal domain (Drover et al. 1994a, Fu et al. 1995). In contrast, NFLD.D10 and NFLD.D2 recognize overlapping epitopes in the DRβ1 domain (Drover et al. 1994b). Thus,

it is possible that weak NFLD.D1 and NFLD.M1 binding to cell surface DR4 was caused by a β2 domain mutation that conformationally altered epitopes recognized by these mAbs.

Alternatively, it is possible that cell surface HLA-DM on BT-20 (see section 3.4.2.2) hindered formation of the epitope recognized by NFLD.D1 (Drover, personal communication).

Another more probable explanation is that β2 domain epitopes were partially masked from antibody recognition on BT-20's cell surface. Antigen masking may be the consequence of an overabundant glycocalyx. In fact, thick glycocalyx have been described on normal human mammary tissue and on primary tumors (Kim et al. 1975, Ghosh et al. 1981). Similarly, the epithelial mucin MUC-1 is known to inhibit cell adhesion by masking cell surface antigens. Since BT-20 expresses an abundance of this polysaccharide (Walsh et al. 1999), it is possible that recognition of membrane proximal HLA-DR epitopes was hindered on these cells due to MUC-1 over-expression.

This theory is supported by several observations. Firstly, NFLD.M1 also recognizes HLA-DR52 and thus should have bound both DR4 and DR52 on BT-20 (Table 2.4). However, as stated above, only weak binding of this antibody was observed. This weak binding in view of strong binding by another DR52 mAb (7.3.19.1) supports the idea that β2 domain epitopes were masked.

Additionally, analysis of intracellular DR4 expression by IC determined that ~60% of BT-20 were DR4 positive using NFLD.D1; ~50% of BT-20 were DR4/DR52 positive using NFLD.M1; ~35% of BT-20 were DR4 positive using NFLD.D2 and ~70% of BT-20

were DR4 positive using NFLD.D10. Low NFLD.D2 binding could be attributed to NFLD.D2 epitope alteration by the fixative acetone (Drover, personal communication).

Taken together, these data suggest that recognition of β2 domain epitopes on BT-20's cell surface were hindered. Since the T cell co-receptor CD4 interacts with residues in the β2 domain (Cammarota et al. 1992, Brogden et al. 1998), inhibition of this binding could deleteriously alter CD4 binding and impair T cell engagement mediated by all class II molecules on BT-20. Future research could strip polysaccharides and/or mucins from BT-20 and re-assess cell surface HLA class II allele expression. For example, O-gylcosidase removes O-linked glycans (Garner et al., 2001) such as those attached to the mucin backbone (Bergeron et al., 1997). Additionally, a DR4 specific antibody recognizing a β1 domain epitope could be used to re-examine DR4 up-regulation on this cell line.

Two additional breast cancer cell lines, MDA-MB-157 and BT-474, did not up-regulate intracellular nor cell surface HLA-DR4 following IFN-γ treatment. This finding was confirmed by two different mAbs, NFLD.D1 and NFLD.M1 and by three different assays, extracellular and intracellular flow cytometry and IC.

Three cell lines typed positive for HLA-DRB1*07, MDA-MB-231, SK-BR-3 and MDA-MB-468. As discussed above, MDA-MB-468 did not up-regulate this HLA-DR type but moderate DR7 up-regulation was detected on MDA-MB-231 and SK-BR-3.

Of the six cell lines which typed positive for HLA-DRB3, MDA-MB-435, BT-20, MDA-MB-231 and SK-BR-3 strongly up-regulated cell surface DR52 and MCF7 moderately up-regulated this DR antigen. As described above, NDS13, the antibody used to characterize

DR52 expression by MCF7, bound DR52 constitutively on this cell line. This is improbable, however, since DRB3 mRNA was not constitutively transcribed in MCF7. The exact epitope recognized by NDS13 has not been mapped. Its specificity as determined by binding to B cell lines is HLA-DRB3, DRB1*03, DRB1*08, DRB1*11, DRB1*12, DRB1*13 and DRB1*14 (Fuggle et al., 1987). Since B cell lines expressing DR3, DR11, DR12, DR13 and DR14 also express DR52, it is probable that NDS13 recognizes DR52 exclusively or together with another DRB1 type (Fuggle et al., 1987) or together with another unidentified molecule. These observations challenge whether DR52 is truly up-regulated by MCF7. Unfortunately, another antibody capable of assessing DR52 expression by MCF7 was not available for our study but could be obtained for future work.

