Effect of Human Epidermal Growth Factor Receptor 2 signalling on the IFN-γ pathway in Luminal B Breast Cancer

by © Louisa L. Wiede B.Sc.

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Abstract

Previous studies showed luminal B (Human epidermal growth factor 2 +(HER2+) Estrogen receptor + (ER+) human breast carcinomas, compared to other breast cancer subtypes, are deficient in human leukocyte antigen (HLA) expression. Similarly, representative breast cancer cell line BT-474 (HER2+ER+) exhibited significantly reduced IFN-γ-inducible HLA expression, as compared to MCF-7 (HER2-ER+) and SKBR3 (HER2+ER-). Therefore, we hypothesized hyperactivation of HER2 signalling in ER+ breast cancer may interfere with interferon gamma (IFN-γ) pathway activation. To test this hypothesis, time course studies, targeted inhibition of HER2 (lapatinib or trastuzumab+pertuzumab) and ER (tamoxifen and ICI) and small interfering RNAs (siRNAs) targeting HER2 (ERBB2) and/or ER (ESR1) pathways were performed. Changes in IFN-γ pathway components, HER2, ER and HLA Class I in BT-474 and SKBR3, were assessed by flow cytometry and western blotting. Kinetic studies showed no clear inverse correlation between HER2 levels and surface HLA Class I while HER2 inhibition or knockdown resulted in reduced surface interferon gamma receptor 1 (IFNGR1) and HLA Class I. Neither phosphorylated signal transducer and activator of transcription (pSTAT-1) nor interferon regulatory factor 1 (IRF-1) were significantly altered in BT-474 or SKBR3. The results suggest expression levels and/or function of IFN-γ pathway proteins may be dysregulated, likely independent of HER2 overexpression, resulting in weak IFN-γ signalling in BT-474 (HER2+ER+) and subsequent poor HLA Class I upregulation.

General Summary

Breast cancer (BC) is the most commonly diagnosed cancer in women worldwide. BC is typically tested for two factors that help them grow, estrogen receptor (ER) and Human Epidermal Growth factor receptor 2 (HER2). HER2 is often produced at higher amounts in BC than healthy cells, which is called HER2 overexpression.

Previous work in our laboratory discovered that BC tissues positive for HER2 and ER (HER2+ER+) possibly expressed dysfunctional small proteins called interferon gamma (IFN-γ), which help the body to form an immune response against disease including cancer by activating other proteins.

Here, we investigated the influence of HER2 overexpression on IFN-γ functionality in HER2+ER+ BC and found that HER2 is likely not responsible for IFN-γ dysfunction in these cells.

We concluded that the weak immune response in HER2+ER+ BC might be, in part, due to mutations affecting functionality of proteins involved in immune responses activated by IFN-γ.

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Finally, I would like to thank my family for their endless love and support from across the Atlantic Ocean throughout my academic endeavours. I would like to dedicate this thesis to the loving memory of my grandpa (Opa), Erwin Wiede – until we meet again.

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List of Abbreviations

%:	Percentage
%+:	Percentage of positive
+:	Positive
4EBP1:	Eukaryotic initiation factor 4E-binding protein 1
A/A:	Antibiotic/antimycotic
AA:	Amnio acid
aCARD:	Atypical caspase activation and recruitment domain
AD:	Autosomal dominant
ADCC:	Antibody-dependent cell-mediated cytotoxicity
AF:	Activation function
AIB:	Amplified in breast cancer
AKT:	Serine/threonine-protein kinase
AP:	Activator protein
APC:	Antigen presenting cell
APM:	Antigen processing machinery
APS:	Ammonium persulfate

AR:	Autosomal recessive
ATCC:	American Tissue Culture Collection
ATF1:	Activating transcription factor 1
ATP:	Adenosine triphosphate
β_2M:	β_2-microglobulin
BCA:	Bicinchoninic acid
BCCL:	Breast cancer cell line
BCL-2:	B-Cell Leukemia/Lymphoma 2
BIM:	BCL-2 interacting mediator of cell death
BSA:	Bovine serum albumin
cAMP:	Cyclic adenosine monophosphate
CDK:	Cyclin-dependent kinase
CDK:	Cyclin-dependent kinase
cDNA:	Complementary deoxyribonucleic acid
CIITA:	Class II transactivator
CITA:	Class I transactivator
CM:	Complete medium

CREB:	cAMP response element binding
CRT:	Calrecticulin
CTL:	Cytotoxic T cells
CYP2D6, CYP3A, CYP2C:	Hepatic cytochrome P450 enzymes
D:	DMSO *referred to in Figure 3.7 and 3.8
DBD:	DNA binding domain
DCCR:	DharmaFECT cell culture reagent
dH_2O:	Distilled H_2O
DMSO:	Dimethyl sulfoxide
DUX:	Double Homeobox
E:	Ethanol*referred to in Figure 3.7 and 3.8
E1, E2, E3, E4:	Ubiquitination-facilitating enzymes
$E_{2:}$	Estradiol
EDTA:	Ethylenediaminetetraacetic acid
EGFR:	Epidermal growth factor receptor
ER:	Estrogen receptor

ER:	Endoplasmic reticulum*only referred to in Section 1.2.2 and Figure 1.1
ERAP:	Endoplasmic reticulum aminopeptidase
ERBB:	Epidermal growth factor receptor * gene nomenclature of HER
ERE:	Estrogen response element
ERK:	Extracellular signal-regulated kinase
ERα:	Estrogen receptor alpha
ERβ:	Estrogen receptor beta
ESCC:	Esophageal squamous cell carcinoma
ESR:	Estrogen receptor * gene nomenclature of ER
EZH:	Enhancer of zeste homolog
FACS:	Fluorescence activated cell sorting
FBS:	Fetal bovine serum
FOXO:	Forkhead box O protein
GAB:	GRB2-associated binding protein
GAS:	Gamma interferon activation site

GRB:	Growth factor receptor bound protein
H:	Hours
HC10:	Antibody clone recognizing HLA-B and/or HLA-C heavy chains
HCA2:	Antibody clone recognizing HLA-A heavy chains
H-chain:	Heavy chain
HER2:	Human epidermal growth factor receptor 2
HLA:	Human leukocyte antigen
HPR:	Horseradish peroxidase
I:	ICI *referred to in Figure 3.7 and 3.8
IFN:	Interferon
IFNAR:	Interferon alpha receptor
IFNGR:	Interferon gamma receptor
IFNLR:	IFNλ receptor
IFN-α:	Interferon alpha
IFN-β:	Interferon beta
IFN-γ:	Interferon gamma
IFNε:	Interferon epsilon

IFNκ:	Interferon kappa
IFNλ:	Interferon lambda
IFNω:	Interferon omega
IGF:	Insulin-like growth factor
IGFR:	Insulin-like growth factor receptor
IKK:	IκB kinase
IL:	Interleukin
IL-10Rβ:	IL-10 receptor subunit-β
IMDM:	Iscove's Modified Dulbecco's Medium
IRF:	Interferon regulatory factor
ISG:	Interferon-stimulated gene
ISGF3:	Interferon-stimulated gene factor 3
ISRE:	Interferon stimulated response element
IκB:	Inhibitor of kappa B
JAK:	Janus kinase
L:	Lapatinib *referred to in Figure 3.7 and 3.8
Lap:	Lapatinib

LBD:	Ligand binding domain
LMP:	Low molecular weight protein
LOF:	Loss-of-function
LRR:	Leucine-rich repeat
mAb:	Monoclonal antibody
MAPK:	Mitogen-activated protein kinase
MEK:	Mitogen-activated protein kinase kinase
MFI:	Mean fluorescence intensity
MSMD:	Mendelian Susceptibility to Mycobacterial Disease
mTORC:	Mammalian target of rapamycin complex
NaCl:	Sodium chloride
NBD:	Nucleotide-binding domain
NCOR:	Nuclear receptor corepressor
NEMO:	NF-κB essential modulator
NFY:	Nuclear transcription factor Y
NF-κB:	Nuclear factor-κB
NK:	Natural killer

NLR:	NOD-like receptor
NLRC5:	NLR family CARD domain containing 5
NLS:	Nuclear localization sequence
NTP:	Nucleotide triphosphate
p70S6K:	70kDa ribosomal protein S6 kinase
PA28αβ:	Proteasome activator 28 αβ
PBMC:	Peripheral blood mononuclear cell
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PD-1:	Programmed cell death protein 1
PDK1:	Phosphoinositide-dependent kinase 1
PD-L1:	Programmed cell death ligand 1
PE:	R-Phycoreythrin
PFA:	Paraformaldehyde
PI3K:	Phosphoinositide 3-kinase
PI3KCA:	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PIP2:	Phosphatidylinositol 4,5-diphosphate
PIP3:	Phosphatidylinositol 3,4,5-triphosphate
PLC:	Peptide loading complex
PMSF:	Phenylmethylsulfonyl fluoride
PR:	Progesterone receptor
pSTAT:	Phosphorylated STAT
PTEN:	Phosphatase and tensin homolog
PTPN:	Tyrosine-protein phosphatase nonreceptor type
qPCR:	Quantitative PCR
RAF:	Rapidly accelerated fibrosarcoma
RAS:	Rat sarcoma
RFX:	Regulatory factor X
RFXANK:	RFX associated ankyrin containing protein
RFXAP:	RFX-associated protein
RHEB:	Ras homolog enriched in brain
Ripa:	Radioimmunoprecipitation assay
RISC:	RNA-induced silencing complex

RPKM:	Reads per kilobase per million reads mapped
RTK:	Receptor tyrosine kinase
SDS:	Sodium dodecyl sulfate
SEM:	Standard errors of the mean
SERD:	Selective estrogen receptor down-regulators
SERM:	Selective ER modulators
SH2:	Src Homology 2
SHC:	SH2-conatining protein
siRNA:	Small interfering RNA
SMRT:	Silencing-mediator for retinoid/thyroid hormone receptors
SOS:	Son of sevenless
SP:	Specific protein
Src:	Sarcoma
STAT:	Signal transducer and activator of transcription
t:	Time
T:	Tamoxifen *referred to in Figure 3.7 and 3.8
T/P:	Trastuzumab + pertuzumab (dual therapy)

T/U	‘Treated’ over ‘untreated’
TAA:	Tumour associated antigen
TAM:	Tamoxifen
TAP:	Transporter associated with antigen processing
TBS:	Tris buffered saline
TBS-T:	TBS-Tween
T-DM1:	Trastuzumab emtansine
TEMED:	Tetramethylethylenediamine
TGFα:	Transforming growth factor-alpha
TIL:	Tumour-infiltrating lymphocyte
TKI:	Tyrosine kinase inhibitor
TME:	Tumour microenvironment
TN:	Triple negative
TNFR:	TNF receptor
TNF-α:	Tumour necrosis factor alpha
TPN:	Tapasin
TRAF:	TNF receptor-associated factor

TSC2:	Tuberous sclerosis complex
Tyk2:	Tyrosine kinase 2
W6/32:	Antibody clone recognizing HLA-ABC conformational epitope also known as HLA-ABC conformers
WCL:	Whole cell lysates

List of Appendices

Co-Authorship Statement

I, Louisa L. Wiede, designed and analyzed the experiments herein in collaboration with my supervisor, Dr. Sheila Drover. I performed all experiments and wrote all chapters of this thesis with edits from my supervisor.

Portions of this thesis including slightly modified version of Figures 3.7B and 3.13B as well as Figure 3.8A and 3.14A were edited for publication by Dr. Sheila Drover and are currently published in the following two book chapters as part of the 'Methods of Molecular Biology' book series:

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2. Wiede, L.L., Drover, S. (2022). *Western Blot Analysis of Lapatinib-Mediated Inhibition of the Epidermal Growth Factor Receptor 2 (HER2) Pathway in Breast Cancer Cells*. In: **Christian, S.L. (eds) Cancer Cell Biology. Methods in Molecular Biology**, vol 2508. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-2376-3_14

1 Chapter 1: Introduction

1.1 Breast Cancer

Breast cancer has the highest incidence rate among women worldwide and is the leading cause of cancer-related death in women. Breast cancer is a heterogeneous disease and can be divided into five major subtypes based on their molecular profile (e.g. estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and proliferation marker Ki67 status) (Table 1.1). Classification of breast cancer subtypes plays an important role in assessment of prognosis, disease progression, risk of metastasis, appropriately targeted therapy, therapy response as well as potential therapeutic resistance and overall survival [1–6].

Table 1.1: Classification of Breast Cancer Subtypes [1,3].

Breast Cancer Subtype	Molecular profile	Prognosis	Prevalence
Luminal A	ER+, PR+, HER2−, Ki67 low	Good	40%
Luminal B	ER+, PR+, HER2−, Ki67 high ER+, PR+, HER2+, Ki67 any	Intermediate Poor	20%
HER2 overexpression	ER−, PR−, HER2+, Ki67 any	Poor	12-21%
Triple negative (TN)/Basal	ER−, PR−, HER2−	Poor	11 to 23%
Normal-like	ER+, PR+, HER2−, Ki67 any	Intermediate	3 to 10%

Luminal tumour cells are found in the inner (luminal) lining of the mammary duct while TN/Basal tumour cells originate from the outer lining surrounding the mammary duct [2]. Luminal A, the most common type of breast cancer, responds well to endocrine therapy, is associated with a good prognosis, low recurrence and high survival rates. In comparison, Luminal B tumours have a poorer prognosis and are traditionally treated with a combination of chemotherapy and hormonal treatments; however, anti-HER2 monoclonal antibodies are now commonly added to these treatments [7]. HER2-overexpressing tumours have a less favourable prognosis compared to the two luminal subtypes but respond well to targeted therapy using anti-HER2 monoclonal antibodies. TN/Basal breast cancer is characterized by lack of ER, PR and HER2 overexpression, which subsequently leads to limited therapy options. These tumours are more aggressive and more common in younger individuals and African American women. Reduced disease-specific survival and increased risk of local and regional relapse greatly contribute to the overall poor prognosis in patients with TN/Basal tumours. Incomplete eradication of tumour cells increases the risk of early relapse among women with HER2-overexpressing and TN/Basal breast cancer and, therefore, will benefit the most from chemotherapy. Although normal-like tumours share the same molecular profile as Luminal A tumours, (Table 1.1) they exhibit differences in expression patterns and have a poorer prognosis than luminal A cancers [1–3].

The level of tumour-infiltrating lymphocytes (TILs) within the tumour microenvironment (TME) is an essential prognostic marker for overall prognosis. The

TME consists of a complex and extensive interplay between tumour cells and surrounding stroma, which contains immune cells, endothelial cells and fibroblasts [8]. High levels of CD8+ cytotoxic T cells (CTLs) correlate with a better prognosis in different tumour types including breast cancer. CTL activation is induced by presentation of tumour antigens via Human Leukocyte Antigen (HLA) Class I molecules leading to immune-mediated elimination and control of tumour growth [8]. Factors secreted by CTLs, cancer cells, and other elements of TME influence the makeup and function of the cancer milieu and therefore, modulate the route of breast cancer progression. Paradoxically, the frequency of T cells was found to increase as mammary tumour formation progresses [9].

1.2 Human Leukocyte Antigen

1.2.1 Overview

The HLA complex is a highly polymorphic region of the human genome encoding HLA Class I and HLA Class II proteins responsible for antigen presentation on the cell surface [10]. HLA Class I molecules are expressed on nearly all nucleated cells while HLA Class II molecules are constitutively expressed by so-called professional antigen presenting cells (APCs) such as B cells, macrophages and dendritic cells [8,10,11]. Upon exposure to interferon gamma (IFN-γ), epithelial, endothelial cells and fibroblasts display cell surface expression of HLA Class II within a few days [11]. HLA Class I expression is usually upregulated within 12-24 hours following IFN-γ stimulation [12]. Expression

levels of HLA Class I can also be increased and/or induced by other cytokines such as type I interferons (Interferon alpha (IFN-α) and Interferon beta (IFN-β)) and tumour necrosis factor α (TNF-α) [13,14].

HLA Class I proteins are heterodimers consisting of a heavy chain, a transmembrane glycoprotein and an invariant subunit β_2-mircoglobulin (β_2M) [12,15]. HLA Class I molecules are categorized into three classical HLA Class Ia proteins (HLA-A, HLA-B, and HLA-C) and three non-classical HLA Class Ib proteins (HLA-E, HLA-F, and HLA-G). The HLA Class Ia proteins play an essential role in immune surveillance by presenting antigen-derived peptides to CD8+ T Lymphocytes. This leads to T cell activation and induction of immune responses during viral and bacterial infection, organ transplantation as well as cancers [12,16,17]. Presentation of tumour associated antigens (TAAs) to HLA-restricted CD8+ T cells can trigger a specific adaptive immune response resulting in CTL-mediated cancer elimination and prevention of tumour progression [18]. Loss or downregulation of HLA Class I molecules is often found in human cancers such as breast cancer and is associated with tumour aggressiveness in terms of invasiveness, differentiation in histology and metastatic potential. Tumour cells utilize this mechanism to escape immune recognition and subsequent CTL-mediated immune destruction [19,20].

HLA Class II proteins are heterodimeric glycoproteins on the cell surface composed of an alpha and a beta chain. The classical HLA Class II molecules (HLA-DR, HLA-DQ, and HLA-DP) are crucial for antigen presentation to HLA-restricted CD4+ T cells. In response to HLA Class II-bound pathogenic or tumorigenic peptides, CD4+ T-Lymphocytes undergo activation and clonal expansion leading to synthesis and secretion of cytokines and, thereby triggering CTL activation and/or antibody production by B cells [8,11].

In order to elicit an effective TAA-specific immune response both, CD4+ and CD8+ T cells, are required. Expression levels of HLA Class II differ in malignant cells based on their molecular phenotype and/or origin. The majority of ductal breast carcinoma lesions were found to lack HLA Class II proteins while melanoma tumours gained HLA Class II expression [21]. A previous study from the Drover laboratory showed that ER- tumours were more likely to have upregulated HLA Class II, especially compared to other tumour types [22]. However, in our current study we investigate the expression of HLA Class I as a downstream gene of IFN activation, thus the remaining part of this section will be focussing on HLA Class I antigen presentation and regulation.

1.2.2 HLA Class I antigen processing and presentation

For antigen presentation by HLA Class I molecules, degradation of proteins into peptides occurs via the ubiquitin-proteasome pathway. This pathway preferentially degrades cytosolic proteins consisting of regulatory proteins, misfolded and damaged proteins (e.g. defective ribosomal products), as well as mutated and viral proteins in cancer cells and virus-infected cells, respectively [16].

Protein ubiquitination and peptide generation via proteasome

Ubiquitination is carried out by the following enzymes: Ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), substrate-specific ubiquitin-protein ligase (E3), and under certain circumstances additional conjugation factor (E4) [16]. Polyubiquitinated proteins are then further processed by the proteasome, a barrel-shaped multi-catalytic enzyme complex found in the cytosol [8,16]. The proteasome is composed of a catalytic core, also known the 20S proteasome, which consists of seven α subunits (α1- α7) in the two outer rings and seven β subunits (β1- β7) in the two inner rings. The α subunits regulate access to the proteasome together with regulatory particles such as 19S (major regulatory particle) and proteasome activator 28 αβ (PA28αβ) while the β subunits represent the proteolytic activity of the catalytic core. The presence of type I and type II interferons (IFNs) promote the upregulation of low molecular weight protein 2 (LMP2), LMP7 and LMP10, which replace the β subunits in the 20S proteasome to form the so called immunoproteasome. The immunoproteasome develops in intensified immune responses and displays increased capacity to generate

antigenic peptides as well as to cleave after hydrophobic residues resulting in the formation of optimal C-terminal anchor residues for stable HLA Class I binding [8,16]. Once activated the (immune-)proteasome unfolds, deubiquitinates and cleaves proteins into peptides of 2-25 amino acids (aa) in length, which are then released into the cytosol [16] (Figure 1.1).

Peptide transport

The peptides are then transported from the cytosol into the endoplasmic reticulum (ER) through transporter associated with antigen processing (TAP), which consists of two transporters, TAP1 and TAP2. These transporters are composed of a hydrophobic transmembrane domain and a cytosolic nucleotide-binding domain thereby forming a membrane-spanning pore in the ER membrane. Peptides with 8-12 aa are preferably transported by TAP while the transport of longer peptides is less efficient. Peptides too long to fit and stably bind to the HLA Class I binding groove require further trimming, which is facilitated by endoplasmic reticulum aminopeptidase 1 (ERAP1) and ERAP2 [8,16] (Figure 1.1).