HLA-DR52 was weakly up-regulated by MDA-MB-436 as assessed using NFLD.D7 and NDS13. Immunocytochemical analysis of DR52 expression using NFLD.D7 confirmed this weak DR52 up-regulation. However, using NDS13, no DR52 protein was detected. As described in section 3.3, the epitope recognized by NDS13 may have been altered by acetone fixation or by detergent used in IC.

Seven breast carcinoma cell lines typed positive for HLA-DRB4 (DR53). Cell surface HLA-DR53 was strongly up-regulated by MDA-MB-435 and BT-20. In contrast, MDA-MB-468 (see above), MDA-MB-157 and BT-474 did not up-regulate cell surface DR53. While no intracellular DR53 was observed in BT-474 and MDA-MB-468, weak intracellular DR53 expression was detected in MDA-MB-157. This may simply reflect differences in the sensitivity of these two assays. Alternatively, this could suggest that cell

surface transport of DR53 was impeded in MDA-MB-157. Perhaps, misfolded DR53 protein was retained in the ER or Golgi (Dusseljee et al., 1998).

It was not possible to detect protein expression of all HLA-DR types in breast carcinoma cell lines (see section 3.7.3.1) since DR53 protein expression could not be assessed in DR7 expressing cell lines and DR51 protein expression could not be determined in any cell line. Thus, it is possible that additional HLA-DR types were not up-regulated or were weakly up-regulated by these cells.

4.4.3. Mechanisms Potentially Implicated in Selective HLA-DR Expression

To accurately assess HLA-DR expression on breast cancer cells, it is important to consider whether cells not expressing or weakly expressing HLA-DR alleles do so as a result of naturally occurring HLA-DRB promoter polymorphism or as a result of tumorigenesis. The class II promoter is characterized by a conserved set of *cis*-acting regulatory elements which include the W/S box, the X1 box, the X2 box and the Y box (van den Elsen et al. 1998). Sequencing of numerous HLA-DRB allele promoters has revealed sequence variations within X and Y boxes and spacing differences between regulatory elements (Emery et al. 1993, Perfetto et al. 1993, Singal et al. 1993, Louis et al. 1994, Singal & Qiu 1995). These variations alter binding of transcription factors and thus alter gene transcription (Louis et al. 1994, Singal & Qiu 1995).

Using CAT reporter assays, it has been found that DRB promoters have different transcriptional activities (Emery et al. 1993, Louis et al. 1994, Singal & Qiu 1995). For the most part, these transcriptional activities correlate with levels of steady state DRB mRNA

(Vincent et al., 1994). Since DRB3 and DRB1*01 promoters have high transcriptional activity (Louis et al., 1994), the weak up-regulation of DR52 protein detected in MDA-MB-436 and the lack of DR1 protein in Hs578T were most likely not attributable to naturally occurring promoter polymorphisms. Despite observations that DRB1*04 promoters have low transcriptional activity (Louis et al. 1994), Vincent et al. (1996) observed a relatively high steady state level of DRB1*04 mRNA. Thus, whether poor promoter activity contributed to the lack of DR4 up-regulation in 2/4 breast cancer cell lines is not known. Louis et al. (1994) have also shown that DRB4 promoters have low transcriptional activity. Thus, it is possible that cell lines not expressing or weakly DR53 did so as a result of naturally occurring promoter polymorphism.