Assembly of the peptide loading complex

Peptide loading onto nascent HLA Class I molecules involves four chaperone proteins: thiol oxidoreductase ERp57, calnexin, calreticulin (CRT) and tapasin (TPN). Along with HLA Class I molecules and TAP, these chaperone proteins form the peptide loading complex (PLC). Interaction of calnexin with the HLA Class I heavy chain ensures

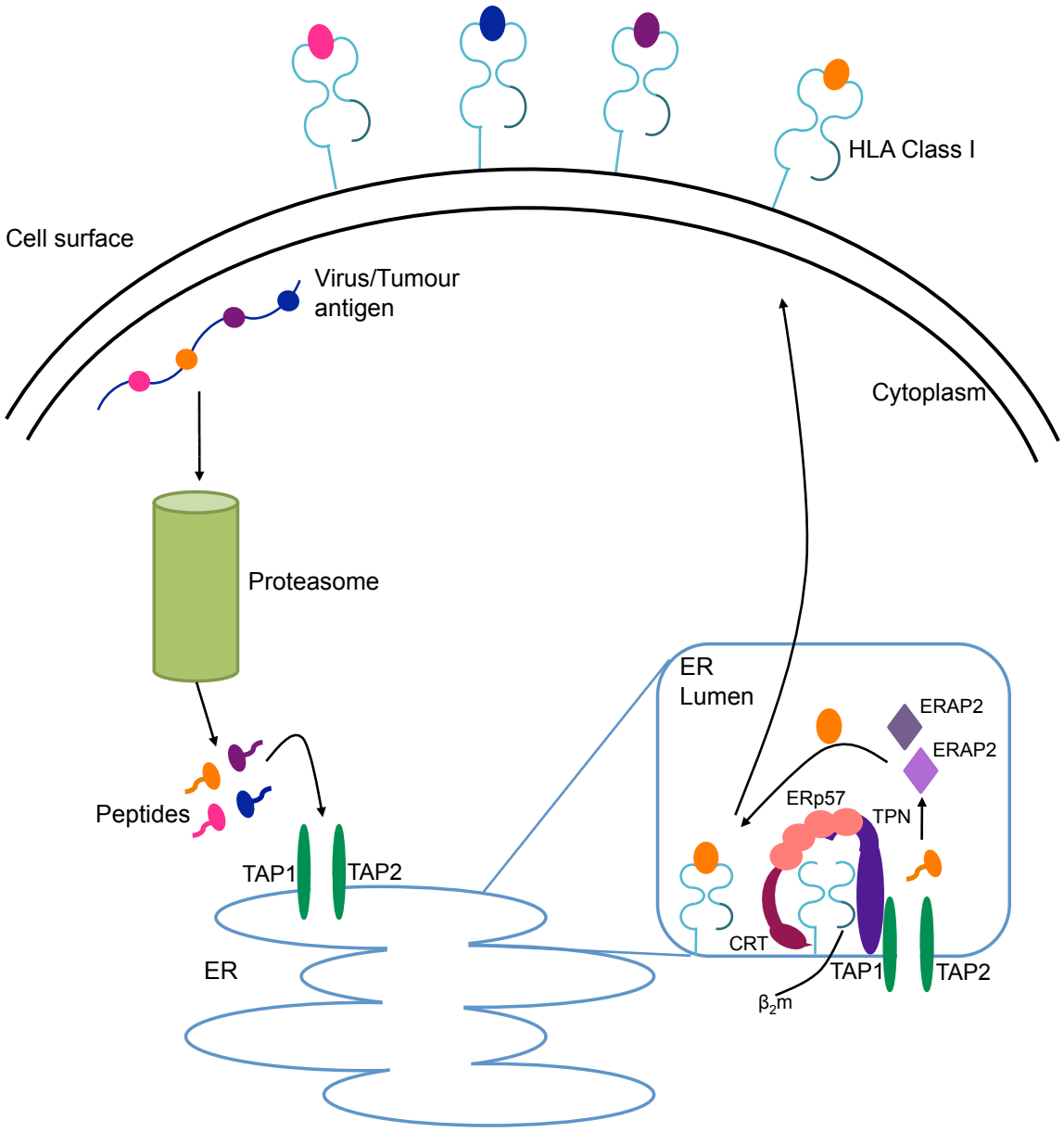
complete and stable folding and, together with ERp57, correct oxidation. This HLA Class I heavy chain conformation is now recognizable by β_2-microglobulin resulting in the formation of the heavy chain/β_2-microglobulin heterodimer. Calnexin is then released allowing access to the heavy chain/β_2-microglobulin heterodimer for CRT binding. Along with TAP and TPN, CRT facilitates peptide binding to heavy chain/β_2-microglobulin heterodimers. In this process TPN functions as bridge between HLA Class I molecules and TAP giving peptides access to ER for HLA Class I binding. It also ensures HLA Class I loading with the best available peptides to form the most stable peptide-HLA Class I complex [8,16] (Figure 1.1).

Antigen presentation

Following successful peptide loading onto HLA Class I molecules, PLC proteins dissociate from the stable peptide-HLA Class I complexes, which then migrate to the cell membrane via the Golgi apparatus. The complexes then fuse with the cell membrane in order to present bound peptides in the HLA Class I groove on the cell surface making it accessible for CD8+ T cells recognition and binding [8,16] (Figure 1.1).

Figure 1.1: HLA Class I antigen processing and presentation.

Following ubiquitination, the proteins are cleaved into peptides by the proteasome. Transport of antigenic peptides to the ER is facilitated by TAP1 and TAP2. In the ER the peptides are then loaded onto HLA Class I molecules, which consist of a heavy chain and β_2m. Peptide loading occurs via macromolecular PLC encompassing CRT, ERp57, and TPN. ERAP1 and ERAP2 are responsible for additional peptide trimming if peptides are too long for HLA Class I binding. Peptide loaded HLA Class I molecules are then released from PLC and transported to the cell surface in order to present peptides to circulating CD8+ T cells. Figure is adapted with permission from references [8] and [17].


HLA Class I
Cell surface
Virus/Tumour
antigen
Cytoplasm
Proteasome
ER
Lumen
ERAP2
ERAP2
ERp57
TPN
Peptides
TAP1
TAP2
CRT
TAP1
TAP2
ER
β_2m

1.2.3 Regulation of HLA Class I expression

NOD-like receptor (NLR) family CARD domain containing 5 (NLRC5) is the key class I transactivator (CITA) responsible for regulation of HLA Class I expression. It belongs to the NLR family and possesses a tripartite structure similar to other NLR proteins. NLRC5 is composed of a N-terminal atypical caspase activation and recruitment domain (aCARD) containing a bipartite-type nuclear localization sequence (NLS) followed by a centrally located nucleotide-binding domain (NBD) and the C-terminal leucine-rich repeats (LRRs). Walter A and B motif are localized within NBD and facilitate nucleotide triphosphate (NTP)-binding and hydrolysis, respectively. Walter A motif is essential for both nuclear translocation and HLA Class I transactivation [17,23]. Various human and mouse tissues express NLRC5 with highest expression levels found in hematopoietic cells. Nonhematopoietic cells exhibit low NLRC5 but it can be upregulated in response to type I and II IFNs. NLRC5 also plays a key role in the regulation of HLA Class I antigen processing and presentation pathway due to its ability to induce expression of pathway components β_2m, TAP1 and LMP2 [17,23,24].

The HLA Class I promoter is composed of the following cis-regulatory elements: enhancer A, interferon stimulated response element (ISRE), and the SXY module, which includes the W/S, X1, X2 and Y boxes. These elements are essential for constitutive and induced expression of HLA Class I. Enhancer A encompasses one to two nuclear factor-κB (NF-κB) binding sites while ISRE is targeted by interferon regulatory factor 1 (IRF-1). Both domains contribute to HLA Class I expression following stimulation of type I and II

IFNs or activation of the NF-κB pathway [17,23,24]. HLA-A levels are predominantly increased by NF-κB due to two NF-κB binding sites in the enhancer A region while HLA-B only contains one NF-κB binding site and other loci are not activated by NF-κB at all [23]. The regulatory factor X (RFX) complex, comprised of DNA-binding unit RFX5, RFX-associated protein (RFXAP), and RFX associated ankyrin containing protein (RFXANK), binds to the X1 box while the X2 box is bound by the transcription factors cAMP response element binding (CREB) or activating transcription factor 1 (ATF1). The Y box is targeted by the nuclear transcription factor Y (NFY) complex encompassing the NFYa, NFYb and NFYc subunits, whereas the binding proteins for the S box remain unknown [17,23,24].

Following translocation to the nucleus NLRC5 associates with RFX complex as well as CREB/ATF1 and the NFY complex to form the HLA Class I enhanceosome, which is crucial for activation of HLA Class I transcription. NLRC5 is dependent on the enhanceosome in order to connect to the HLA Class I promoter region since it lacks a DNA-binding domain. In turn, NLRC5 then serves as a platform for the CITA enhanceosome and is responsible for recruitment of chromatin remodeling enzymes. Additionally, HLA Class I expression can be further regulated by the binding of NF-κB and IRF-1 to enhancer A and ISRE, respectively [17,23] (Figure 1.2).

1.2.3.1 Upregulation of HLA Class I expression by IFN-γ

Following IFN-γ binding to interferon gamma receptor 1 (IFNGR1) and IFNGR2, signal transducer and activator of transcription 1 (STAT-1) is phosphorylated and subsequently activated by Janus kinase 1 (JAK1) and JAK2 leading to the formation of STAT-1 homodimers. These dimers then bind to the gamma interferon activation site (GAS) in the promoter region of NLRC5 and IRF-1 inducing their transcription. NLRC5 and IRF-1 target their binding sites in the HLA Class I promoter and activate HLA Class I transcription as described in section 1.2.3 (35, 38, 42) (Figure 1.2).

1.2.3.2 Upregulation of HLA Class I expression by IFN-α/β

Upon IFN-α/β binding to the interferon alpha receptor 1 (IFNAR1) and IFNAR2, the receptors dimerize causing an increased kinase activity of the receptor-associated kinases JAK1 and tyrosine kinase 2 (TYK2) via juxtapositioning and transphosphorylation. In turn JAK1 and TYK2 then phosphorylate IFNAR1 and IFNAR2 on tyrosine residues providing docking sites for STAT-1 and STAT-2, which results in recruitment of these STATs [25]. Once bound to IFNAR1/2, both STAT-1 and STAT-2, are phosphorylated leading to heterodimerization, association with IRF-9 and subsequent formation of the interferon-stimulated gene factor 3 (ISGF3). Following nuclear translocation ISGF3 binds to the ISRE domain in the HLA Class I promoter and activates HLA Class I transcription (Section 1.2.3) [25–27]. Alternatively, an ISGF3-like complex can be formed by the interaction of STAT-2 homodimer with IRF-9 in the absence of STAT-1 [25]. Additionally,

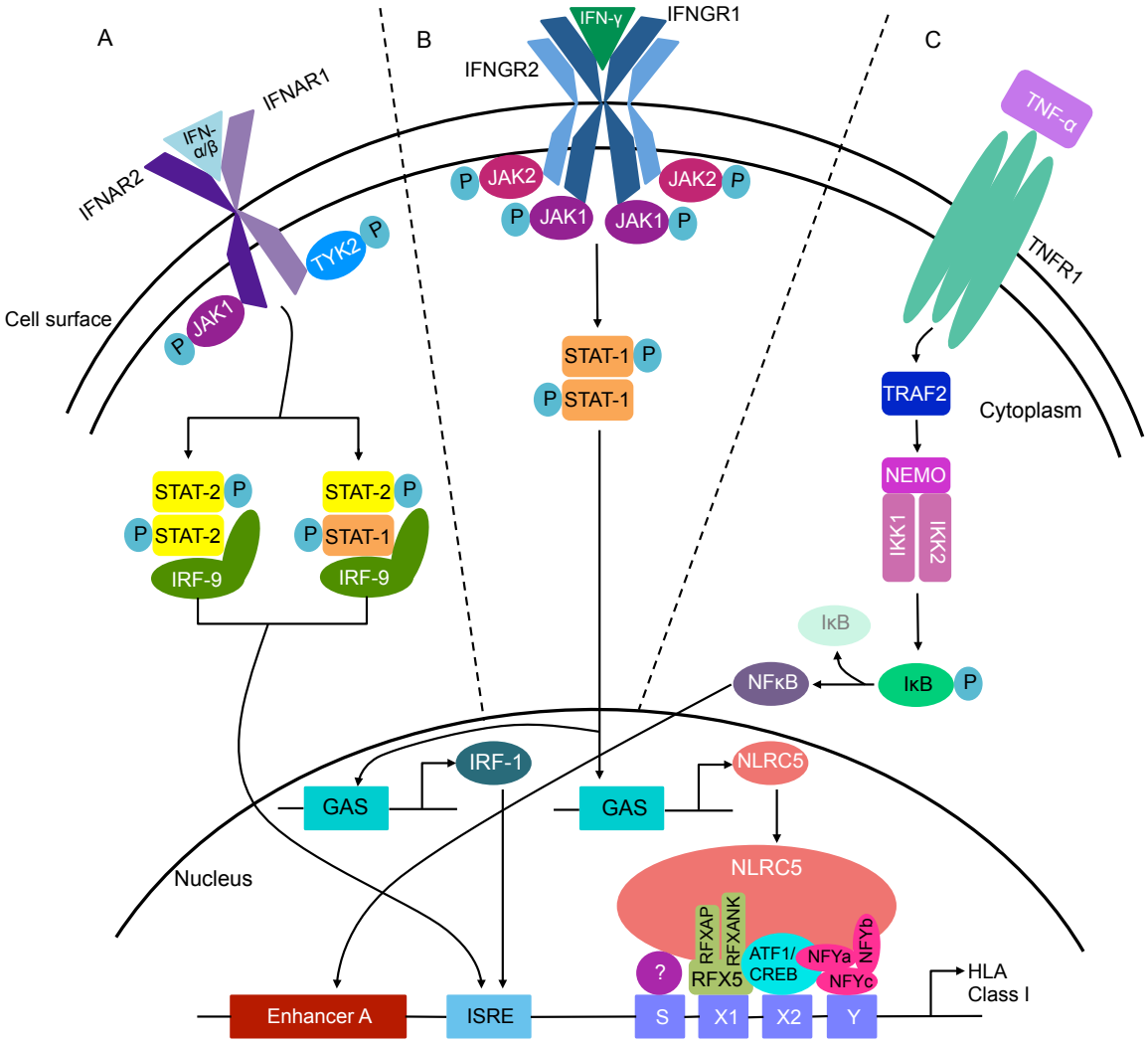
IFN type I stimulation can also result in formation of STAT-1 homodimers, which shuttle to the nucleus and bind to the GAS site in the promoter regions of IRF-1 and NLRC5 causing IRF-1 and NLRC5 production and subsequent HLA Class I transactivation [23,25]. Due to direct competition with STAT-1 - STAT-2 heterodimerization as well as ISGF3 formation, IFN type I induces STAT-1 homodimerization at lower rates compared to IFN type II [25] (Figure 1.2).

1.2.3.3 Upregulation of HLA Class I expression by TNF-α

The engagement of TNF receptor 1 (TNFR1) with TNFα results in activation of TNF receptor-associated factor 2 (TRAF2), which leads to the subsequent stimulation of the IκB kinase (IKK) complex. This complex consists of two related kinases IKK1 and IKK2 as well as NF-κB essential modulator (NEMO) and is responsible for the phosphorylation of inhibitor of kappa B (IκB) and initiation of their proteasomal degradation. Consequently, NF-κB switches to an active state and is now able to translocate into the nucleus and bind to enhancer A in the HLA Class I promoter causing HLA Class I transcription (Section 1.2.3, Figure 1.2)[28–30].

Figure 1.2: Transcriptional regulation of HLA Class I expression via IFN-α/β, IFN-γ, and TNF-α stimulation.

(A) IFN-α/β binding to IFNAR1/2 and receptor dimerization is followed by JAK1 and TYK2 activation and subsequent STAT recruitment. Receptor-bound STAT-1 and STAT-2 are then phosphorylated resulting in heterodimerization and association with IRF-9 forming ISGF3, which then binds to the ISRE binding site in the HLA Class I promoter. An alternative complex consisting of STAT-2 homodimer and IRF9 can be formed, which exhibits the same mode of action. (B) Upon IFN-γ stimulation IFNGR1/2-associated JAK1 and JAK2 are activated leading to STAT-1 phosphorylation and homodimerization. The activated STAT-1 homodimer then translocates to the nucleus and binds to the GAS site in the IRF-1 and NLRC5 promoter inducing IRF-1 and NLRC5 transcription. (C) TNFα-induced TNFR1 signalling triggers TRAF2 recruitment and subsequent activation of the IKK complex composed of NEMO as well as IKK1 and IKK2. Active IKK complex then phosphorylates the IκB inhibitor leading to its degradation permitting NF-κB activation, translocation to the nucleus, and binding to enhancer A region in the HLA Class I promoter. HLA Class I gene transcription is facilitated by the NLRC5/CITA enhanceosome bound to the SXY module in the HLA Class I promoter. NRLC5 serves hereby as platform for transcription factors including RFX trimeric complex, ATF1/CREB, and NYF factors. HLA Class I transcriptional activity can be further modulated by the binding of NF-κB to enhancer A and/or IRF-1 or ISGF3 to ISRE. Figure is adapted with permission from references [17,25,26,28,29].


A
B
C
IFN-γ
IFNGR1
IFNGR2
IFNAR1
IFNAR2
IFN-α/β
TNF-α
TNFR1
Cell surface
JAK2
JAK1
TYK2
P
STAT-1
STAT-2
IRF-9
TRAF2
Cytoplasm
NEMO
IKK1
IKK2
IκB
NFκB
IRF-1
GAS
NLRC5
Nucleus
RFXAP
RFXANK
RFX5
ATF1/
CREB
NFYa
NFYb
NFYc
?
S
X1
X2
Y
Enhancer A
ISRE
HLA
Class I

1.3 Interferons (IFNs)

1.3.1 Overview

Interferons belong to a group of pleiotropic cytokines and are essential for communication between innate and acquired immune responses eliciting a host defense against viral and bacterial pathogens as well as tumour surveillance [26,31,32]. IFNs are classified into three types: IFN type I, IFN type II and IFN type III. The members of type I IFN are IFNα/β including 13 subtypes of IFNα and IFNε, IFNκ, IFNω, which all bind to IFNAR1 and IFNAR2 while IFN-γ is the single member of type II IFN binding to IFNGR1 and IFNGR2 [26,32]. IFNλ1, IFNλ2, IFNλ3 (also known as interleukin-29 (IL-29), IL-28A and IL-28B respectively) and IFNλ4 are type III IFNs engaging with IFNλ receptor 1 (IFNLR1) and IL-10 receptor subunit-β (IL-10Rβ) [26]. Type I IFNs are primarily produced by dendritic cells but can be induced in different cell types such as T cells, monocytes, fibroblasts, and epithelial cells while the major sources of IFN-γ are natural killer (NK) cells and T cells [27]. IFNs are known to be involved in tumour development and treatment by regulating expression of genes affecting tumour proliferation, differentiation, survival and migration [26].

1.3.2 The classical IFN-γ pathway

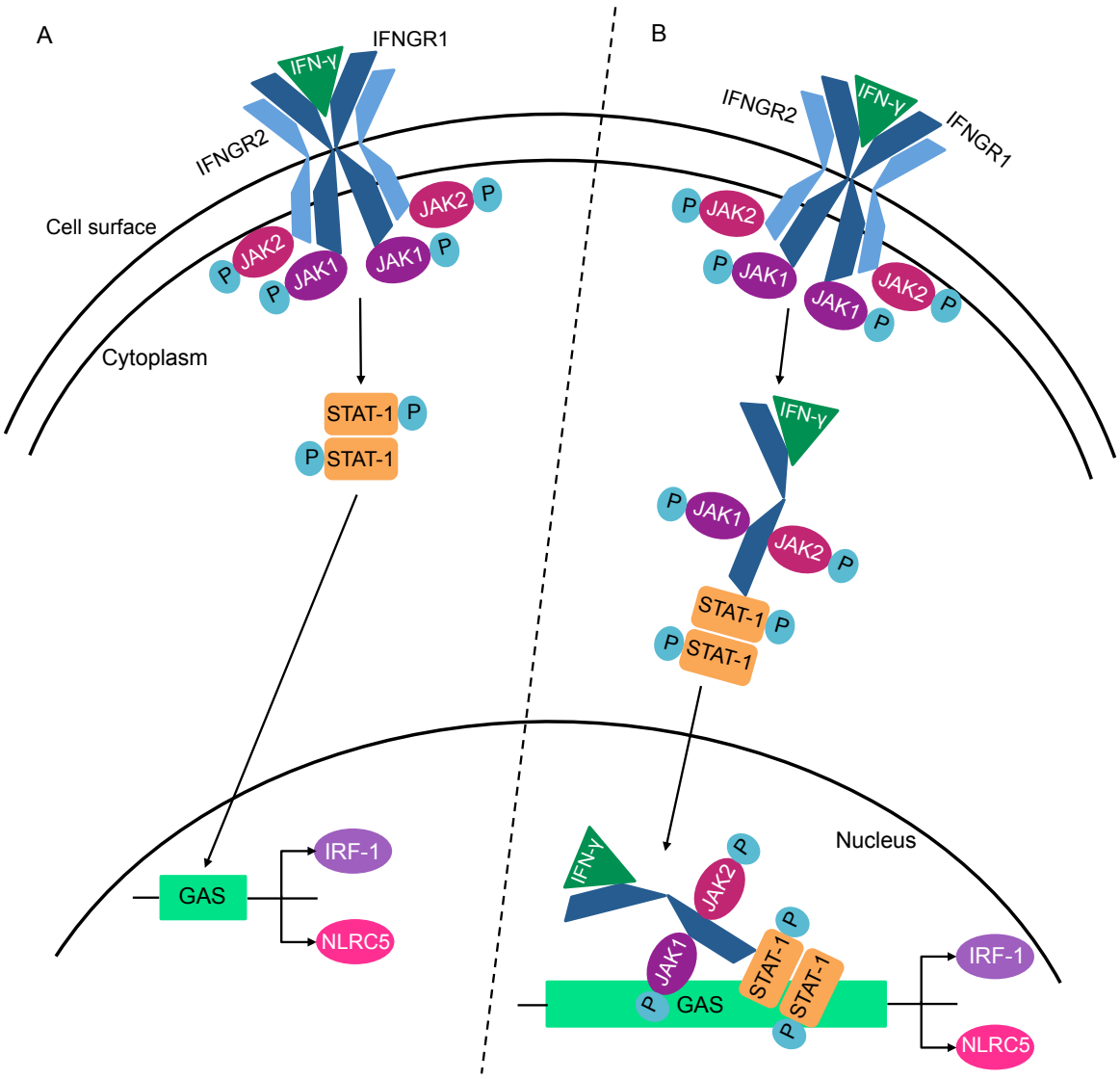
Binding of IFN-γ to the heterodimeric receptors IFNGR1 and IFNGR2 activates allosteric changes in their cytoplasmic domain allowing receptor-associated JAK1 and JAK2 in close proximity to each other causing transphosphorylation and activation of these kinases. Activated JAK1 and JAK2 phosphorylate IFNGR1 providing a docking site for STAT-1 leading to STAT-1 recruitment and phosphorylation. Phosphorylated STAT-1 (pSTAT-1) forms a homodimer, dissociates from IFNGR1 and translocates to the nucleus through its NLS to bind to GAS resulting in initiation of interferon-stimulated genes (ISGs) such as IRF-1 and NLRC5/CITA [17,23,33,34]. Secondary response genes such as HLA Class I are then activated by IRF-1 and NLRC5/CITA (Section 1.2.3,Figure 1.3) [17,23].