Mechanisms responsible for the lack of HLA-DR up-regulation by breast cancer cell lines were not elucidated. However, when DR protein was not up-regulated, DRA and DRB genes were transcribed in response to IFN-γ. Interestingly, HLA-DRB transcription, assessed using HLA-DRB generic primers coincided with DRB transcription assessed using allele specific primers with two notable exceptions. Non-induced MDA-MB-157 did not transcribe DRB as assessed using generic DRB primers but did transcribe DRB4 mRNA. Similarly, non-induced MDA-MB-468 did not transcribe DRB as assessed using generic DRB primers but did transcribe DRB1*01 mRNA. Perhaps, the allele specific primers were better at amplifying DRB genes than the generic DRB primer.

There are several possible explanations as to why certain DR allelic products were not up-regulated. For example, since primers used to assess DR allele transcription were

specific for sequences within exon 2, it is possible that mutations resulting in premature stop codons in other exons or that gene deletions prevented successful transcription of the entire DR gene. These phenomena has been implicated in class I allele down-regulation in cervical cancers (Koopman et al., 1999, Brady et al. 2000, Koopman et al., 2000). For example, the lack of HLA-A2 expression in the cervical cancer cell line 808 was caused by a point mutation in exon 3 which encoded a premature stop codon (Brady et al., 2000). Alternatively, mutations in splice sites within introns may prevent proper mRNA processing. Aberrant mRNA splicing was determined to be the mechanism responsible for selective loss of HLA-A2 in 624MEL28 melanoma cells (Wang et al., 1999). Future research could sequence DR genes not up-regulated by breast cancer cells to identify putative gene mutations.

Inherited mutations in some HLA-DRB4 alleles may prevent their translation. One such null allele, HLA-DRB4*0103102N, has been found in association with DRB1*0401, DQB1*0301 positive individuals (Voorter et al. 2000). Since BT-474 typed positive for DRB1*0401 and DQB1*03, the presence of this B4 null allele could explain the lack of DR53 expression in this cell line. Using sequence specific primers, a future study could determine whether BT-474 has a DRB4 null allele.

Cancer induced mutagenesis of regulatory elements in the HLA-DRB promoter could have hindered DRB transcription such that mRNA levels were too low for efficient gene translation. This is plausible since sequence variations in class II regulatory regions influence binding of TFs and thus influence gene transcription (Louis et al. 1994, Singal &

Qiu 1995). Other possible mechanisms impeding up-regulation of HLA-DR allelic products could include instability of HLA-DRB mRNA or rapid degradation of HLA-DR protein.

Finally, while shedding of HLA class II molecules by cancer cells has been described, selective shedding of class II antigen has not (Ziai et al., 1985, Maio et al., 1989). However, selective secretion of class II antigens has been demonstrated. Briata et al. (1986) described B cell lines that secreted HLA-DQB. Of four cell lines analyzed for DQB secretion, 2/4 secreted DQB, suggesting allele specific secretion. These cell lines displayed alternative splicing of DQB which deleted DQB's transmembrane portion and resulted in its secretion. All of these cells produced normal DQB transcripts as well. Thus, it is possible that HLA-DR allelic products were selectively shed or selectively secreted. To investigate these possibilities, future experiments could assess cell line supernatant for DR allelic products.

4.4.4. Significance of Selective HLA-DR Expression in Breast Carcinoma Cells

As stated above, selective expression of HLA alleles is not without precedence, since selective loss of HLA class I alleles occurs frequently in human cancers including breast carcinomas (Perez et al. 1986, Moller et al. 1989, Concha et al. 1991a, Moller & Hammerling 1992, Cabrera et al. 1996, Hicklin et al. 1999, Algarra et al. 2000, Marincola et al. 2000). The biological relevance of class I allele down-regulation is inferred by the observation that metastases have a higher frequency of class I allele loss than primary tumors (Tait, 2000). Furthermore, several investigations have linked loss of class I allele expression with an unfavorable clinical outcome. For example, van Driel et al. (1996) reported that the loss of HLA-A2 expression in cervical carcinomas was associated with accelerated disease

progression. The characterization of HPV peptides capable of binding HLA-A2 (Yoon et al., 1998, Rudolf et al., 2001) and HLA-A2-restricted HPV peptide-specific CTLs (Evans et al., 1997, Yoon et al., 1998) support the involvement of HLA-A2 in the immune response towards HPV-induced cervical carcinoma. Additionally, the loss of HLA-A2 expression in melanoma cells was shown to prevent HLA-A2-restricted cytotoxic T cell (CTL) recognition of these cells. Intriguingly, re-expression of HLA-A2 in these melanoma cells partially restored immunological recognition by CTLs (Pandolfi et al., 1991).