1.3.3 The non-classical IFN-γ pathway

Similar to the classical pathway IFN-γ engages with IFNGR1 and IFNGR2 leading to activation of JAK1 and JAK2, phosphorylation of IFNGR1 cytoplasmic domain as well as phosphorylation and homodimerization of STAT-1 [34,35]. Unlike the classical IFN-γ pathway, the non-classical pathway involves receptor endocytosis of IFNGR1 followed by nuclear translocation of the IFN-γ/IFNGR1/pSTAT-1/JAK1/JAK2 complex via NLS of IFN-γ [34–36]. This complex binds to GAS element and activates the transcription of IRF-1 and NLCR5/CITA and secondary response genes including HLA Class I (Section 1.2.3, Figure 1.3) [17,34,35].

Figure 1.3: Classical and non-classical IFN-γ pathway.

(A) The classical IFN-γ signalling pathway is induced by IFN-γ binding to IFNGR1 and IFNGR2 causing allosteric changes in the receptor cytoplasmic domain leading to transphosphorylation and activation of receptor-bound JAK1 and JAK2. Activated JAKs then phosphorylate the IFNGR1 cytoplasmic domain resulting in STAT-1 recruitment, phosphorylation and homodimerization. Active STAT-1 homodimer dissociates from IFNGR1, translocates into the nucleus, and binds to the GAS site in the promoter region of IRF-1 and NLRC5 leading to IRF-1 and NLRC5 transcription. IRF-1 and NLRC5 can then induce transcription of secondary response genes including HLA Class I (Figure 1.2). (B) In the non-classical model of IFN-γ signalling JAK1/2 activation, IFNGR1 phosphorylation and STAT-1 homodimerization is accompanied by IFNGR1 endocytosis. Nuclear transport of the IFNGR1/STAT-1/JAK1/JAK2 complex results in binding to the GAS site in the IRF-1 and NLRC5 promoter inducing IRF-1 and NLRC5 transcription followed by transcription of secondary response genes such as HLA Class I (Figure 1.2). Figure is adapted with permission from references [17,34,35].


A
IFNGR1
IFN-γ
IFNGR2
Cell surface
JAK2
P
JAK1
Cytoplasm
STAT-1
GAS
IRF-1
NLRC5
B
IFNGR2
IFN-γ
IFNGR1
Nucleus

1.4 Cell Signalling pathways in ER+HER2+ Breast Cancer

1.4.1 Estrogen receptor signalling

ER signalling is facilitated by two different ER types: ERα and ERβ, which belong to the nuclear receptor superfamily. These receptors are encoded by two different genes, estrogen receptor 1 (ESR1) and ESR2, respectively, but share the same five distinct structural domains: a DNA binding domain (DBD), a ligand binding domain (LBD), a hinge region bearing the nuclear localization signal and a ligand-independent activation function (AF-1) as well as a ligand-dependent transactivation function (AF-2) [37–39]. On the functional level, ERα is known to play an important role in cell proliferation especially in response to estradiol (E_2) in both normal and cancerous breast tissue. The function of ERβ in breast cancer remains elusive and it may have a bi-directional effect since some studies indicate antagonistic effects of ERβ towards ERα while others show proliferative effects of ERβ in the absence of ERα [38–40]. ERα signalling can occur through the following two major pathways [37–39,41]:

Genomic signalling pathway:

ERα undergoes specific conformational changes following ligand binding (e.g. E_2), which induce receptor homo- and heterodimerization and results in nuclear translocation and subsequent binding to specific estrogen response elements (EREs) in the promoter region of target genes. DNA-bound ERα facilitates gene expression modification by recruiting distinct co-regulatory proteins (both co-activators and co-

repressors) [37–39,41]. Interaction of nuclear ERα with other transcription factors such as activator protein 1 (AP-1) or specific protein 1 (SP-1) leads to transcriptional regulation of non-ERE regulatory genes, which is referred to as the 'non-classical genomic pathway' of ER. The so-called 'estrogen-independent pathway' involves ER activation via phosphorylation by epidermal growth factor receptor-dependent (EGFR) kinases such as extracellular signal-regulated kinase (ERK) and serine/threonine-protein kinase (AKT) [37–39].

Non-genomic signalling pathway:

The non-genomic action of estrogen-bound ERα is mediated by ERα interaction with receptor tyrosine kinases associated with EGFR, insulin-like growth factor receptor (IGFR) and/or HER2 receptors. These interactions induce the activation of signalling cascades involving rat sarcoma (RAS) and mitogen-activated protein kinase kinase (MEK) as well as phosphoinositide 3-kinase (PI3K) and AKT, which in turn activate ERα [38,39,41].

1.4.2 HER2 signalling pathway

HER2 is a member of the EGFR family, which consist of HER1 (EGFR, ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (EbrB4) [42–45]. These receptor tyrosine kinases play a central role in regulation of cell proliferation, differentiation and survival [42,45]. The HER2 receptor is a 185kDa membrane-spanning glycoprotein, which is encoded by the HER2 proto-oncogene. All four HER receptors are composed of an extracellular ligand-

binding site, a lipophilic transmembrane segment and an intracellular cytoplasmic domain containing the tyrosine kinase catalytic activity [42,43,45,46].

As they exist as inactive monomers on the cell surface, homo- or heterodimerization of the HER receptors following ligand binding is essential for signalling activity. HER2 does not have a known ligand of its own and, therefore, may be in a constitutively activated state, which makes it the preferred dimerization partner for other family members. HER3 is only able to form a heterodimer as it lacks the tyrosine kinase activity. Heterodimers involving HER2 exhibit especially high ligand binding resulting in a high signalling potency. The HER2-HER3 homodimer has the highest signalling potency and, therefore, is believed to be an important oncogenic signalling unit [42,43,45]. Following receptor dimerization, the tyrosine domain gets activated by autophosphorylation resulting in recruitment of various adapter proteins, which leads to the initiation of two main downstream signalling pathways: PI3k/AKT and mitogen-activated protein kinase (MAPK) pathway [42,43,45,46] (Figure 1.4).

PI3K/AKT pathway:

The PI3K/AKT pathway is activated by recruitment of PI3K to the activated HER receptors, which then phosphorylates phosphatidylinositol 4,5-diphosphate (PIP2) to form phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 activates phosphoinositide-dependent kinase 1 (PDK1) leading to the recruitment of protein kinase B, also known AKT, to the membrane [44–46]. PDK1 and mammalian target of rapamycin complex 2

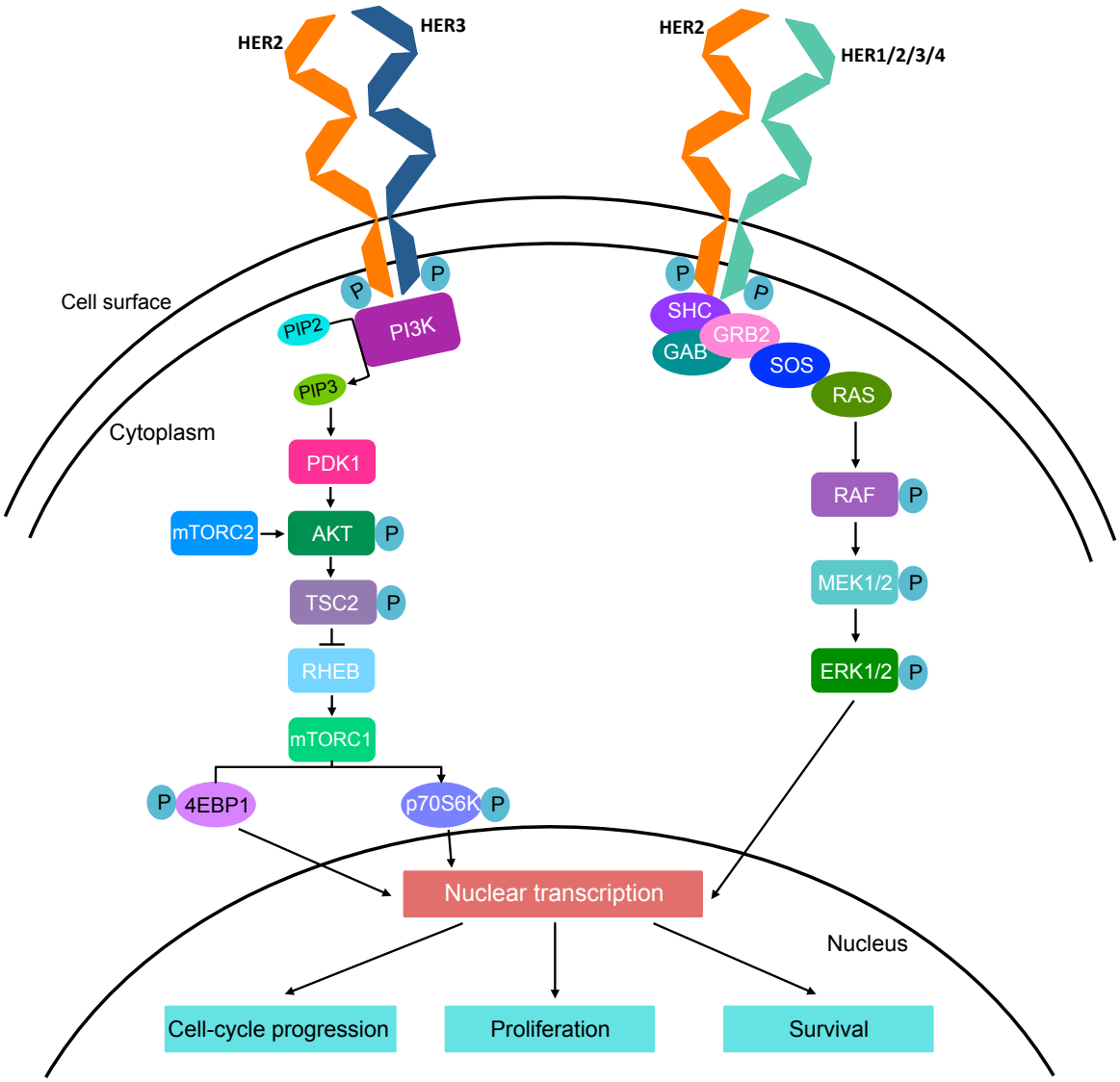
(mTORC2) phosphorylate and activate AKT, which then phosphorylates tuberous sclerosis complex 2 (TSC2). This causes destabilization of TSC2 and subsequent inhibition of GTB-binding protein RAS homolog enriched in brain (RHEB) resulting in activation of mTORC1 [46,47]. In turn mTORC1 phosphorylates eukaryotic initiation factor 4E-binding protein 1 (4EBP1) and 70kDa ribosomal protein S6 kinase (p70S6K) promoting transcription and regulating ribosomal gene transcription [47] (Figure 1.4).

MAPK pathway:

The initiation of the MAPK pathway is facilitated by formation of receptor complexes containing SH2-conatining protein (SHC), growth factor receptor bound protein 2 (GRB2) and GRB2-associated binding protein (GAB). Son of sevenless (SOS), an exchange factor, is then constitutively bound by GRB2 leading to activation of RAS. Active RAS recruits rapidly accelerated fibrosarcoma (RAF) from the cytoplasm to the membrane and activates its kinase activity through phosphorylation resulting in activation of MEK1/2. Phosphorylation of ERK1/2 by active MEK1/2 leads to stimulation of cell proliferation and survival [46,48] (Figure 1.4).

Figure 1.4: HER2 signalling pathway.

HER2, lacking a known ligand of its own, forms homo- or heterodimers with HER1/2/3/4 of which the HER2-HER3 heterodimer is the most potent signalling dimer. Receptor dimerization induces the activation of the downstream signalling pathways PI3K/AKT and MAPK. PI3K/AKT signalling (left) is initiated by the recruitment of PI3K to the activated HER receptors leading to induction of a signalling cascade involving PIP2/3, PDK1, AKT, TSC2, and RHEB, which ultimately leads to the activation of mTORC1. mTOCR1 then phosphorylates and activates 4EBP1 and p70S6K. MAPK pathway (right) is induced by formation of a receptor complex including SHC, GAB, and GRB2 leading to RAS activation via GRB2-bound SOS. RAS activation is responsible for the initiation of a signalling cascade ultimately resulting in ERK1/2 activation after RAF and MEK1/2 phosphorylation and activation. Both downstream signalling pathways lead to transcriptional modulation of various genes involved in cell-cycle progression, proliferation, and survival. Figure is adapted with permission from references [42,45,48,49].


HER2
HER3
HER2
HER1/2/3/4
Cell surface
Cytoplasm
P
PIP2
PI3K
PIP3
PDK1
mTORC2
AKT
TSC2
RHEB
mTORC1
4EBP1
p70S6K
SHC
GAB
GRB2
SOS
RAS
RAF
MEK1/2
ERK1/2
Nuclear transcription
Nucleus
Cell-cycle progression
Proliferation
Survival

1.4.3 ERα - HER2 crosstalk

An inverse relationship between ERα and HER2 in breast cancer has been shown by preclinical as well as clinical studies. Increased HER2 signalling can negatively influence the expression of ERα on the genomic and protein level through AKT-mediated inhibition of forkhead box O protein 3a (FOXO3a), a key regulator of ERα gene transcription, or through MAPK activation directly resulting in ERα degradation [50,51]. This can lead to a loss of ERα and subsequent reduced sensitivity to endocrine therapy. Therefore, ERα levels can be increased/restored by inhibiting HER2 signalling in preclinical models of HER2+ breast cancer [50]. On the other hand, activation of the HER2 pathway can also enhance and modulate genomic and non-genomic signalling of ERα and its ligand dependency. Consequently, HER2 overexpression can activate ERα in a ligand-independent fashion (Section 1.4.1) through ERα phosphorylation by receptor tyrosine kinase (RTK)-dependent kinases such as p38 MAPK, PI3K/AKT, and p43/42 MAPK [41,50,51]. These kinases can also activate ER co-activators such as amplified in breast cancer 1 (AIB1) causing an increased transcriptional activity of ERα. This ligand-independent ERα activation can result in a switch of selective ER modulators (SERMs) pharmacological actions from antagonistic to agonistic leading to endocrine resistance. Activation of HER2 signalling can be induced directly by ERα or indirectly by its interaction with the G protein. ER can stimulate downstream signalling cascades including receptor tyrosine kinases and cause increased expression of transforming growth factor-α (TGFα) and insulin-like growth factor 1 (IGF1). On the contrary ERα

signalling is also able to decrease HER1 and HER2 levels while increasing expression of IGF1 receptor. Following anti-HER2 therapy, ERα signalling can be reactivated in ER+HER2+ preclinical models representing an escape mechanism that can 'bypass effective HER2 inhibition' [50,51]. Due to this intense bidirectional crosstalk between ERα and HER2 therapy observed in preclinical and clinical studies, treatment strategies are focussing on combined blockade of ERα and HER2 signalling since targeting one pathway often only leads to the overexpression of the other one [41,50].

1.5 Targeted therapy of ER+ and HER2+ breast cancer

1.5.1 ER+ breast cancer

1.5.1.1 Tamoxifen

Tamoxifen (TAM) is used as adjuvant endocrine therapy for ER-positive breast cancer in premenopausal and postmenopausal women [52–54]. Additionally, TAM is used as a therapy for *in situ* ductal carcinoma and as a preventive treatment for women at high risk of developing breast cancer [52]. TAM is a member of the SERM drug group, which are antiestrogens that compete with estrogen and alter ERα activity via recruitment of different cofactors associating with ERα [53]. Based on its pharmacological profile, TAM is a prodrug and its anticancer properties stem from its active metabolites 4-hydroxy TAM and endoxifen. These metabolites are generated by the transformation of TAM via hepatic cytochrome P450 enzymes (CYP2D6, CYP3A and CYP2C) and their binding causes ERα to change into an antagonistic conformation. This

initiates the recruitment of the corepressors nuclear receptor corepressor 1 (NCOR1) and silencing-mediator for retinoid/thyroid hormone receptor (SMRT) to ERE resulting in suppression of ER signalling [53,54]. Therefore, TAM inhibits cell growth and cell proliferation as well as induces apoptosis. While TAM has antagonistic effects in breast tissues, it exhibits agonistic actions in uterus, bone and heart leading to the development of uterine cancer [52,54]. Chronic exposure to TAM can cause changes in cancer cells enabling them to recognize the Erα - TAM complex as transcriptionally 'active' resulting in ERα signalling [55]. Despite these adverse events TAM remains the standard of care for treatment of ER+ breast cancer [53]. The patient's cancer may recur and progress due to TAM resistance, which leads to the use of second-line hormonal therapy drugs such as fulvestrant [55,56].

1.5.1.2 Fulvestrant (ICI)

Fulvestrant, also known as ICI 182,780, is used in the treatment of postmenopausal women with ER+, locally advanced and metastatic relapsed breast cancer during or after adjuvant antiestrogen therapy as well as tumour progression on antiestrogen therapy using SERMs such as TAM [53,56]. ICI is an analogue of estradiol with a substitution at the 7α position. It belongs to the group of selective estrogen receptor down-regulators (SERDs). These antiestrogens competitively bind with a 100 times greater affinity to ER than TAM causing impaired dimerization, increased ER turnover, and disrupted nuclear localization [53,54,56]. As consequence, ERα is unable to inhibit or activate gene transcription. In comparison to TAM, ICI binding to ERα results

in a rapid degradation of ERα and subsequent loss of ERα protein in cancer cells rendering it unavailable or unresponsive to estrogen or estrogen agonists. Another advantage of ICI is its ability to consistently decrease ERα and PR levels in the tumour, which also distinguishes it from the mode of action of TAM [56,57]. ICI is also described as pure antiestrogen due to its lack of agonism in all ER+ tissues [53]. Treatment with ICI leads to antiproliferative and antiestrogenic effects as well as induction of apoptosis. Similar to TAM, continuous administration of ICI results in acquired resistance in most patients with advanced breast cancer via a poorly understood mechanism [54].

1.5.2 HER2+ breast cancer

1.5.2.1 Lapatinib

Lapatinib (Lap), a reversible tyrosine kinase inhibitor (TKI), is typically used in combination with chemotherapy drug capecitabine to treat HER2+ metastatic breast cancer patients, previously treated with trastuzumab and chemotherapeutic agents anthracycline and taxane [42,58]. These small TKIs target the intracellular domain of HER2 and EGFR by competing for the adenosine triphosphate (ATP)-binding domain of protein kinases, thereby interrupting receptor phosphorylation and subsequent activation of downstream signalling pathways such as MAPK and PI3K/AKT pathways [42,58,59]. This leads to decreased cell proliferation and cell migration as well as apoptosis due to increased transcriptional activity of pro-apoptotic protein BCL-2 interacting mediator of cell death (BIM) and downregulation of the apoptosis inhibitor

survivin [58,59]. Lap is also able to prevent the phosphorylation of HER3. Acquired resistance to Lap treatment is mediated by modifications of various proteins involved in downstream signalling pathways of target receptors such as mutations in the catalytic subunit of PI3K (PI3KCA). These mutations can render Lap administration less effective [58].

1.5.2.2 Trastuzumab (Herceptin)

Trastuzumab (or Herceptin) is a humanized monoclonal antibody (mAb) used to treat HER2 overexpressing breast cancer in both adjuvant and metastatic setting. Monotherapy as well as combined therapy with chemotherapy drugs were shown to have clinical benefits in metastatic breast cancer patients. Combination treatments including trastuzumab and chemotherapy are considered standard of care for HER2-postive breast cancer patients [60–62]. Trastuzumab targets the extracellular domain IV of HER2 and exhibits antitumor properties by triggering the following mechanisms: inhibition of either HER2 homodimerization or ligand-independent HER2/HER3 heterodimerization, prevention of HER2 activation via extracellular domain shedding, induction of cell cycle arrest by accumulation of the cyclin-dependent kinase 2 (CDK2) inhibitor p27, and inhibition of angiogenesis [60,63–66]. The main mechanisms involved in trastuzumab-mediated anticancer effect include interference in HER2 downstream signalling pathways MAPK and PI3K/AKT pathways as well as antibody-dependent cell-mediated cytotoxicity (ADCC) [62–65]. Binding of trastuzumab to HER2 leads to inhibition of AKT phosphorylation and tyrosine kinase sarcoma (src) signalling, thereby

increasing phosphatase and tensin homolog (PTEN) expression causing suppression of PI3K/AKT signalling. Inhibition of MAPK and PI3K/AKT pathways leads to reduction in cell growth, cell proliferation and survival [62]. ADCC is facilitated by binding of immune cells, mainly NK cells, via their Fcγ receptor to the Fc region of the trastuzumab antibody [62,65]. Despite its effectiveness, less than 35% of HER2-positive breast cancer patients initially respond to trastuzumab treatment while the majority of patients experience recurrence and disease progression due to acquired trastuzumab resistance [62,63,65,67]. This resistance mainly occurs via steric effects such as HER2 mutations, overexpression of alternative tyrosine kinase receptors such as HER3 and IGFR3, or intracellular changes of HER2 downstream signalling such as PTEN loss [62,63]. Trastuzumab is unable to prevent ligand-activated HER2 dimerization with EGFR and especially HER3 leading to the activation of downstream signalling, thus, providing a way for cancer cells to escape trastuzumab-induced inhibition [60,62,64,66,67]. In order to overcome this escape mechanism, a novel therapeutic drug called pertuzumab was designed [67].

1.5.2.3 Pertuzumab

The humanized monoclonal antibody pertuzumab used in combination with trastuzumab and chemotherapy agent docetaxel is now considered to be first-line therapy for the treatment of HER2-overexpressing metastatic breast cancer. This triple-drug combination also has clinical benefit as neoadjuvant therapy for HER2-positive breast cancer patients with locally advanced, inflammatory, or early-stage breast cancer [65,67]. In contrast to trastuzumab, pertuzumab binds to the extracellular domain II of HER2, the dimerization epitope of this receptor leading to inhibition of ligand-activated HER2 homo- and heterodimerization and subsequent downstream signalling [63,65–67]. Pertuzumab especially targets the potent HER2/HER3 interaction and efficiently prevents its dimerization [63,67]. Administration of pertuzumab not only results in inhibition of HER2 downstream signalling but also induces apoptosis and ADCC [63,66,67]. The combined therapy of pertuzumab and trastuzumab exhibits synergistic effects in HER2-overexpressing breast tumours leading to inhibition of HER2-associated growth and enhanced antitumor activity as compared to single-drug treatment [63,65–67].

1.6 Rationale and hypothesis

Previous studies in our laboratory involving breast cancer tissues showed low IFN-γ -inducible HLA expression on breast carcinoma even in the presence of high IFN-γ levels suggesting a dysregulation of HLA expression [22]. TNBC and HER2-overexpressing breast cancers had the highest HLA expression among the four breast cancer subtypes followed by luminal A (ER+HER2-) and luminal B (ER+HER2+). Notably, luminal B breast cancers did not show any HLA-DR expression while the expression levels in the other breast cancer subtypes ranged from 30-60% [68]. Another study from our laboratory revealed reduced IFN-γ response in BT-474 (ER+ HER2+) and reduced IFN-γ-inducible HLA expression as compared to MCF-7 (ER+HER2-) and T47D (ER+HER2-) (Matthew Young, BSc Honors thesis and unpublished data).

Similarly, downregulation of HLA Class I expression is a common mechanism by which cancer cells escape immune recognition. Moreover, HER2 expression has been linked to an immune escape phenotype [69,70].

Defective IFN-γ signalling not only prevents IFN-γ-induced HLA Class I upregulation and, thereby impairing immune responses to cancer, but has also been associated with resistance to checkpoint immunotherapy in melanoma. Downregulation or loss of HLA Class I has clinical implications as HLA Class I-low cancers have been correlated with less TILs, worse clinical outcome, resistance to adoptive immunotherapy, and metastases progression during immunotherapy [71].

Based on these previous reports, we hypothesized that **HER2 overexpression in ER+ breast cancer may interfere with activation of the IFN-γ pathway, and consequently HLA upregulation**.

Objectives:

1. Compare IFN-γ signalling kinetics by assessing expression levels of IFNGR1, IFNGR2, HER2 and IFN-γ secondary response gene, HLA Class I, in BT-474 (ERα-HER2-overexpressing) and SKBR3 (HER2-overexpressing).
2. Compare the kinetics of IFN-γ-inducible HLA Class I expression in BT-474 and SKBR3 to that in MCF-7/HER218, a transfectant overexpressing ERα and HER2.
3. Compare IFN signalling by assessing HLA Class I expression following IFN-α and IFN-γ stimulation in HER2-overexpressing BCCLs.
4. Analyze alterations in IFN-γ pathway components in HER2-overexpressing BCCLs in response to:
    A) Endocrine therapy
    B) Small interfering RNAs (siRNAs) treatment
    C) Targeted therapy

2 Chapter 2: Materials and Methods

2.1 Cell culture

Human breast cancer cell lines were initially purchased from the American Tissue Culture Collection (ATCC) and include BT-474 (ATCC® Number: HTB-20) and SKBR3 (ATCC® Number: HTB-30) (Table 2.1). These cell lines were authenticated at The Center for Applied Genomics, Toronto, Canada. MCF-7/HER218 and its control transfectant MCF7/neo were a kind gift from Dr. Rachel Schiff. MCF-7/HER2-18 was generated by transfecting MCF-7 cells with full-length HER2 cDNA, which exhibits a 45-fold greater p185HER2 surface receptor expression as compared to the parental and transfectant control (MCF-7/neo) while the ER content was identical to parental and control transfected cells [72]. The B-cell line SAVC was obtained from 10th International Histocompatibility Workshop.

All materials and chemicals used in this study are listed in Appendix 1.

Table 2.1: Human Breast Cancer Cell Lines used in this study [1,72–75].

Breast Cancer Cell Line	Type of Cancer	Classification	Molecular profile	Reference
MCF-7/neo	Adenocarcinoma	Vector transfectant	ER+, PR+, HER2	[72]
MCF-7/HER218	Adenocarcinoma	HER2 transfectant	ER+, PR+, HER2+	[72]
BT-474	Invasive Duct Carcinoma	Luminal B	ER+, PR+, HER2+	[73]
SKBR3	Adenocarcinoma	HER2	ER−, PR−, HER2+	[74]
SAVC	EBV-transformed lymphoblastoid line	Blood sample	Not applicable (N/A)	[75]

BCCL were grown as adherent cells in tissue culture dishes and maintained in complete medium (CM), which consists of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and antibiotic-antimycotic mixture, all from Invitrogen. Cells were grown at 37 °C in a 5% CO_2 atmosphere and refreshed with CM if required, prior to harvesting and re-seeding when 80-90% confluent, as determined by phase contrast microscopy.

To harvest the cells, medium was removed by aspiration, cells were washed in phosphate buffered saline (PBS), followed by addition of 0.25%-Trypsin-EDTA to detach the cells. After detachment, trypsin was deactivated by CM, cells were pipetted back and forth, then added to a 15 mL conical tube and centrifuged at 1400 rpm for 5 minutes at 10°C. The supernatant was decanted, the cell pellet was washed once more, then resuspended in 6 mL CM and the cell number was determined using a hemocytometer and phase contrast microscopy. Cells were reseeded into a 10 cm tissue culture dish (Falcon) for continuous cell culture with 10ml CM or set up in a 6-well plate at $5x10^5$ cells/well for BT-474, at $3.5x10^5$ cells/well for SKBR3 and at $2.5x10^5$ cells/well for MCF-7/neo, and MCF-7/HER218 for experimental treatments. The B-cell line SAVC was cultured in suspension with CM.

2.2 Treatment

2.2.1 IFN-γ time course

To assess the influence of HER2 overexpression on IFN-γ signalling and subsequent HLA Class I expression a time course was performed in which BT-474, SKBR3, MCF-7/neo and MCF-7/HER218 were treated with human recombinant IFN-γ for 15 minutes, 30 minutes, one hour, six hours, 12 hours, 24 hours, 48 hours and 72 hours. Cells were harvested and seeded as described in Section 2.1, allowed to adhere for 24 hours, after which IFN-γ (100 units/mL) was added and left to incubate for the appropriate time point. Unstimulated cells at 24 hours and 72 hours served as controls. B-cell line SAVC was used as control to assess HLA induction by IFN-γ and functionality of HLA-binding antibodies. The concentration of IFN-γ is based on optimization experiments performed by Dr. Drover's laboratory for every new batch of IFN-γ.

2.2.2 IFN-α and IFN-γ stimulation

To determine the effect of IFN-α and IFN-γ on HLA Class I induction, SKBR3 and BT-474 were treated with human recombinant IFN-α and IFN-γ. Cells were harvested and seeded as described in Section 2.1, allowed to adhere for 24 hours and stimulated with IFN-α (100 units/mL) or IFN-γ (100 units/mL) for 24 hours. Unstimulated cells at 24 hours served as a control. IFN-α was kindly gifted to us from Dr. Hirasawa and concentration was optimized by Dr. Hirasawa's laboratory.

2.2.3 Targeted knockdowns

Endocrine and targeted therapy drugs were used to interrupt ER and/or HER2 signalling in order to determine the effect of ER and/or HER2 knockdown on activation of the IFN-γ pathway in BT-474 and SKBR3 cells. Cells, seeded as described in Section 2.1, were incubated for 24 hours, followed by treatment with TAM, ICI, Lap for 24 hours. For dual-targeted therapy with trastuzumab and pertuzumab (T/P) BT-474 and SKBR3 cells were plated into a 6-well plate at $3x10^5$ cells/well and $2x10^5$ cells/well, respectively, and allowed to adhere for 24 hours before cells were treated with drugs for 24, 48, 72 and 96 hours. Drug concentrations are shown in Table 2.2.

Table 2.2: Endocrine and targeted drug concentrations for the treatment of BCCLs BT-474 and SKBR3.

Breast Cancer Cell Line	**Endocrine drug concentrations**		**Targeted drug concentrations**	
	Tamoxifen	**Fulvestrant (ICI)**	**Lap**	**T/P**
BT-474	10^-6 M	10^-6 M	0.5 µM	10 µg/mL each
SKBR3	10^-6 M	10^-6 M	0.5 µM	10 µg/mL each

The concentration of ICI, TAM, and Lap were based on optimization experiments performed by Ahmed Mostafa, a former PhD student, and other students in Dr. Drover's laboratory. The concentration of T/P was based on physiologically relevant values. Two 6-well plates were set up for each cell line and treated with endocrine and targeted drugs. One plate was stimulated with IFN-γ (100 units/mL) one hour after drug treatments, while the other served as a control and to assess the effect of drug treatments on constitutive HLA Class I expression. All experiments included untreated cells not exposed to any IFN-γ or drugs but to an equal concentration of diluents, ethanol, dimethyl sulfoxide (DMSO) or PBS, used in preparation of the drugs.

To distinguish the effect of T/P on IFN-γ signalling from the effect of IFN-γ treatment itself, experiments included PBS controls treated with IFN-γ to match drug treatments with IFN-γ for all the time points tested.

2.2.4 Knock down using small interfering RNAs (siRNA's)

Knockdowns were performed to assess effects of HER2 and/or ER loss and moreover, to determine the effect of the knockdowns on IFN-γ pathway components. Therefore, BT-474 and SKBR3 cells were harvested and plated (2 plates/cell line) in CM without antibiotics at $3x10^5$ cells/well in 6-well plates or at $5x10^4$ cells/well in 24-well plates and allowed to adhere for 24h. BT-474 cells were treated with siRNA for ESR1 (targeting ER) and ErbB2 (targeting ErbB2), a combination of both siRNA's, non-targeting siRNA (scrambled) or 1x siRNA buffer (Mock). Since SKBR3 only expresses HER2, these cells were treated with siRNA for ErbB2, scrambled siRNA and Mock. Both targeting and scrambled siRNA were re-suspended in 1x siRNA buffer to create a stock concentration of 20 µM, which was then aliquoted and stored at -80°C. At the time of the knockdown siRNA aliquots were thawed and diluted in 1x siRNA buffer to a final concentration of 5 µM. The transfection complex was prepared by diluting siRNAs or 1x siRNA buffer in DharmaFECT cell culture reagent (DCCR) (1/20 dilution). In a second tube, DharmaFECT 1 (all purpose transfection reagent) was diluted in DCCR (1/50 dilution) and incubated for 5 minutes at room temperature. The contents of both tubes were then mixed by pipetting and allowed to incubate for 20 minutes at room temperature. The appropriate volume of antibiotic-free CM was added to each tube to generate a total volume of 2 mL for 6-well plate or 50 0µL for 24-well plate. The medium in the 6-well or 24-well plate was then aspirated, and the transfection complex was added (and 2 mL/well for 6-well plate and 500 µL/well for 24-well plate). The following day, the transfection complex

was replaced with regular CM in one plate, which served as a control, and with CM containing 100 units of IFN-γ in the other plate.

2.3 Western blot

2.3.1 Whole cell lysates (WCLs)

Radioimmunoprecipitation assay (Ripa) buffer (PBS pH 7.4, NP-40 1%, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate) was completed by adding the following protease inhibitors: aprotinin (5 mg/mL), leupeptin (10 mg/mL), pepstanin A (2 mg/mL), phenylmethylsulfonyl fluoride (PMSF) (2 mg/mL) and a Halt Phosphatase Inhibitor cocktail and then used for cell lysis.

To prepare WCLs, medium was removed by vacuum from each well, followed by washing with ice cold sterile PBS. The PBS was aspirated and depending on the cell confluence, 100 µL, 150 µL or 200 µL of complete Ripa buffer was added to each well of 6-well plate while 100 µL of complete Ripa buffer was added to the wells of the 24-well plate. A plastic cell scraper was used to detach the cells in 6-well plate, whereas the cells in 24-well plate were detached by pipetting up and down. The lysate was collected into 1.5 mL Eppendorf tubes and centrifuged for 15 minutes at 14,000xg at 4°C. The supernatant was transferred into new Eppendorf microcentrifuge tubes and stored at -80°C.

2.3.2 Protein assay

To determine the protein concentration of each lysate, reagents and standards from the bicinchoninic acid (BCA) assay kit were used. To prepare standards, bovine serum albumin (BSA) was diluted to concentrations of 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL and 31.2 5µg/mL in distilled H_2O (dH_2O). Samples were also diluted in dH_2O at a ratio of 1:5 for the lysates from the knockdown with siRNA's and at a ratio of 1:10 for the lysates from the targeted knockdown. Standard and sample (50 µL) were transferred to plastic centrifuge tubes. Standards were done in duplicates. Reagent A and B were mixed in a 50:1 ratio. One millilitre of mixture was added to each tube, incubated for 30 minutes at 37°C in a water bath, contents transferred to 1.5 mL plastic cuvettes and read at 562 nm in a spectrometer.

2.3.3 Sodium dodecyl sulfate (SDS) gel electrophoresis

A discontinuous polyacrylamide gel, consisting of 8% resolving gel and a stacking gel, was prepared to separate protein samples. Once solidified, gels were loaded with protein samples and then placed in a cell electrophoresis chamber filled with 1x running buffer (25 mM Tris HCL pH 8.8, 0.1% SDS, 190 mM glycine) and run at 100 V for two hours.

For sample preparation an appropriate amount of lysate was diluted in reducing buffer (2-mercaptoethanol, 5% SDS, 10% glycerol and bromphenol blue to color) to a final concentration of 10 µg/mL of protein and denatured by boiling at 100°C for 7

minutes. To determine the protein size a pre-stained protein ladder was loaded as a standard.

2.3.4 Electrophoretic transfer

After electrophoresis, the proteins were transferred onto nitrocellulose membranes. Filter paper, membranes and transfer pads were soaked in transfer buffer (25 mM Tris HCL pH 8.8, 0.1% SDS, 190 mM glycine, 20% methanol) for 15 minutes prior to use. Gel was removed from cell electrophoresis chamber and stacking gel was separated from resolving gel. The remaining gel was then placed in the plastic grid with the following sandwich arrangement: transfer pad, 2 sheets of filter paper, gel, membrane, 2 sheets of filter paper, transfer pad. Grids were then put into Trans-Blot cell filled with transfer buffer. A small stir bar and an ice tray were placed into Trans-Blot cell and transfer was carried out at 100 V for one hour.

2.3.5 Immunoassay

Once the protein transfer was completed, the membranes were removed and incubated in blocking buffer (5% milk powder in Tris buffered saline (TBS)-Tween (0.15M sodium chloride (NaCl), 0.05M Tris pH 7.4-7.6, 0.05% Tween 20)) for one hour on a rotator. Primary antibodies (Table 2.3) were diluted in blocking buffer, added to trays containing membranes and left rocking overnight at 4°C. The membranes were then washed three times with TBS-Tween (TBS-T) for 5 minutes each while mixing on shaker. Horseradish peroxidase (HPR) conjugated secondary antibodies (Table 2.4) were

prepared in blocking buffer, added to membranes and left to incubate at room temperature for 1 hour on rotating mixer. Following the incubation period the membranes were washed again with TBS-T for 10 minutes each. Chemiluminescent HPR substrate was used for signal detection. Blots were imaged on ImageQuant LAS 4000 and protein band densities were determined using spot density analysis software (ImageQuant TL8.1). Antibodies against housekeeping proteins, GAPDH and α-Tubulin, were used as controls to ensure equivalent protein loading in each lane.

2.3.6 Re-probing membranes

To probe for multiple targets on the same blot membranes were washed three times for 10 minnutes with stripping buffer (TBS pH 2.0) followed by three washes with TBS-T for 10 minutes each.

2.3.7 Interpretation of western blot data

Protein expression was semi-quantified by scanning densitometry using image GE software (GE Healthcare). The relative expression was calculated as follows: density of relevant protein / density of loading control.

Table 2.3: Primary antibodies used for western blotting.

Antibody	Clone	Isotype	Concentration	Sources
α-Tubulin	B7	Mouse IgG2a	1/800	Santa Cruz
Phospho-AKT (Ser473) XP	D9E	Rabbit IgG	1/1000	Cell Signalling
AKT (pan)	C67E7	Rabbit IgG	1/1000	Cell Signalling
GAPDH	6C5	Mouse IgG1	1/20,000	Abcam
IRF-1	20	Mouse IgG1	1/1000	BD Pharmigen
Phospho-HER2 (pTry1248)	PN2A	Mouse IgG1	1/1000	ThermoFisher Scientific
HER2	e2-4001	Mouse IgG1	1/1000	ThermoFisher Scientific
HLA Class I	HC10	Mouse IgG2a	1/1000	Nordic-Mubio
ERα	HC-20	Rabbit IgG	1/800	Santa Cruz
Phospho-ERK (Try204)	E-4	Mouse IgG2a	1/500	Santa Cruz
ERK-1	K-23	Rabbit IgG	1/5000	Santa Cruz
Phospho-STAT-1 (Tyr701)	4a	Mouse IgG2a	1/1000	BD Pharmigen
STAT-1 (Clone 1/STAT-1)	1/STAT-1	Mouse IgG1	1/1000	BD Pharmigen

Table 2.4: Secondary antibodies used for western blotting.

Antibody	Clone	Isotype	Concentration	Sources
HRP-conjuagted affiniPure $f(ab)_2$ fragment goat anti-mouse (GAM) Fc specific	Polyclonal	Goat IgG	1/10,000	Jackson ImmunoResearch
HRP-conjuagted affiniPure $f(ab)_2$ fragment goat anti-rabbit (GAR) Fc specific	Polyclonal	Goat IgG	1/10,000	Jackson ImmunoResearch

2.4 Flow cytometry

2.4.1 HLA Class I status of BCCL and HLA Class I detection

Three different HLA Class I antibodies were included in flow cytometric analysis of IFN-γ time course experiments of BCCLs (Section 2.2.1) to assess the effect of HER2 overexpression on expression levels of different HLA Class I alleles. HLA Class I antibody specificities, HLA Class I status of BCCLs based in TRON database and expected recognition of BCCLs expressed HLA Class I alleles by HLA Class I antibodies are shown in Table 2.5–Table 2.8. HLA Class I status of MCF-7 transfectant (MCF-7/HER218) and its vector control (MCF-7/neo) is expected to be same as their parental control MCF-7, which was used in TRON database.

Table 2.5: HLA Class I antibody specificities [76–78].

HLA Class I Type	HLA Class I (W6/32)	HLA Class I (HCA2)	HLA Class I (HC10)
A	All HLA A alleles	HLA heavy chains (except HLA-A24)	HLA-A10, HLA-A28-HLA-A33
B	All HLA B alleles	N/A	All HLA B alleles
C	All HLA C alleles	N/A	All HLA C alleles

Table 2.6: HLA Class I status of BT-474 and expected recognition by HLA Class I antibodies.

HLA Class I Type	Allele 1	Allele 2	HLA Class I (W6/32)	HLA Class I (HCA2)	HLA Class I (HC10)
A	29:01	N/A	++	++	+
B	07:02’	N/A	++	N/A	++
C	07:02	16:01’	++	N/A	++

Table 2.7: HLA Class I status of SKBR3 and expected recognition by HLA Class I antibodies.

HLA Class I Type	Allele 1	Allele 2	HLA Class I (W6/32)	HLA Class I (HCA2)	HLA Class I (HC10)
A	02:01’	02:01++++	++	++	N/A
B	14:02’	39/50/55	++	N/A	++
C	03:04’	03:04++++	++	N/A	++

Table 2.8: HLA Class I status of MCF-7 and expected recognition by HLA Class I antibodies.

HLA Class I Type	Allele 1	Allele 2	HLA Class I (W6/32)	HLA Class I (HCA2)	HLA Class I (HC10)
A	02:01’	02:01	++	++	N/A
B	18:01	44:02	++	N/A	++
C	05:01	05:01	++	N/A	++

2.4.2 Cell surface protein expression by flow cytometry

Flow cytometry was used to assess the effect of cell treatments (Section 2.2) on cell surface expression of IFNGR1, IFNGR2, HLA Class I and HER2 receptor in BT-474, SKBR3, MCF-7/neo and MCF-7/HER218.

Adherent cells in experimental wells were harvested and counted as described in section 2.1. Cells were washed once with fluorescence activated cell sorting (FACS) buffer. After resuspending cells, 100 µL containing a minimum of $1x10^5$ cells was added to 5mL polystyrene round-bottom tubes. 25 µL of primary antibody (Table 2.9), optimally diluted in FACS buffer, was added to appropriate tube and incubated at 4^oC on ice for 30 minutes. Cells were then washed twice by adding FACS buffer and centrifuged at 1550 rpm for 5 minutes each. 25 µL of secondary antibody (R-Phycoreythrin (PE)-conjuagted affiniPure $f(ab)_2$ fragment goat anti-mouse (GAM) Fcγ fragement, 1/40 dilution, Jackson ImmunoResearch) was added to each tube and then incubated for 30 minutes in the dark at 4^oC. Following two washes with FACS buffer, 150 µL of 1% paraformaldehyde (PFA) diluted in PBS was added to each tube. Cell analysis of 10,000 events was performed by FACSCalibur flow cytometer and Kaluza Software.

2.4.3 Intracellular protein expression by flow cytometry

Intracellular expression of IFNγR1, IFNγR2, HLA Class I and HER2 receptor on BT-474, SKBR3, MCF-7/neo and MCF-7/HER218 was detected by flow cytometry to determine the effects of cell treatments (Section 2.2). Notably, intracellular flow cytometry, described below, is expected to mostly detect cytoplasmic proteins and only small amounts of nuclear proteins if at all.

Adherent cells were harvested and counted as described in section 2.1. Cells were then fixed in 2% PFA for 15 minutes on ice. Following fixation, the cells were washed once in IMDM and then washed in PBS. Permeabilization of cells was performed by incubating them in 0.2% Tween-20 in PBS for 15 minutes on ice. The cells were then washed in intracellular FACS buffer (0.5% FCS and 0,2% Tween-20 in PBS) and distributed into 5 mL polystyrene tubes round-bottom tubes as described in section 2.4.2. The remaining protocol was identical to cell surface flow cytometry except the primary antibodies (Table 2.9) and secondary antibody (PE-GAM) were diluted in intracellular FACS buffer.