The physiological importance of selective HLA-DR allele expression in breast carcinomas is not known. In fact, the significance of HLA-DR expression by breast carcinomas remains controversial. While several investigations have correlated HLA-DR expression with well differentiated breast tumors and favorable prognosis (Zuk & Walker, 1988, Brunner et al., 1991, Concha et al., 1991b, Concha et al., 1995) others have found no such correlation (Moller et al., 1989, Wintzer et al., 1990, Lucin et al., 1994, Maiorana et al., 1995). Since these studies assessed DR expression using pan anti-DR antibodies, it is not known whether breast carcinomas up-regulated all HLA-DR types or select DR types. We propose that class II positive tumors not associated with a favorable clinical outcome did not up-regulate the HLA-DR allele that was particularly adept at presenting tumor antigen. This theory is now supported since selective HLA-DR up-regulation was documented in our study.

The implication that HLA-DR positive carcinomas are associated with a favorable clinical outcome suggests that these tumors are recognized and eradicated by CD4+ T cells.

Indeed, evidence of HLA class II restricted tumor antigen presentation exists (see section 1.7.3). Therefore, if like class I allele loss, lack of class II allele expression impairs T cell recognition, then it may represent another mechanism by which tumors evade immune surveillance. It is intriguing to speculate that failure to up-regulate a particular HLA-DR type by breast cancer cell lines was the result of selective immunological pressure encountered *in vivo*. For example, one of the cell lines in our study, BT-474, that did not up-regulate DR4, has been shown to over-express Her-2/neu (Brodowicz et al., 1997). Since DRB1*04 is known to present Her-2/neu peptides (Kobayashi et al., 2000), its lack of expression may have allowed this tumor to evade a Her-2/neu specific CD4+ T cell immune response.

This study revealed that certain breast carcinoma cell lines did not up-regulate several different HLA-DR types (see Table 4.3). More subtle changes in DR expression on tumor cells may also impede their recognition by CD4+ T cells. For example, DR52 was weakly up-regulated by MDA-MB-436. As stated previously, failure to up-regulate HLA or even its weak up-regulation may impair the tumor antigen-specific T cell response (Costello et al.,1999). Further, CD4+ T cell recognition of class II molecules on BT-20 may be hindered by polysaccharides or mucins. Since one DR allotype was not consistently absent in breast cancer cells, it suggests that different tumors require different class II molecules for tumor antigen presentation. This is not surprising given that multiple tumor antigens exist (Renkvist et al., 2001, Scanlan & Jager, 2001).

To ensure that selective HLA-DR up-regulation is not a phenomenon restricted to cultured breast carcinoma cell lines, ongoing work in our laboratory is assessing DR allele

expression *in situ*. Since *in situ* tumor cells are under more selective immunological pressure than *in vitro* established cell lines (Ferrone & Marincola, 1995), it is predicted that a higher frequency of selective DR allele up-regulation will be observed.

4.5. Summary and Potential Implications of Findings

Some breast carcinoma cell lines constitutively expressed HLA class II, class II co-chaperones and the co-stimulatory molecule CD40. Whether the expression level of these molecules would be sufficient to engage CD4+ T cells is not known. CIITA independent regulation of constitutive HLA class II and co-chaperone expression was described in our study and may provide insight into different mechanisms of class II regulation in non-conventional APCs.