2.4.4 Interpretation of flow cytometry data

To determine the amount of cell surface and intracellular expression of IFNGR1, IFNGR2, HLA Class I and HER2 receptor, mean fluorescence intensity (MFI) and percentage of positive (%+) cells were analyzed by Kaluza software. Protein expression was determined by using the following formula using Microsoft Excel 2011:

MFI Test – MFI Negative control.

Test results were considered positive if MFI was at least twice the background in three independent experiments.

For cell treatments with T/P the ratio of treated/untreated was obtained according to the following formula using Microsoft Excel 2011:

(MFI treatment – MFI Negative control) / (MFI PBS control– MFI Negative control)

Test values were compared to the values of the relative control, which were given the value of 1. The test values were then interpreted as follows:

Test value = 1: 'No effect'

Test value > 1: Positive or up-regulatory effect

Test value < 1: Negative or down-regulatory effect

Table 2.9: Primary antibodies and concentrations used for flow cytometry.

Antibody	Clone	Isotype	Concentration	Sources
CD119 (IFNGR1)	GIR-94	Mouse IgG2b	1/100	BioLegend
CD119 (IFNGR1)	GIR-94	Mouse IgG2b	1/100	BD Pharmigen
IFNGR2	MMHGR-2	Mouse IgG1	1/125	Abcam
IFNGR2	MMHGR-2	Mouse IgG1	1/125	LSBio
IgG1	P3.6.2.8.1	Mouse IgG1	1/200	eBioscience
IgG2a	NSG2a	Mouse IgG2a	1/200	Local Source
IgG2b	A-1	Mouse IgG2b	1/200	Southern Biotech
HER2	9G6	Mouse IgG1	1/200	Santa Cruz
HLA-ABC	W6/32	Mouse IgG2a	1/25	In house
HLA-BC	HC10	Mouse IgG2a	1/100	Nordic-Mubio
HLA-A	HCA2	Mouse IgG1	1/100	Nordic-Mubio

2.5 Statistical analysis

The average of three independent experiments is shown as mean ± standard errors of the mean (SEM). HER2 and/or ER inhibition experiments and IFN-α – IFN-γ comparison experiments were analyzed using one-way ANOVA combined with Tukey HSD test to compare cell treatment to control treatment within a single cell line. IFN-γ time course experiments were analyzed using two-way ANOVA paired with Tukey HSD test to compare cell treatment to control treatment between two or three different cell lines. Statistical analysis was carried out using GraphPad prism and a *p*-value of ≤ 0.05 was considered to be of significance.

3 Chapter 3: Results

3.1 Response to cytokine stimulation in HER2 overexpressing and luminal B BCCLs

3.1.1 Kinetics of the expression of IFN-γ receptors and HLA Class I molecules in IFN-γ treated BT-474 (HER2+ER+) and SKBR3 (HER2+ER-) cells

Previous studies in the Drover laboratory revealed reduced IFN-γ response in BT-474 (HER2+ER+) and reduced IFN-γ-inducible HLA expression as compared to other BCCL (Matthew Young, BSc Honors thesis and unpublished data). To explore this further, time course experiments were performed to compare expression of IFN-γ receptors and inducibility of HLA Class I expression in BT-474 and SKBR3 cells, untreated or treated with IFN-γ at the indicated times (Figure 3.1-Figure 3.3). All proteins were stained simultaneously for flow cytometry. Cells were analyzed to assess cell surface and intracellular expression of IFNGR1 and IFNGR2. Since overexpressed HER2 may contribute to immune escape by impairing expression of individual HLA Class I alleles [79,80], we also investigated expression of all HLA-ABC conformers (W6/32), HLA-A heavy chains (HCA2) and HLA-B and/or HLA-C heavy chains (HC10). HER2 expression was determined to assess whether it was altered by IFN-γ. SAVC, a B-cell line, was used to confirm functionality of all antibodies used in flow cytometry (Appendix 2).

3.1.1.1 Kinetics of IFNGR1 and IFNGR2 expression in IFN-γ treated BT-474 and SKBR3

The percentage (%) of IFNGR1 positive (+) cells and MFI of cell surface expression was greater on SKBR3 than on BT-474 while intracellular levels were almost identical (Figure 3.1A). Similar to literature reports [34–36], we found decreased %+ IFNGR1 cells at 15 minutes for both cell lines post IFN-γ stimulation inferring receptor mediated internalization. However, the kinetics of receptor internalization and re-expression were markedly different for each cell line. For example, the %+ IFNGR1 SKBR3 cells declined from time 0 to 15 minutes, increased at 30 minutes, peaked sharply at 1 hour, decreased at 6 hours, then increased to nearly basal levels by 24 hours, whereas for BT-474, the %+ IFNGR1 cells continued to decline up to 1 hour, peaked at 6 hours, decreased at 12 hours and increased at 24 hours. This was accompanied by fluctuating intracellular expression levels, peaking at 15 and 30 minutes for SKBR3 and BT-474, respectively, with both falling at one hour and rising at later time points.

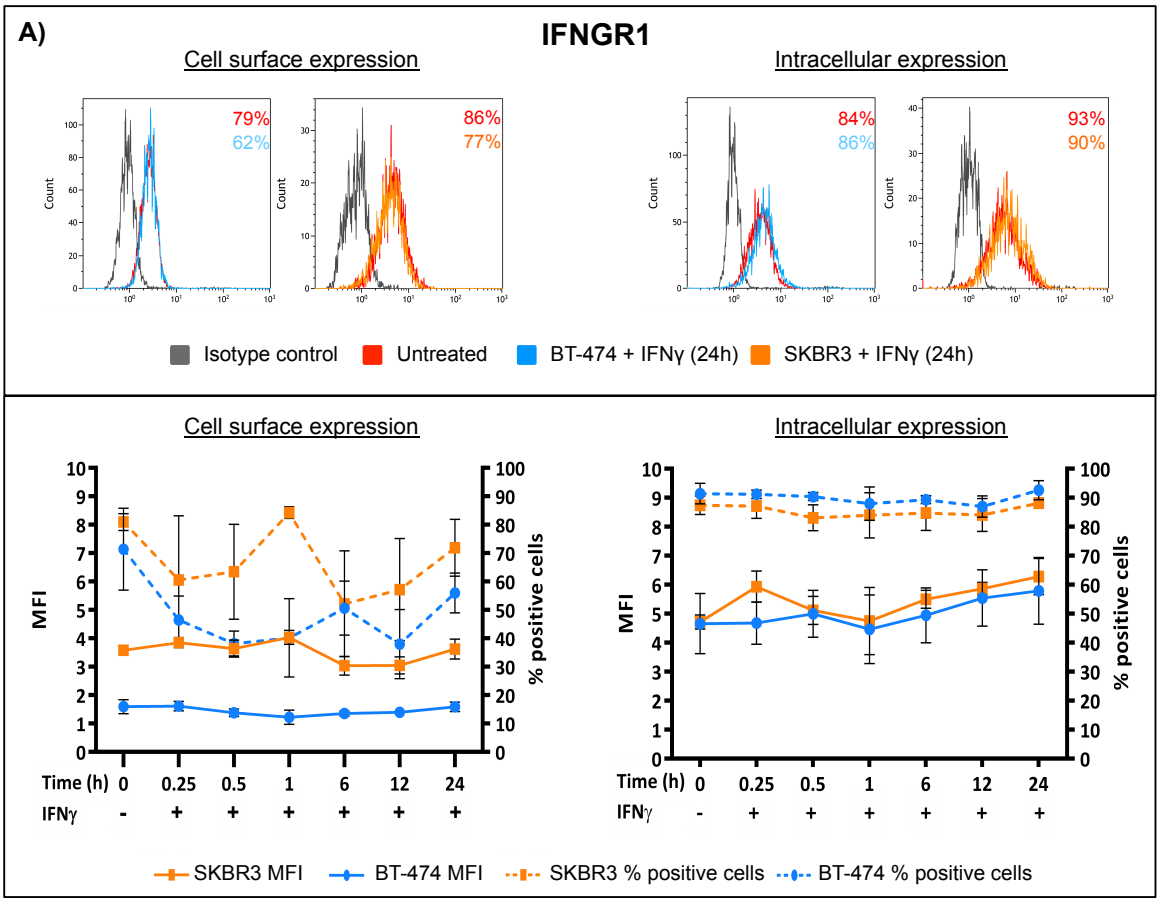
Cell surface IFNGR2 was significantly less on BT-474 (~5% + cells) compared to SKBR3, which weakly expressed IFNGR2 on 20-30% of cells (Figure 3.1B). Negligible surface expression was not unexpected as the antibody poorly detects naïve surface IFNGR2 [81,82] likely due to an insufficient amount of native IFNGR2 as suggested by Paus et al [82]. Based on our results the IFNGR2 antibody appears to bind better to intracellular INFGR2 as protein levels are higher inside the cells. Nearly 100% of SKBR3 and BT-474 cells contained high intracellular IFNGR2 levels with the latter displaying considerably higher amounts than SKBR3 (Figure 3.1B). Again, the kinetics were

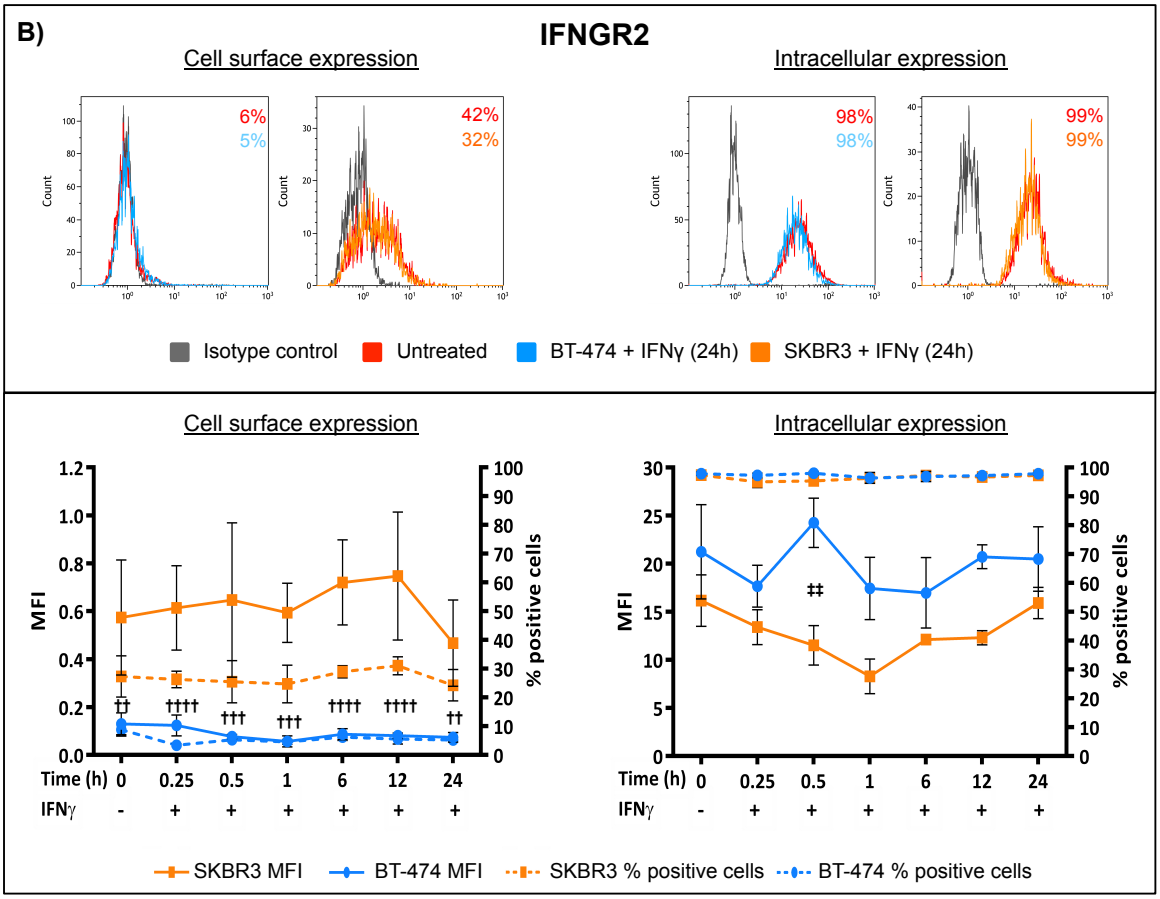
different, with levels in SKBR3 falling at 15, 30 and 60 minutes and then continuing to rise to t=0 hours levels by 24 hours, whereas levels in BT-474 decreased at 15 minutes, rose significantly at 30 minutes compared to SKBR3, decreased at 1 and 6 hours and then increased to nearly t=0 hours levels at 24 hours.

In summary, the reduced numbers and expression levels of surface IFNGR1 and IFNGR2 receptors on BT-474 as compared to SKBR3 may explain the reduced IFN-γ response that was previously identified by Young (Matthew Young, BSc Honors thesis and unpublished data).

Figure 3.1: BT-474 and SKBR3 differently modulate IFN-γ receptors in response to IFN-γ stimulation over time.

BT-474 and SKBR3 cells were treated with IFN-γ (100 U/mL) at the indicated time points and subjected to cell surface and intracellular flow cytometric analysis to assess IFNGR1 (A) and IFNGR2 (B) Top: Histogram overlays are representative of the 24 hour time point for one experiment. Percentage of positive cells is colour-matched accordingly. Bottom: Line graphs represent the mean MFI or %+ cells with error bars ± SEM of three independent experiments. Statistical analysis was done by two-way ANOVA and Tukey HSD test (‡ indicates a significant difference in MFI between cell lines, ‡‡$p<0.01$; † indicates a significant difference in % positive cells between cell lines, ††$p<0.01$, †††$p<0.001$, and ††††$p<0.0001$).


A)
IFNGR1
Cell surface expression
Intracellular expression
79%
62%
86%
77%
84%
86%
93%
90%
Count
Isotype control
Untreated
BT-474 + IFNγ (24h)
SKBR3 + IFNγ (24h)
Cell surface expression
Intracellular expression
MFI
% positive cells
Time (h) 0 0.25 0.5 1 6 12 24
IFNγ - + + + + + +
SKBR3 MFI
BT-474 MFI
SKBR3 % positive cells
BT-474 % positive cells


B)
IFNGR2
Cell surface expression
Intracellular expression
6%
5%
42%
32%
98%
98%
99%
99%
Count
Isotype control
Untreated
BT-474 + IFNγ (24h)
SKBR3 + IFNγ (24h)
Cell surface expression
Intracellular expression
MFI
% positive cells
Time (h) 0 0.25 0.5 1 6 12 24
IFNγ - + + + + + +
SKBR3 MFI
BT-474 MFI
SKBR3 % positive cells
BT-474 % positive cells

3.1.1.2 Kinetics of IFN-γ-induced HLA Class I expression in BT-474 and SKBR3

Compared to BT-474, IFN-γ-induced expression of HLA-ABC conformers is stronger on SKBR3. Furthermore, HLA-ABC surface expression on BT-474 remains low over the time course, whereas it is significantly increased on SKBR3 at 12 and 24 hours post IFN-γ treatment (Figure 3.2A, left panel). Intracellular HLA-ABC also increased in SKBR3 between 6 and 24 hours (Figure 3.2A, right panel). Interestingly, nearly 100% of BT-474 cells contained intracellular HLA-ABC molecules but were unaffected by IFN-γ stimulation. By contrast, the %+ HLA-ABC SKBR3 cells fell at 15 minutes post IFN-γ treatment and were even significantly reduced compared to BT-474; however, both, %+ cells and expression in SKBR3, increased at 12 and 24 hours, indicating responsiveness to IFN-γ stimulation.

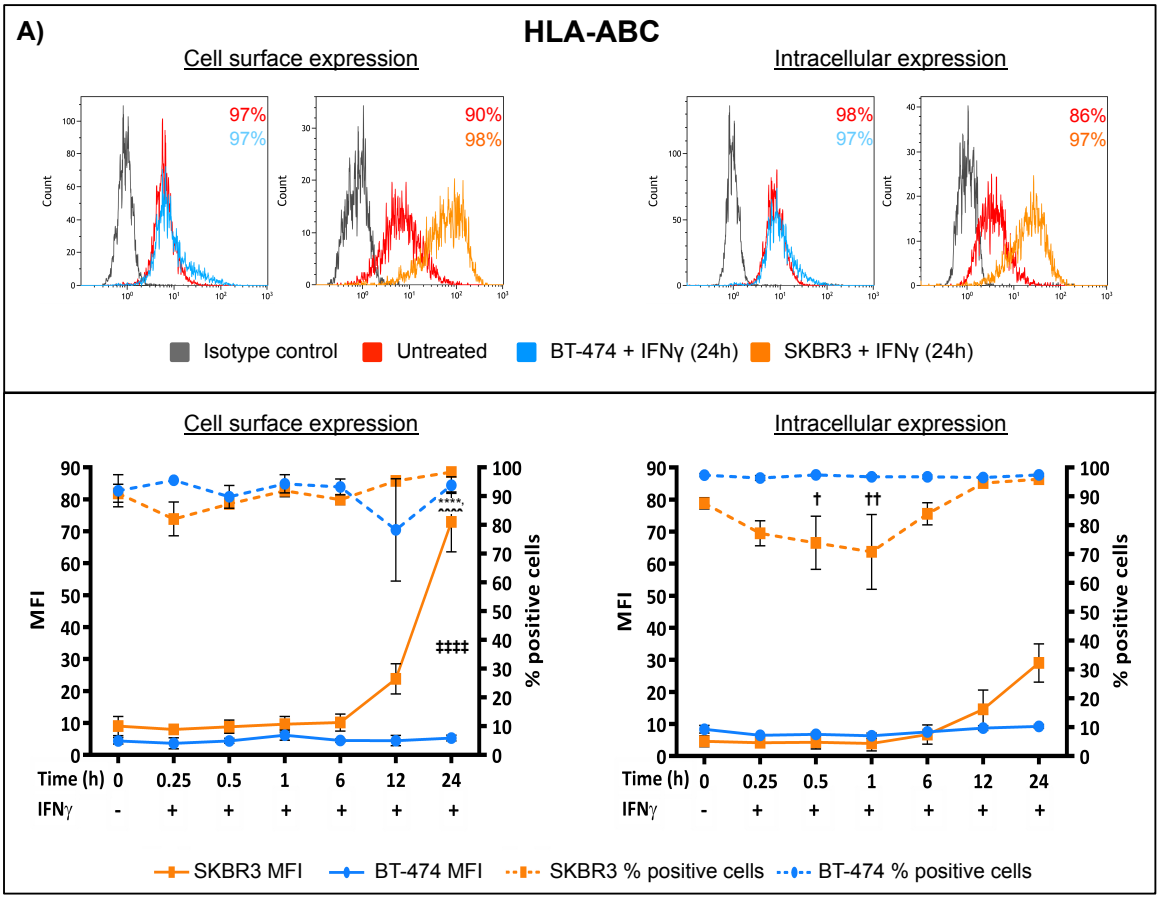
As shown in Figure 3.2B (left panel), BT-474 and SKBR3, untreated or treated with IFN-γ, displayed no cell surface HLA-A molecules. Possibly the HCA2 epitope is not detectable on the cell membrane of BCCL, although it was clearly present on SAVC cells (Appendix 2). HCA2 detected intracellular HLA-A expression in SKBR3 in a time-dependent manner, with the number of HLA-A positive SKBR3 cells significantly increasing at 12 and 24 hours as compared to untreated cells. BT-474 cells were negative for intracellular HLA-A (Figure 3.2B, right panel). The difference in IFN-γ-induced HLA-A expression and kinetics between the two cell lines is significant at 6, 12 and 24 hour time points.

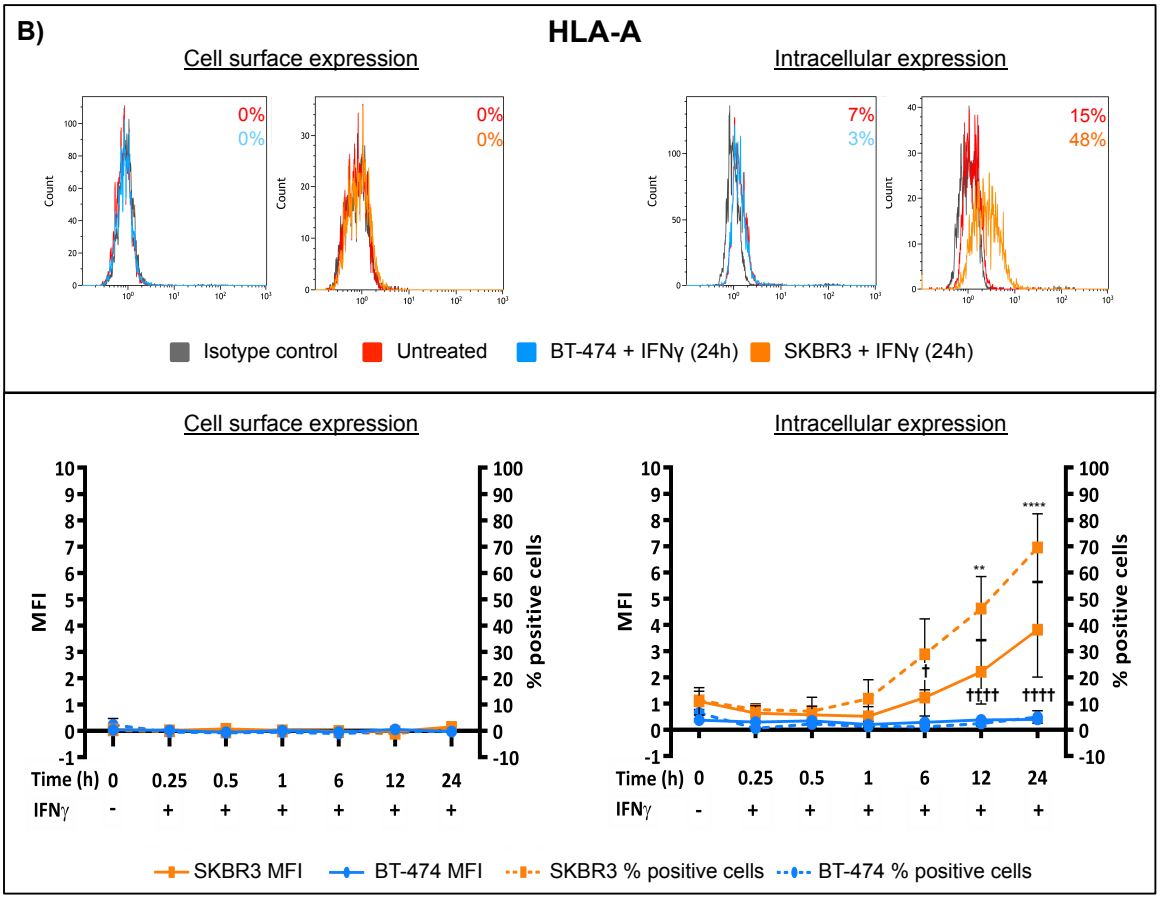
Similar to HLA-A, no or very low HLA-BC cell surface molecules were detected on BT-474 or SKBR3 (Figure 3.2C, left panel) and, as previously noted, they were highly expressed on SAVC (Appendix 2). Although intracellular levels of HLA-BC were detected in both cell lines, SKBR3, compared to BT-474, had significantly reduced expression and numbers of HC10 positive cells up to six hours post IFN-γ stimulation, after which HLA-BC expression surpassed that of BT-474. Thus, while BT-474 cells did not change expression in response to IFN-γ, SKBR3 cells clearly upregulated intracellular HLA-BC levels in a time-dependent manner (Figure 3.2C, right panel).

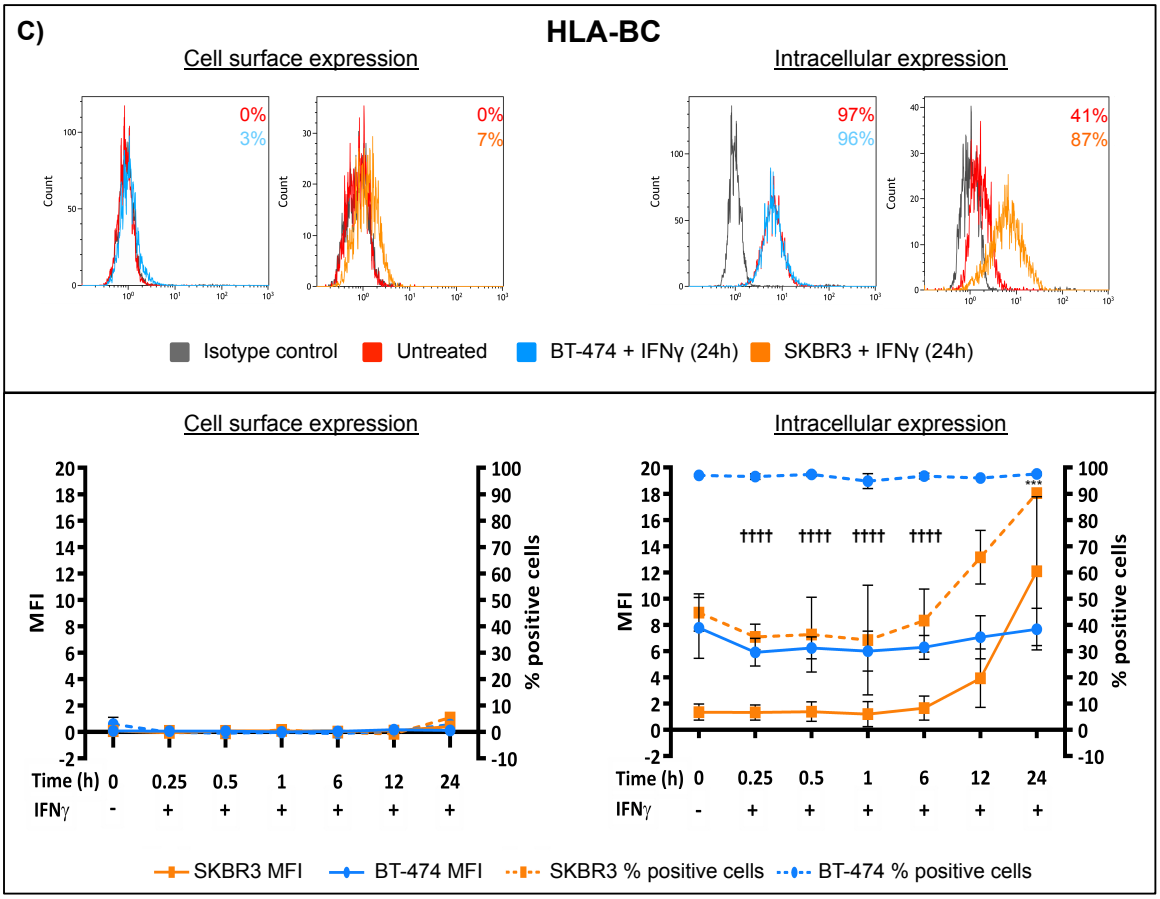
Taking all the data together, we conclude that total HLA Class I expression (HLA-ABC, HLA-A and HLA-BC) is poorly if at all induced by IFN-γ in BT-474, at least up to 24 hours.

Figure 3.2: BT-474 and SKBR3 differently modulate HLA Class I induction in response to IFN-γ stimulation over time.

BT-474 and SKBR3 cells were treated with IFN-γ (100 U/mL) at the indicated time points and subjected to cell surface and intracellular flow cytometric analysis to assess HLA-ABC (A), HLA-A (B), and HLA-BC (C) expression. Top: Histogram overlays are representative of the 24 hour time point for one experiment. Percentage of positive cells is colour-matched accordingly. Bottom: Line graphs represent the mean MFI or %+ cells with error bars ± SEM of three independent experiments. Statistical analysis was done by two-way ANOVA and Tukey HSD test (* indicates a significant difference in MFI or %+ cells within a single cell line compared to baseline (0 hours), **ρ<0.01, ***ρ<0.001; ****ρ<0.0001; ^ indicates a significant difference in MFI or %+ cells of an time point compared to its previous time point within a single cell line, ^^^^ρ<0.0001; ‡ indicates a significant difference in MFI between cell lines, ‡‡‡‡ρ<0.0001; † indicates a significant difference in %+ cells between cell lines, †ρ<0.05, ††ρ<0.01, and ††††ρ<0.0001).


A)
HLA-ABC
Cell surface expression
Intracellular expression
97%
97%
90%
98%
98%
97%
86%
97%
Count
Isotype control
Untreated
BT-474 + IFNγ (24h)
SKBR3 + IFNγ (24h)
Cell surface expression
Intracellular expression
MFI
% positive cells
Time (h) 0 0.25 0.5 1 6 12 24
IFNγ - + + + + + +
SKBR3 MFI
BT-474 MFI
SKBR3 % positive cells
BT-474 % positive cells


B)
HLA-A
Cell surface expression
Intracellular expression
0%
0%
0%
0%
7%
3%
15%
48%
Count
Isotype control
Untreated
BT-474 + IFNγ (24h)
SKBR3 + IFNγ (24h)
Cell surface expression
Intracellular expression
MFI
% positive cells
Time (h) 0 0.25 0.5 1 6 12 24
IFNγ - + + + + + +
SKBR3 MFI
BT-474 MFI
SKBR3 % positive cells
BT-474 % positive cells

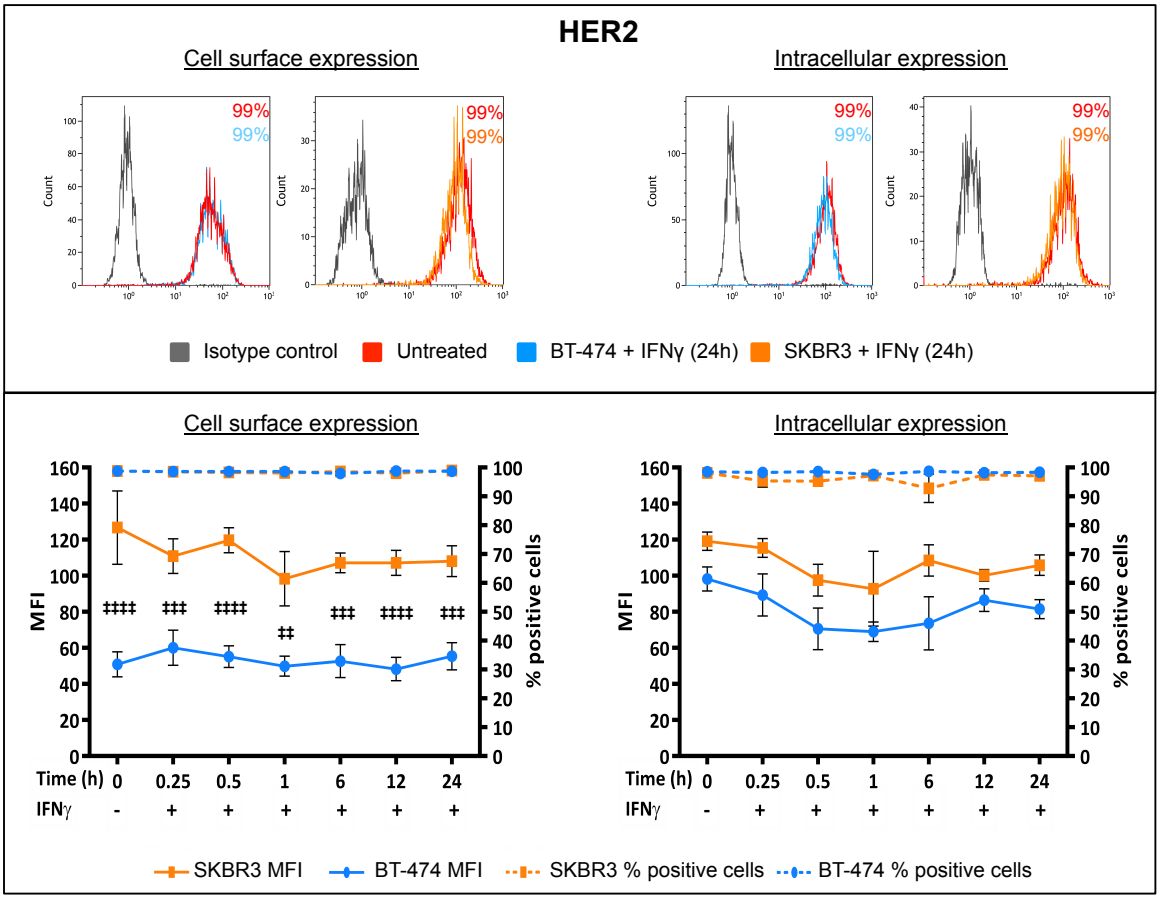

C)
HLA-BC
Cell surface expression
Intracellular expression
0%
3%
0%
7%
97%
96%
41%
87%
Count
Isotype control
Untreated
BT-474 + IFNγ (24h)
SKBR3 + IFNγ (24h)
Cell surface expression
Intracellular expression
MFI
% positive cells
Time (h) 0 0.25 0.5 1 6 12 24
IFNγ - + + + + + +
SKBR3 MFI
BT-474 MFI
SKBR3 % positive cells
BT-474 % positive cells

3.1.1.3 Effect of IFN-γ on HER2 expression in BT-474 and SKBR3

Nearly all SKBR3 and BT-474 cells expressed cell surface HER2 with significantly higher amounts on SKBR3 at all time points tested. A similar trend was detected for intracellular HER2 expression in both cell lines. IFN-γ did not affect surface or intracellular HER2 levels in either cell line over the time course. Interestingly, the amounts of surface and intracellular HER2 in SKBR3 were comparable, whereas intracellular HER2 was higher than surface expression for BT-474 (Figure 3.3).

Figure 3.3: BT-474 and SKBR3 do not alter HER2 expression in response to IFN-γ stimulation over time.

BT-474 and SKBR3 cells were treated with IFN-γ (100 U/mL) at the indicated time points and subjected to cell surface and intracellular flow cytometric analysis to assess HER2 expression. Top: Histogram overlays are representative of the 24 hour time point for one experiment. Percentage of positive cells is colour-matched accordingly. Bottom: Line graphs represent the mean MFI or %+ cells with error bars ± SEM of three independent experiments. Statistical analysis was done by two-way ANOVA and Tukey HSD test (‡ indicates a significant difference in MFI between cell lines, ‡‡$p<0.01$, ‡‡‡$p<0.001$, and ‡‡‡‡$p<0.0001$).


HER2
Cell surface expression
Intracellular expression
99%
99%
Count
Isotype control
Untreated
BT-474 + IFNγ (24h)
SKBR3 + IFNγ (24h)
MFI
% positive cells
Time (h)
IFNγ
SKBR3 MFI
BT-474 MFI
SKBR3 % positive cells
BT-474 % positive cells

3.1.2 Kinetics of IFN-γ-induced HLA Class I expression in BCCLs with various levels of ER and HER2

The aims of this experiment were: 1) to extend the time course to 72 hours to determine a possible delayed response as there was little to no HLA Class I upregulation within 24 hours in BT-474; 2) to indirectly assess whether co-expression of ERα and HER2 altered IFN-γ-induced HLA Class I expression. Therefore, in addition to BT-474 (HER2+ER+) and SKBR3 (HER2+ER-), we included HER2 transfectant, MCF-7/HER218 (HER2+ER+), and its vector control transfectant MCF-7/neo (HER2-ER+). MCF-7/HER218, generated by transfecting MCF-7 cells with full-length HER2 cDNA, was reported to display a 45-fold greater p185HER2 surface receptor expression as compared to parental and vector control (MCF-7/neo) while the ERα content was identical to parental and control transfected cells [72]. The five cell lines were either unstimulated (t = 0 hours) or stimulated with IFN-γ for 24 and 72 hours, followed by flow cytometry to determine expression of HLA-ABC, HLA-A, and HLA-BC, respectively, as described in the previous section. Surface and intracellular expression of HER2 was analyzed on all cells.

3.1.2.1 IFN-γ-induced HLA Class I expression at 72 hours is significantly lower in BT-474 as compared to SKBR3

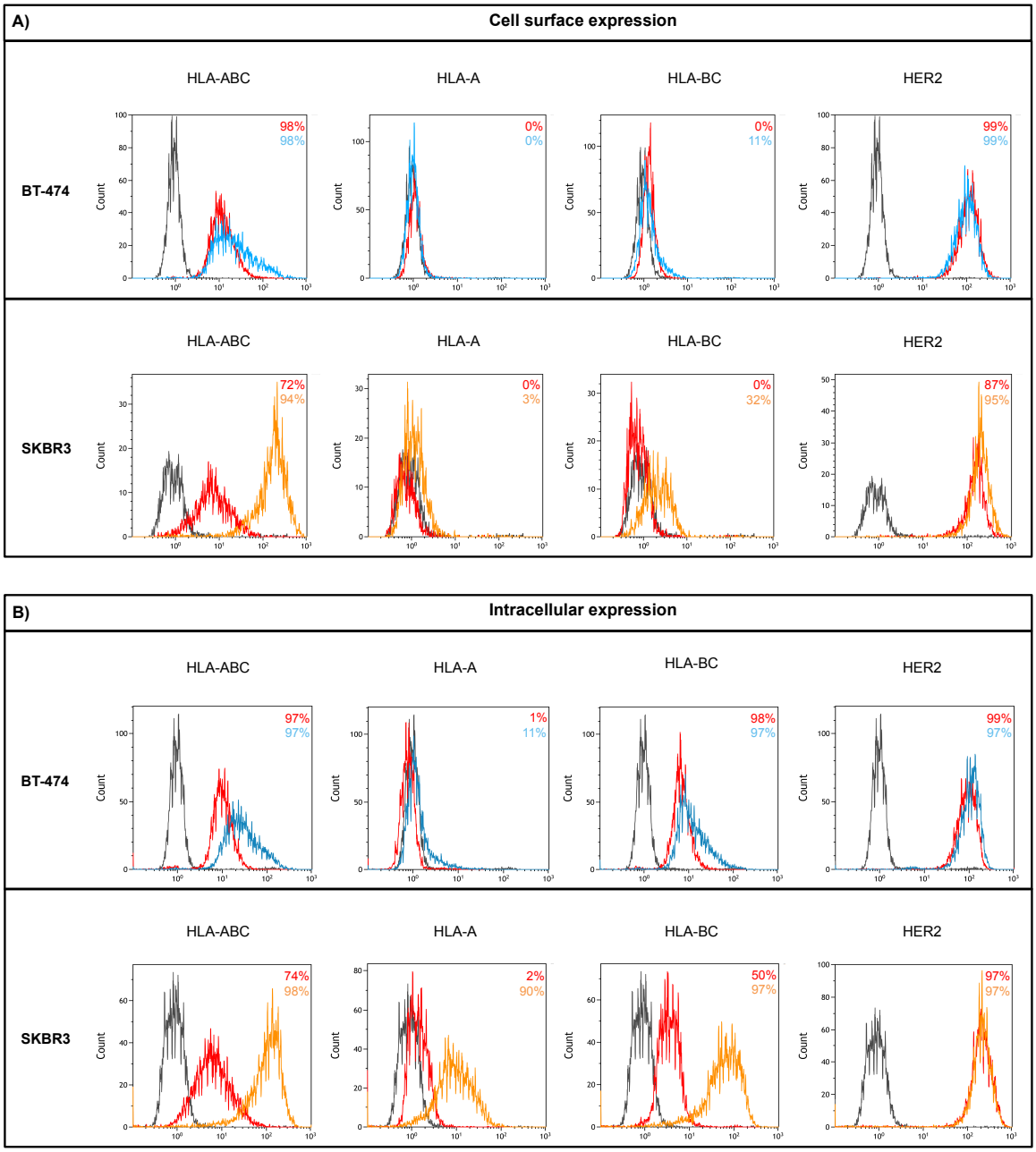
Consistent with results described in the previous section (Section 3.1.1.2), SKBR3 responds to IFN-γ stimulation in a time-dependent manner, with significantly increased numbers and levels of HLA-ABC positive cells, both at the cell surface and intracellularly (Figure 3.4C). Almost all BT-474 cells expressed some HLA-ABC conformers on their cell surface with the %+ cells in untreated samples significantly higher than in SKBR3; however, there was little IFN-γ-induced expression in BT-474 even at 72 hours. Cell surface HLA-A H-chains were not detected in either BT-474 or SKBR3 at any time point (Figure 3.4C) whereas significant intracellular expression was detected only in IFN-γ-treated SKBR3. Similarly, cell surface HLA-BC chains were not detected on BT-474 or SKBR3 constitutively or after IFN-γ stimulation (Figure 3.4C, left panel). Intracellular HLA-BC, detected in both cells, was significantly upregulated in a time-dependent manner only in SKBR3 cells (Figure 3.4C, right panel).

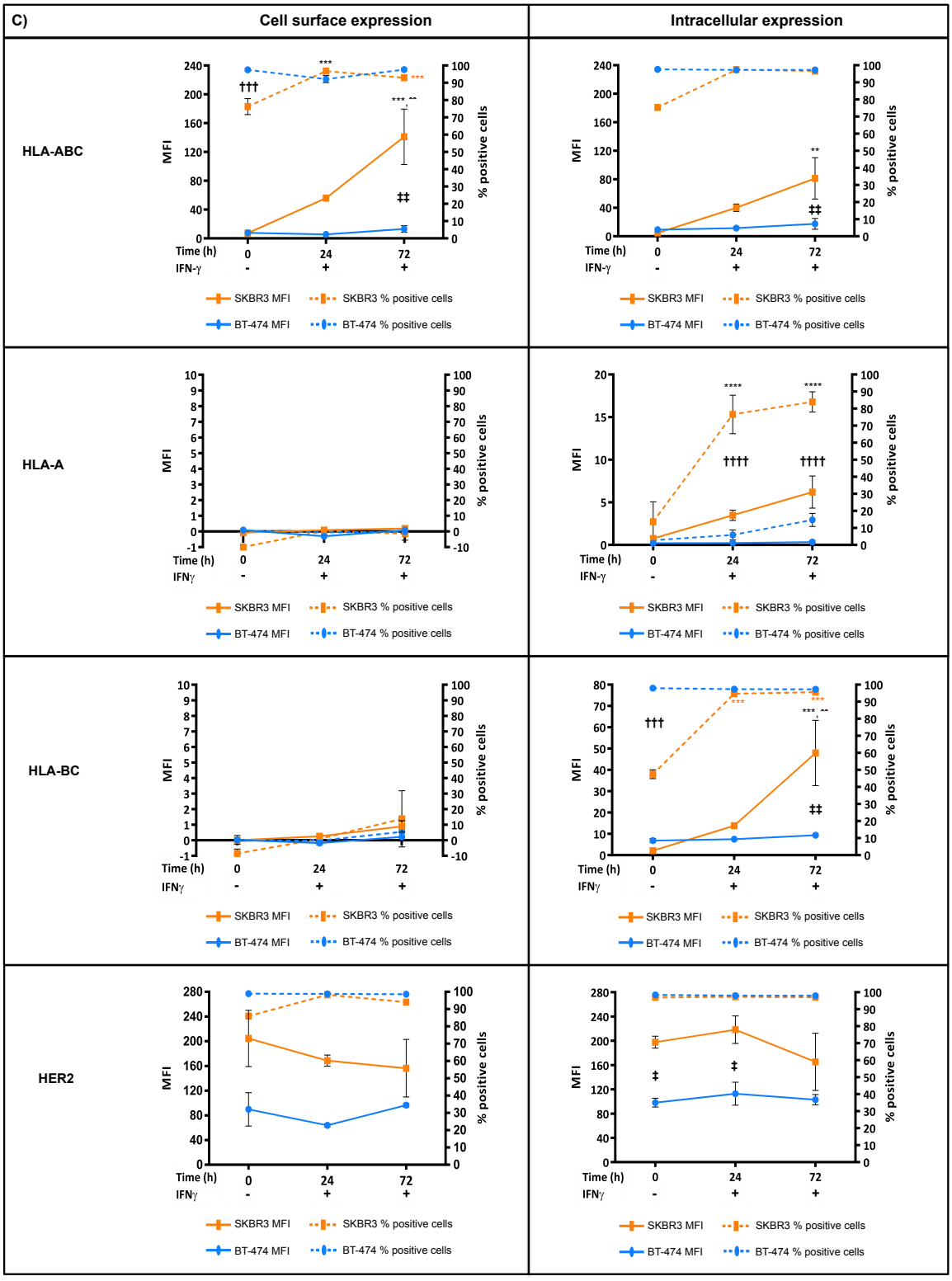
In summary, BT-474 compared to SKBR3, has significantly reduced IFN-γ-induced HLA-ABC even after 72 hours of stimulation. While surface expression of HLA-A and HLA-BC heavy chain molecules were not detected by the HCA2 and HC10 antibodies, respectively, positive binding by W6/32 which recognizes HLA-ABC conformers, suggests at least one type of HLA Class I molecule is present on the cell surface of these cells. As noted previously, SKBR3 has significantly higher surface (Figure 3.4C, left panel) and

intracellular (Figure 3.4C, right panel) HER2 expression than BT-474 suggesting that HER2 overexpression on its own may not be implicated in reduced global HLA expression.