Following stimulation with IFN-γ, most cell lines up-regulated class II, co-chaperones and the co-stimulatory molecule CD40. This has physiological significance since IFN-γ, along with other immunomodulators, may act *in situ*. Again, it is not known whether these tumor cells would engage CD4+ T cells. However, since several studies have shown that class II positive tumors are associated with a favorable clinical prognosis (Zuk & Walker, 1988, Brunner et al., 1991, Concha et al., 1991b, Concha et al., 1995), it suggests that breast tumors can engage T cells. However, there are several mechanisms by which tumors escape immune recognition. For example, interaction of HLA class II expressing tumors with CD4+ T cells in the absence of concomitant co-stimulation may anergize tumor specific T cells (Croft & Dubey, 1997, Martin et al., 1999). Also, Ii expression may hinder an effective HLA

class II restricted anti-tumor immune response. Presumably, Ii blocks the class II peptide binding groove thus preventing binding of endogenous tumor peptides (Ostrand-Rosenberg, 1994, Armstrong et al., 1997, Xu et al., 2000). Notably, in our study, all class II positive breast carcinoma cell lines also expressed Ii.

For the first time, our study has shown that breast carcinoma cells may differentially express HLA-DR allotypes. Thus, it may be that tumors evade immune recognition by not expressing a particular HLA class II allele adept at tumor antigen presentation. Class I allele down-regulation is common in breast tumors and it has been associated with a poor clinical outcome (Concha et al. 1991a, Cabrera et al. 1996, Algarra et al. 2000, Marincola et al. 2000). Increased understanding of the immune response towards tumors and the mechanisms by which tumors escape this response may be critical for effective immunotherapies.

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APPENDIX A: SOLUTIONS

10x PBS (Phosphate Buffered Saline)

80 g sodium chloride (BDH Inc., Toronto, ON), 2 g potassium chloride (Fisher Scientific, Fair Lawn, NJ), 14.4 g di-sodium hydrogen orthophosphate anhydrous (BDH Inc., Toronto, ON), 2.4 g potassium dihyrdogen phosphate (Fisher Scientific) were dissolved in distilled water (800 ml) and the final volume was corrected to 1000 ml using distilled water. The pH was adjusted to 7.4 using 1N NaOH. 1x PBS was made by diluting 10x PBS 1/10 in distilled water.

4% Paraformaldehyde

8 g paraformaldehyde (Sigma, St. Louis, MO) was added to 100 ml distilled water. The mixture was heated until dissolved and three drops of 1 N NaOH was added to clear the solution. After cooling to room temperature, 4% paraformaldehyde was made by adding 100 ml 2x PBS. The pH was adjusted to approximately 7.4 using 1N NaOH. To make 1% paraformaldehyde, 4% paraformaldehyde was diluted 1/4 in 1x PBS.

10x TBE

53.9 g Tris base (Life Technologies, Gaithersburg, MD) and 3.72 g EDTA (Sigma) were combined in 400 ml distilled water. Slowly, 27.5 g boric acid were added. The final volume was adjusted to 500 ml using distilled water. The pH was adjusted to 8.3 using 1N NaOH. To make 0.5x TBE, 10x TBE was dissolved 1/20 in distilled water.

Loading Buffer

10 ml 50% glycerol in water, 0.01 g bromophenol blue (J.T. Baker Chemical, Phillipsburg, NJ) and 0.01 g xylene cyanol were combined. Loading buffer was stored at -20°C until required.

Mayer's Hematoxylin

1 g haematoxyin (BDH Inc.), 0.2 g sodium iodate (Sigma) and 50 g potassium alum (Sigma) were dissolved overnight in 1000 ml distilled water. 1.09 g citric acid (BDH Chemicals, Toronto, ON) and 50 g chloral hydrate (BDH Laboratory Supplies, Poole, England) were added to the above mixture. The solution was boiled for 5 minutes and cooled to room temperature.

Scott's Tap Water

7 g sodium bicarbonate (Mallinckrodt, Paris, KY) were added to 1 L tap water and 7 g magnesium sulfate 7-hydrate (J.T. Baker Chemical) were added to 1 L tap water. These solutions were combined 1:1 to make Scott's Tap Water.

DEPC Water

0.1% diethyl pyrocarbonate (DEPC) (Sigma) was dissolved in distilled water and incubated for 6 hours at room temperature. The solution was then autoclaved for 20 minutes.