Figure 3.4: HER2 overexpression does not correlate with decreased HLA expression observed in BT-474.

BT-474 and SKBR3 cells were stimulated or not with IFN-γ (100 U/mL) at the indicated time points. Cell surface and intracellular flow cytometry was used to determine expression of HLA-ABC, HLA-A, HLA-BC and HER2. Histogram overlays of cell surface (A) and intracellular (B) expression are representative of one experiment. The percentage of positive cells is colour-matched accordingly. C) Line graphs represent the mean MFI or %+ cells with error bars ± SEM of three independent experiments. Statistical analysis was done by two-way ANOVA and Tukey HSD test (* indicates a significant difference in MFI or %+ cells within a single cell line compared to baseline (0 hours), **ρ<0.01, *** ρ<0.001, ****ρ<0.0001; ^ indicates a significant difference in MFI or %+ cells of an time point compared to its previous time point within a single cell line, ^^ρ<0.01; ‡ indicates a significant difference in MFI between cell lines, ‡ρ<0.05, ‡‡ρ<0.01; † indicates a significant difference in %+ cells between cell lines, †††ρ<0.001 and ††††ρ<0.0001).


A)
Cell surface expression
HLA-ABC
HLA-A
HLA-BC
HER2
BT-474
98%
98%
0%
0%
0%
11%
99%
99%
SKBR3
72%
94%
0%
3%
0%
32%
87%
95%
Count
B)
Intracellular expression
HLA-ABC
HLA-A
HLA-BC
HER2
BT-474
97%
97%
1%
11%
98%
97%
99%
97%
SKBR3
74%
98%
2%
90%
50%
97%
97%
97%
Count


C)
Cell surface expression
Intracellular expression
HLA-ABC
HLA-A
HLA-BC
HER2
MFI
% positive cells
Time (h)
IFN-γ
IFNγ
SKBR3 MFI
SKBR3 % positive cells
BT-474 MFI
BT-474 % positive cells

3.1.2.2 Effect of overexpressing HER2 in ERα+ MCF-7 on HLA Class I expression

To address whether overexpression of HER2 in an ERα positive cell line modulates constitutive and IFN-γ-inducible HLA Class I expression, we used vector control MCF-7/neo and its transfectant MCF-7/HER218 described in section 2.1. Both MCF-7 cells expressed considerably more constitutive and IFN-γ-inducible HLA-ABC than SKBR3 or BT-474 (compare Figure 3.4C to Figure 3.5C). IFN-γ significantly induced cell surface expression over time or with similar kinetics in MCF-7/neo and MCF-7/HER218. Intracellular HLA-ABC levels (Figure 3.5C, right panel) were significantly upregulated by IFN-γ in both MCF-7 cell lines at 24 hours, however, by 72 hours they were still significantly trending upwards in MCF-7/HER218 as compared to declining levels in the vector control resulting in significantly less intracellular HLA-BC in MCF-7/neo than in the transfectant. The %+ HLA-ABC cells remained unchanged in both MCF-7 cell lines for cells surface and intracellular expression.

Cell surface expression of HLA-A was barely perceptible on either MCF-7 cell line at 72 hours post IFN-γ stimulation (Figure 3.5C, left panel) accompanied by low levels of %+cells. Contrastingly, intracellular HLA-A molecules significantly increased over time in MCF-7/HER218 and MCF-7/neo cells (Figure 3.5C, right panel), but there was no significant difference among the two cell lines. Expression of cell surface HLA-BC as well as %+ cells were increased in both IFN-γ-treated MCF-7 cell lines in a time dependent manner, but only %+ cells rose significantly (Figure 3.5C, left panel). Intracellular HLA-BC+ cells were observed constitutively, and expression levels were significantly

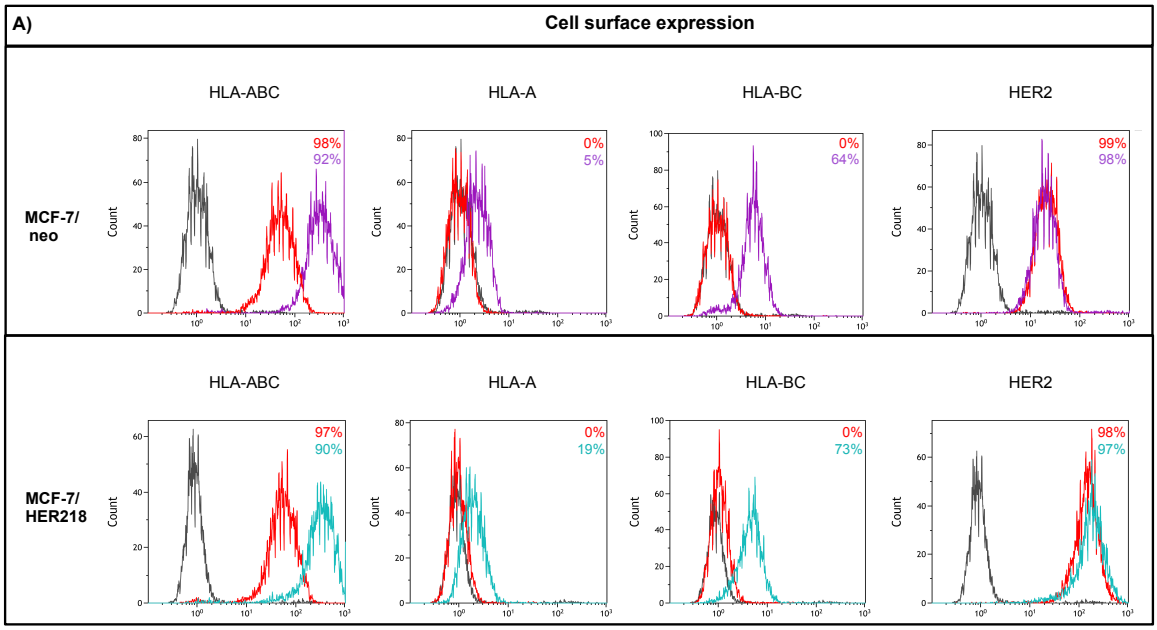
upregulated by IFN-γ stimulation in MCF-7/neo and MCF-7/HER218 (Figure 3.5C, right panel) over time.

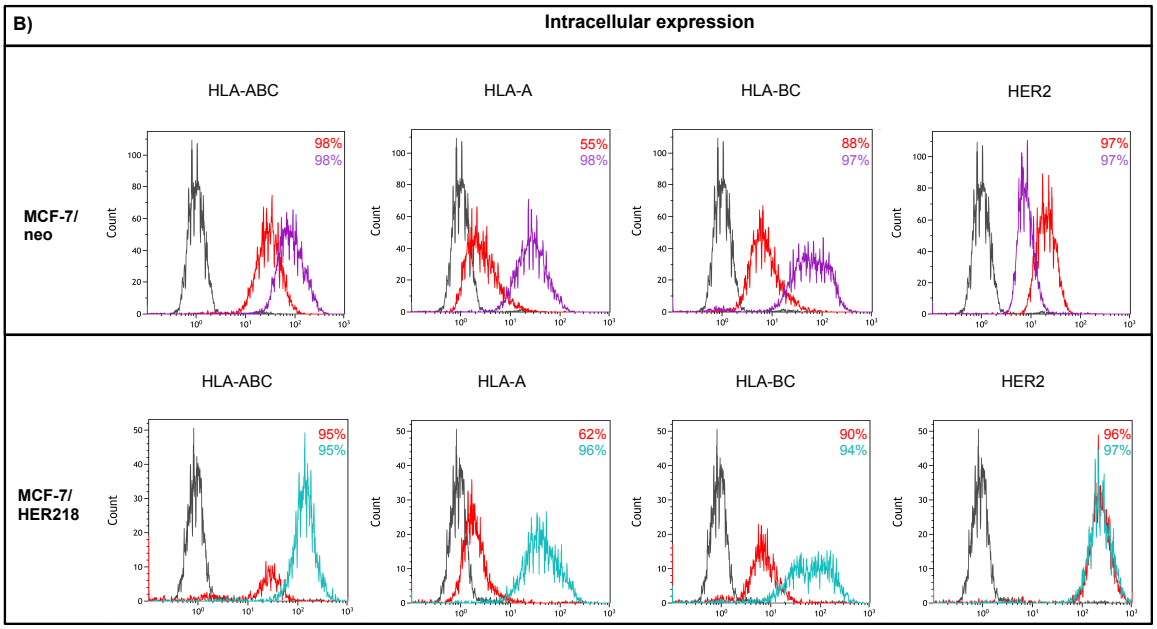
Cell surface and intracellular levels of endogenous HER2 were not markedly affected by IFN-γ treatment (Figure 3.5C). As expected, ectopic HER2 in MCF-7/HER218 cells was expressed at significantly higher surface and intracellular levels compared to endogenous HER2 in MCF-7/neo (Figure 3.5C). MCF-7/HER218 also displayed functional HER2 signalling as indicated by increased pHER2 levels compared to vector control MCF-7/neo (Appendix 3).

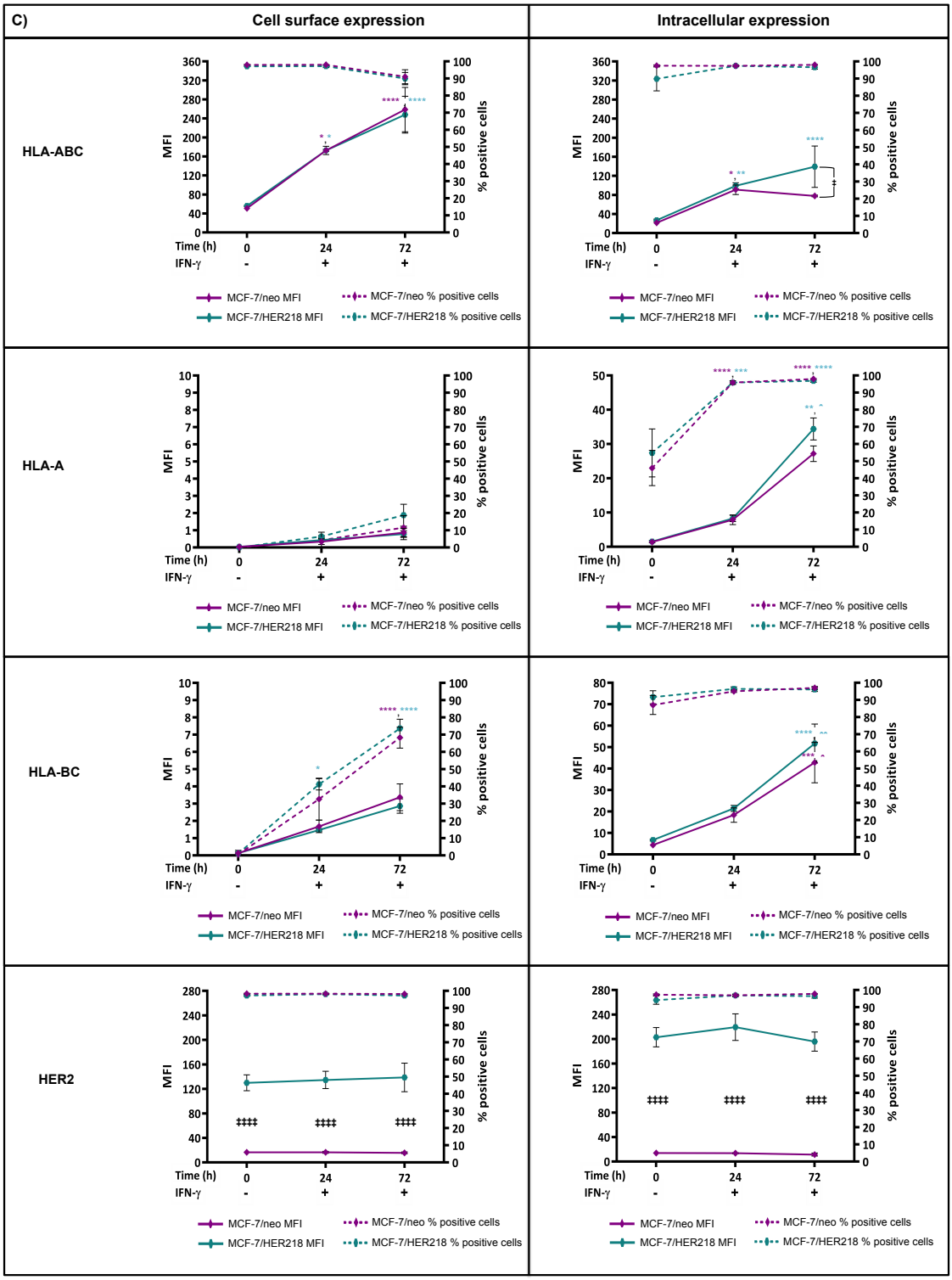
All together the results show MCF-7 cells, with and without overexpressed HER2, have considerably higher levels of constitutive and inducible HLA Class I molecules than SKBR3 or BT-474. Ectopic HER2 expression affected expression of IFN-γ-inducible HLA class conformers, by slightly reducing the surface while significantly increasing intracellular accumulation at 72 hours. A similar trend was observed for expression of HLA-A and HLA-BC heavy chain epitopes. Based on these data, we speculate that HER2 overexpression may have a sequestering effect on intracellular HLA class I molecules with long term IFN-γ induction.

Figure 3.5: Effect of ectopic HER2 overexpression on IFN-γ-inducible HLA Class I in MCF-7/HER128 cells.

MCF-7/neo and MCF-7/HER218 cells were stimulated or not with IFN-γ (100 U/mL) at the indicated time points. Cell surface and intracellular flow cytometry was used to determine expression of HLA-ABC, HLA-A, HLA-BC and HER2. Histogram overlays of cell surface (A) and intracellular (B) expression are representative of one experiment. The percentage of positive cells is colour-matched accordingly. C) Line graphs represent the mean MFI or %+ cells with error bars ± SEM of three independent experiments. Statistical analysis was done by two-way ANOVA and Tukey HSD test (* indicates a significant difference in MFI or %+ cells within a single cell line compared to baseline (0 hours), *ρ<0.05, **ρ<0.01, ***ρ<0.001, ****ρ<0.0001; ^ indicates a significant difference in MFI or %+ cells of an time point compared to its previous time point within a single cell line, ^ρ<0.05, ^^ρ<0.01, ^^^^ρ<0.0001; ‡ indicates a significant difference in MFI between cell lines, ‡ρ<0.05, ‡‡‡‡ρ<0.0001). Symbols are colour-matched accordingly.


A)
Cell surface expression
HLA-ABC
HLA-A
HLA-BC
HER2
MCF-7/ neo
98%
92%
0%
5%
0%
64%
99%
98%
MCF-7/ HER218
97%
90%
0%
19%
0%
73%
98%
97%
Count


B)
Intracellular expression
HLA-ABC
HLA-A
HLA-BC
HER2
MCF-7/ neo
98%
98%
55%
98%
88%
97%
97%
97%
MCF-7/ HER218
95%
95%
62%
96%
90%
94%
96%
97%
Count


C)
Cell surface expression
Intracellular expression
HLA-ABC
HLA-A
HLA-BC
HER2
MFI
% positive cells
Time (h)
IFN-γ
MCF-7/neo MFI
MCF-7/HER218 MFI
MCF-7/neo % positive cells
MCF-7/HER218 % positive cells

3.1.3 Comparison of IFN-α and IFN-γ induction of HLA Class I in BT-474 and SKBR3

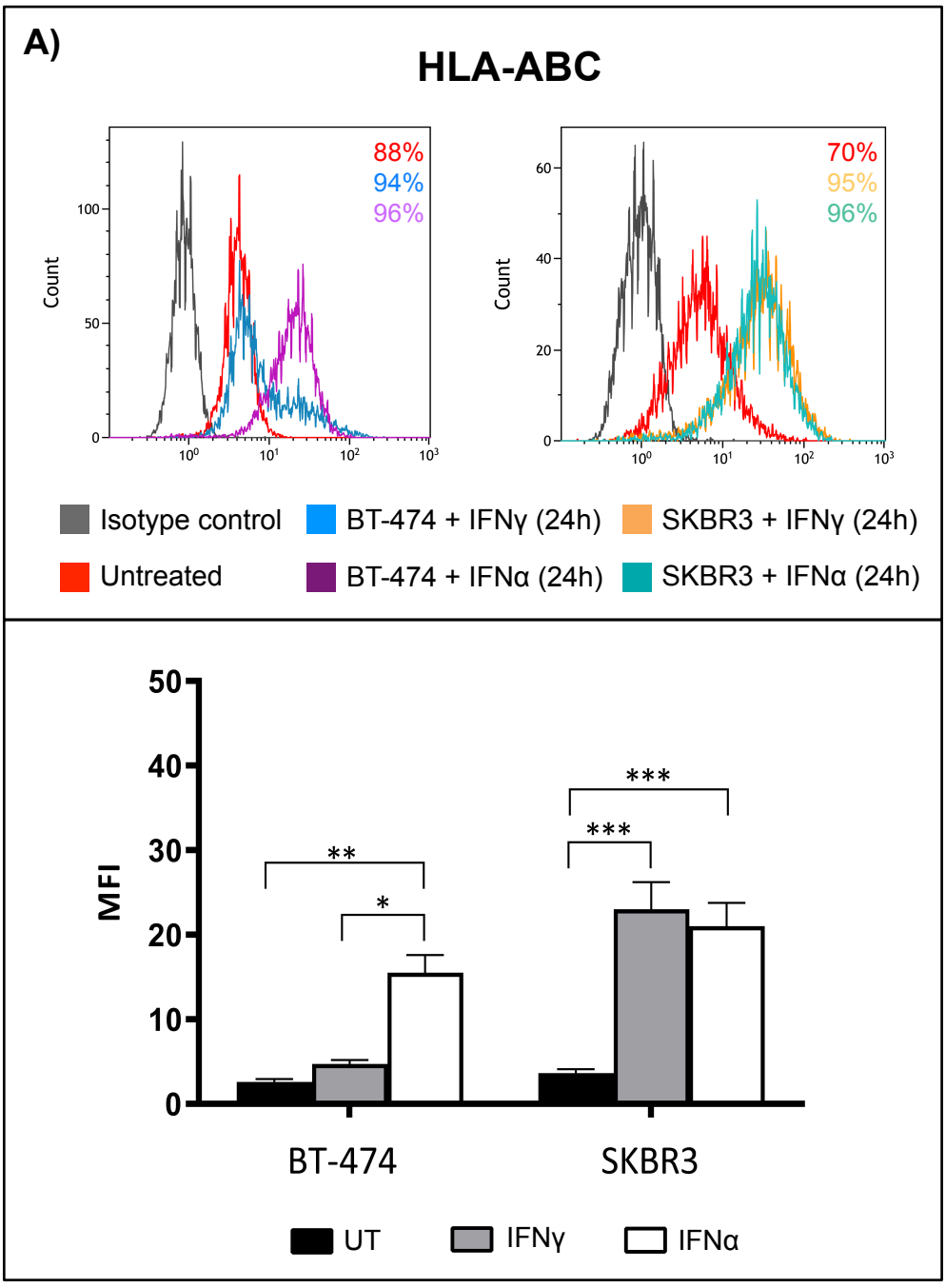
In comparison to SKBR3, BT-474 appears to have weaker IFN-γ signalling as shown by reduced constitutive levels of surface IFNGR1 with reduced internalization as well as negligible HLA Class I induction in response to binding IFN-γ. Next, we wanted to determine whether BT-474 only responded poorly to IFN-γ or also to other cytokines such as IFN-α. Therefore, both cell lines were treated with IFN-α or IFN-γ for 24 hours followed by flow cytometric analysis to determine cell surface expression of HLA-ABC and HER2.

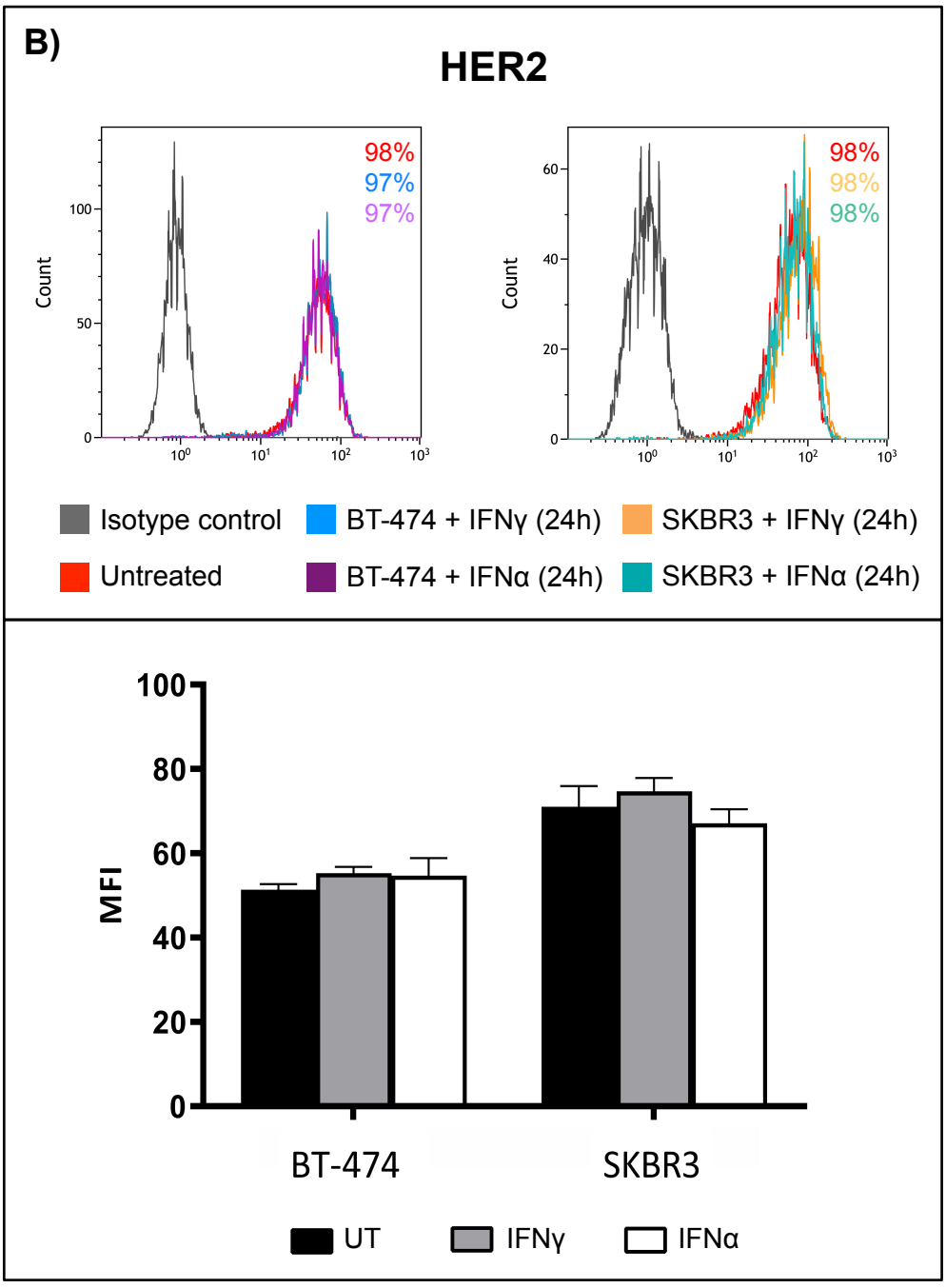
IFN-α stimulation significantly increased surface HLA-ABC compared to untreated and IFN-γ-stimulated cells while IFN-γ treatment only marginally upregulated HLA-ABC in BT-474. This indicates that JAK1 is present and functional in BT-474 as both, IFNAR1 and IFNGR1, are associated with JAK1 on their cytoplasmic domain while TYK2 and JAK2 are bound by IFNAR2 or IFNGR2, respectively. JAKs are activated via phosphorylation following IFN-α or IFN-γ binding with subsequent receptor heterodimerization before phosphorylating and activating STAT-1 thereby triggering HLA Class I induction (Section 1.2.3.1 and 1.2.3.2). In contrast to BT-474, both IFN-α and IFN-γ stimulation, equivalently increased HLA-ABC surface expression in SKBR3 (Figure 3.6A).

As previously shown (Section 3.1.1 and 3.1.2) SKBR3 expressed higher surface HER2 levels than BT-474; however, HER2 expression was not affected by either IFN-α or IFN-γ stimulation in BT-474 or SKBR3 (Figure 3.6B).

Figure 3.6: Comparison of HLA-ABC upregulation in response to IFN-α and IFN-γ.

BT-474 and SKBR3 cells were treated with either IFN-γ (100 U/mL) or IFN-α (100 U/mL) for 24 hours and subjected to flow cytometric analysis to assess HLA-ABC (A) and HER2 (B) cell surface expression levels. Top: Histogram overlays are representative of one experiment. The percentage of positive cells is colour-matched accordingly. Bottom: Bar graphs represent the mean MFI with error bars ± SEM and are indicative of three replicate assays. Statistical analysis was done by one-way ANOVA and Tukey HSD test (*$\rho<0.05$, **$\rho<0.01$, ***$\rho<0.001$).


A)
HLA-ABC
88%
94%
96%
70%
95%
96%
Count
Isotype control
Untreated
BT-474 + IFNγ (24h)
BT-474 + IFNα (24h)
SKBR3 + IFNγ (24h)
SKBR3 + IFNα (24h)
MFI
**
*
***
***
BT-474
SKBR3
UT
IFNγ
IFNα


B)
HER2
98%
97%
97%
98%
98%
98%
Count
Count
Isotype control
Untreated
BT-474 + IFNγ (24h)
BT-474 + IFNα (24h)
SKBR3 + IFNγ (24h)
SKBR3 + IFNα (24h)
MFI
BT-474
SKBR3
UT
IFNγ
IFNα

3.1.4 Discussion

3.1.4.1 Result summary

The work described in this chapter builds on previous observations in the Drover laboratory showing reduced constitutive and negligible IFN-γ inducible HLA Class I on the surface of BT-474 as compared to SKBR3 and MCF-7 (Matthew Young, (BSc Honors Thesis, unpublished data). This initial study further showed the IFN-γ pathway was poorly activated in BT-474, with levels of pSTAT-1 and IRF-1 significantly reduced as compared to other ERα+ BCCL. Another study in the Drover lab showed reduced HLA expression in luminal B breast cancer tissues [22]. Using BT-474 and SKBR3, this study focused on the kinetics of IFNGR1 and IFNGR2 expression, HLA class I inducibility and whether HLA Class I inducibility was affected by overexpression of HER2 in the ERα positive cell line, MCF-7. Key findings include: 1) Surface expression of IFNGR1 and IFNGR2 and IFN-γ-inducible HLA Class I were significantly reduced on BT-474 as compared to SKBR3; 2) Surface expression of HLA Class I isoforms as detected by the antibodies HCA2 and HC10 were poorly induced by IFN-γ on both BT-474 and SKBR3; 3) Overexpression of HER2 in MCF-7 resulted in a small but significant alteration in HLA Class I expression over time.

3.1.4.2 Potential underlying causes of dysregulated IFN-γ signalling in BT-474

The very low levels of membrane IFNGR1 and IFNGR2 on BT-474 as compared to SKBR3 may be the underlying mechanism for reduced IFN-γ signalling and HLA upregulation in BT-474. However, reduced signalling does not appear to be due to lack of IFNGR1 receptor mediated endocytosis as internalization, based on reduced percentages of positive cells occurred after 15 minutes response to IFN-γ (Figure 3.1A). It was more difficult to evaluate IFNGR2 endocytosis as the antibody MMHGR-2, is reported to poorly detect native surface IFNGR2 [81,82]; yet it bound low levels of surface IFNGR2 on 42% of SKBR3 cells as compared to 6% for BT-474, suggesting IFNGR2 is indeed poorly expressed on the cell surface of BT474. Notably, high intracellular expression of IFNGR1 and IFNGR2 in both cells indicate these receptors are transcribed and translated.

There are a number of possibilities for the deficit of surface IFNGR1 on BT-474 including a loss-of-function (LOF) mutation in the IFNGR1 gene, as was described in the context of Mendelian susceptibility to mycobacterial disease [83]. Additionally, mutations in various components of the IFN-γ signalling pathway including IFN-γ receptors as well as JAK1 and JAK2 in several types of cancer are now well documented [71,84–86] and have been proposed as underlying mechanisms for failure to respond to immunotherapies such as anti-programmed cell death ligand 1 (PD-L1) [71,84,85]. Whether such mutations are present in BT-474 would require sequencing, which was not a financial option during this study timeframe.

Moreover, JAK2 plays a crucial role in IFN-γ signalling and subsequent HLA Class I upregulation as its loss diminishes restoration of TAP expression by IFN-γ stimulation [86]; however, we were unable to assess JAK2 expression in our study by flow cytometry or western blotting. Since JAK1 signalling is associated with both IFNAR1 and IFNGR1, we indirectly assessed its function in BT-474 and SKBR3 by stimulating both BCCLs with IFN-α or IFN-γ for 24 hours and assessing HLA-ABC expression. Indeed, the significant upregulation of HLA-ABC on BT-474 in response to IFN-α was equivalent to SKBR3, whereas only SKBR3 responded to IFN-γ. This further supports defective IFN-γ signalling in BT-474, but suggests it is not due to defective JAK1 signalling.

As described in Chapter 1 (Section 1.2.3.3) NF-κb binding to Enhancer A site in the HLA Class I promoter is also essential for basal and IFN-γ-induced HLA Class I [23]. Since downregulation or loss of NF-κb has been identified as an immune escape mechanism for impaired or deficient HLA Class I expression in cancer [71,87], a preliminary experiment (Appendix 4), was performed to assess its function. Equivalent HLA-ABC upregulation by BT-474 in response to TNF-α treatment and IFN-α, but not IFN-γ was observed. SKBR3 also upregulated HLA-ABC via TNF-α albeit to lesser extent than IFN-α and IFN-γ stimulation. All three cytokines are known to stimulate expression of APM components such as LMP2/7/10, TAP1/2, and ERAP1/2; however, IFN-γ has been suggested to be most effective in upregulating APM proteins [16,87,88].

Taken together, the increased HLA-ABC expression in BT-474 in response to IFN-α and TNF-α but not IFN-γ, albeit being most potent APM stimulator [16,88], further

points to dysregulation of IFN-γ signalling through components of the IFN-γ pathway (e.g. IFNGR1/2, STAT-1, IRF-1, NLRC5) and/or IFN-γ-inducible proteins of APM (e.g. TAP1/2, PA28α/β, LMP2, LMP7) [23,89,90]. Investigating the known negative regulators of IFN-γ and APM components such as double homeobox 4 (DUX4), tyrosine-protein phosphatase nonreceptor type 1/2 (PTPN1/2), and enhancer of zeste homolog 2 (EZH2) [84,87] could help determine the cause for the observed weak IFN-γ response in BT-474.

3.1.4.3 Possible correlation between HER2 signalling and HLA Class I induction by IFN-γ

Both BCCLs overexpress HER2, which has been reported by some but not all studies [19,69,70,79,80,91,92] to repress HLA Class I expression. Notably, while SKBR3 consistently displayed higher levels of HER2 and HLA Class I than BT-474, its HLA Class I expression is also lower than that of MCF-7/neo and MCF-7/HER218. However, BT-474, a luminal B BCCL, overexpresses ERα, which is known to modulate HLA Class II [93] on BCCL and was also found associated with poor HLA Class II expression in luminal breast cancer tissues [22]. We therefore queried whether the combination of overexpressed HER2 and ERα might negatively influence HLA Class I by comparing ERBB2-transfected MCF-7 cells (HER2+ER+) and its vector control (MCF-7/neo) to BT-474 and SKBR3. Although both MCF-7 cell lines displayed markedly higher levels of HLA Class I than either BT-474 or SKBR3, MCF-7/HER218 cells showed small, but significantly reduced surface and significantly increased intracellular HLA Class I expression. This tends to support the idea that co-expression of HER2 and ERα is somehow associated with

accumulating HLA Class I molecules in the intracellular compartments after long term IFN-γ exposure.

An intriguing finding by Mimura et al. demonstrated an inverse correlation between HER2 and HLA-A2 [79], suggesting the importance of analysing various isoforms of HLA Class I. In fact, the loss of HLA Class I heterozygosity is well described in many cancers. A study, by Montesion and colleagues found 17% of 59 different cancer types, including breast cancer, lost their HLA Class I heterozygosity demonstrated by either loss of a single copy of HLA Class I heavy chain or β_2M gene possibly as a result of immunoediting [94]. In melanoma patients inactivating mutations were found in the coding regions of individual HLA Class I heavy chain genes leading to a total loss of HLA Class I function if the same cells already exhibit HLA Class I loss of heterozygosity [71].

Based on this literature, we thought it prudent to include analysis of HLA-A and HLA-BC molecules using HCA2 and HC10, respectively, along with the HLA Class I conformer (W6/32), which also binds the non-classical HLA Class I such as HLA-E. The deficit of HLA-A and HLA-BC on BT-474 and SKBR3, as compared to low but positive levels on MCF-7 cells suggest the respective HLA-A and HLA-BC antibodies may not be optimal for detecting low levels of surface molecules. Although both antibodies were reported to poorly bind native, fully assembled stable HLA Class I proteins [78,95], Seitz and colleagues showed HCA2 ability to bind HLA-A molecules on the cell surface of C1R cells [77]. This conforms with our findings of surface HLA-A and HLA-BC in B cell line SAVC (Appendix 2) indicating that antibody reactivity with surface HLA Class I molecules

may be cell-context dependent. Moreover, Seitz et al. found that the HAC2 clone not only detects HLA-A proteins but also recognizes some HLA-B and HLA-C alleles as well as HLA-E, HLA-F and HLA-G molecules, thereby complicating analysis of experiments using HCA2 antibody [77].

Alternatively, HLA Class I isoforms recognized by HAC2 and HC10 may not be expressed on the surface of BT-474 and SKBR3. Indeed transcription rates based on TRON database [96,97] indicate the negligible amount of HLA-A expressed by BT-474 and SKBR3 might not be surprising given the low 'Reads per kilobase per million reads mapped'(RPKM) found in BT-474 (RPKM = 0.18) and SKBR3 (RPKM = 3.97). The TRON database utilizes RNA sequencing data from 1,082 human cancer cell lines to generate gene expression data based on transcript levels, which are quantified as RPKM. Therefore, the number of RPKMs corresponds to gene expression level (e.g. RPKM = 1 corresponds to low gene expression) [96].

Interestingly, SKBR3 and parental MCF-7 express the same alleles for HLA-A (Table 2.7–Table 2.8) with MCF-7 exhibiting higher RPKM for HLA-A (RPKM = 20.19) than SKBR3, which only resulted in marginally higher detectable surface HLA-A in MCF-7. RPKM values are highest for HLA-C in both HER2 expressing cell lines (RPKM = 45.39 and RPKM = 14.33 for BT-474 and SKBR3, respectively) while RPKM value for HLA-C in MCF-7 (RPKM = 20.01) was similar to the one for HLA-A. However, MCF-7/HER218 and its vector control MCF-7/neo showed higher surface HLA-BC than BT-474 or SKBR3 indicating that differences in HLA alleles for HLA-A, HLA-B, and HLA-C among the three

cell lines (Table 2.6–Table 2.8) may affect antibody sensitivity. This suggests a need to develop more HLA allele specific antibodies especially as HLA is known to be extremely polymorphic [10].

3.1.4.4 Conclusion

In summary, IFN-γ kinetics in BT-474, SKBR3, and MCF-7/HER128 as well as comparative study stimulating BT-474 and SKBR3 cells with three different cytokines (IFN-γ, IFN-α, and TNF-α) confirmed that IFN-γ signalling and IFN-γ-inducible HLA Class I is dysregulated in BT-474. Notably, we speculate that HER2 overexpression might have a sequestering effect on intracellular HLA Class I with long term IFN-γ exposure.

A properly functional IFN-γ pathway is crucial as evidenced by the higher incident rate of cancer detected in mice with defective IFN-γ signalling. One way for cancers to evade detection is by interrupting IFN-γ signalling through various mechanism such as mutations in IFNGRs, JAK or STAT proteins thereby preventing HLA Class I upregulation and proper immune surveillance and response via CD8+ T cells. Subsequently, low HLA Class I tumours have been associated with worse clinical outcome, resistance to adoptive and checkpoint immunotherapy as well as tumour progression during immunotherapy [71].

3.2 Influence of HER2 silencing on HER2-overexpressing BCCLs

3.2.1 Effects of HER2 and/or ERα inhibition on IFN-γ signalling in HER2-overexpressing cell line BT-474

The rationale for the following set of experiments stemmed from several observations in the Drover laboratory: 1) reduced HLA Class II expression in breast cancer cells from luminal B (HER2+ER+) human breast carcinomas, as compared to other breast cancer subtypes even in the presence of endogenous IFN-γ [68]; 2) E_2-ER activation attenuated STAT-1 signalling resulting in decreased levels of STAT-1 regulated genes such as IRF-1 and CIITA [93]; 3); significantly reduced responsiveness to IFN-γ stimulation in BT-474 as compared to MCF-7 and T47D (Matthew Young, MSc thesis), potentially explained by reduced IFNGR1 expression on BT-474 (Section 3.1.1.1); 4) modulated IFN-γ-induced HLA Class I expression in MCF-7/HER2 as compared to its vector control (Section 3.1.2.2). Lastly, others [69,70,80,91] have reported HER2 overexpression is linked to downregulation of HLA Class I along with APM deficiencies in human cancers, thereby providing a possible immune escape mechanism. To test whether hyperactivation of HER2 signalling in ER+ breast cancers interferes with the activation of the IFN-γ pathway, we used three different approaches to interrupt HER2 and/or ER signalling in BT-474:

1. Targeted therapy using the anti-estrogens, TAM and ICI, and the tyrosine kinase inhibitor, Lap

2. Gene silencing using anti-ER (ESR1) and anti-HER2 (ERBB2) siRNAs
3. Combination therapy using two anti-HER2 antibodies, trastuzumab and pertuzumab (T/P)

3.2.2 Inhibition of ERα or HER2 pathways with targeted therapies in BT-474 differently affects IFN-γ signalling and induction of HLA Class I

BT-474 cells treated with TAM (10^{-6}M), ICI (10^{-6}M), Lap (0.5μM), or controls (ethanol and DMSO), with and without IFN-γ for 24 hours, were analyzed by flow cytometry to determine surface expression of IFNGR1, HLA-ABC and HER2. TAM and ICI treatments slightly increased constitutive and IFN-γ-activated IFNGR1 but had a negligible effect on HLA-ABC expression (Figure 3.7A-B). In contrast, Lap treatment significantly reduced % and expression (MFI) of IFNGR1+ and HLA-ABC+ cells in untreated and IFN-γ-stimulated cells. Neither ER inhibitors nor Lap significantly altered the numbers of HER2+ cells, but Lap increased the amount of cell surface HER2 (Figure 3.7C). As anti-estrogens and Lap target intracellular activation of ER and HER2 signalling, respectively, it was important to confirm their efficacies and to examine their downstream effects on IFN-γ signalling by western blot analysis.

Western blot analysis of WCLs prepared from BT-474 cells, (Figure 3.8A-B) showed TAM treatment increased constitutive and IFN-γ-induced ERα expression while ICI treatment only decreased constitutive ERα as compared to the ethanol control. Since TAM is a partial ER agonist and ICI is an ER antagonist, the results are as expected;

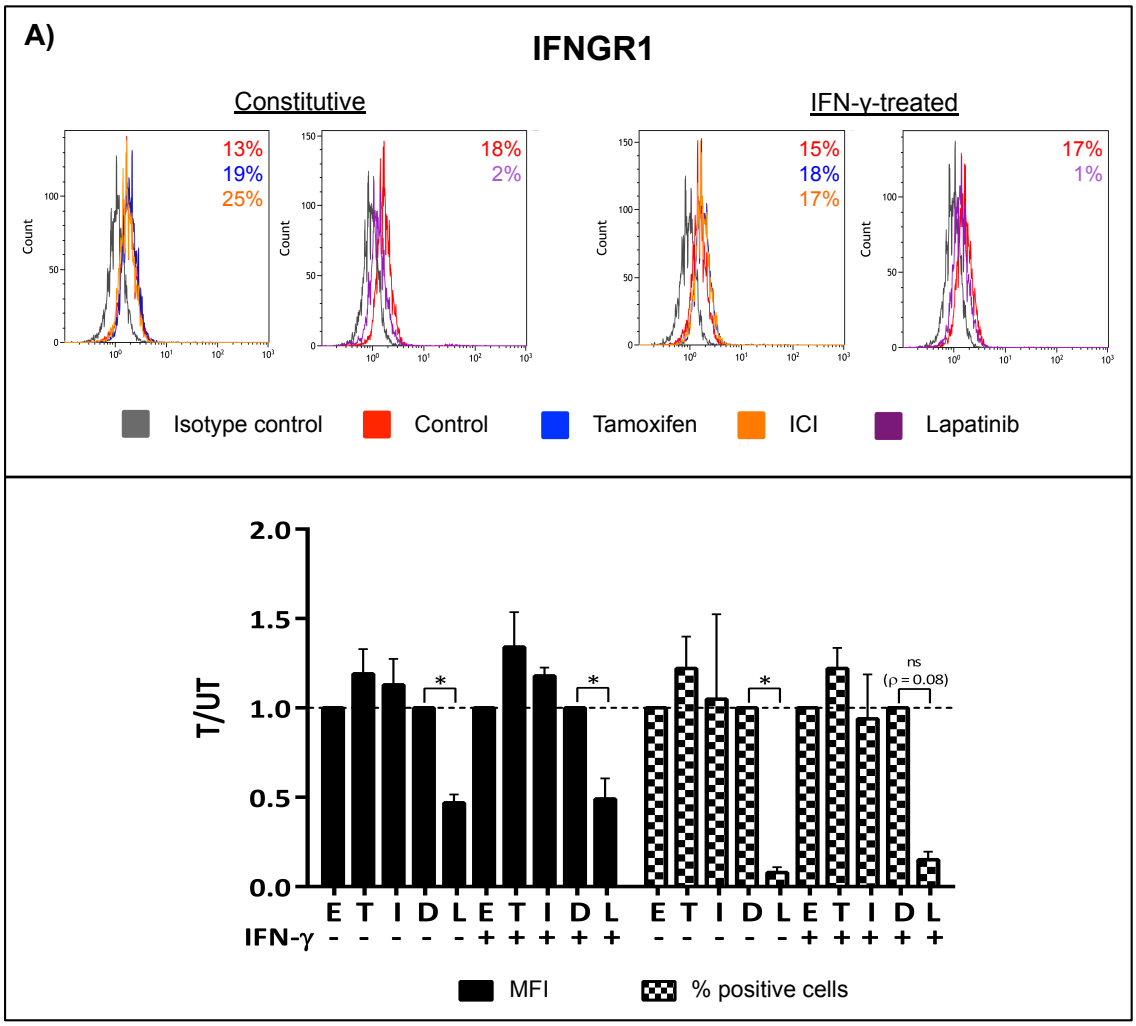
however, in retrospect, a better indicator of changes in ERα expression and signalling would have been to use nuclear extracts and a phosphorylated anti-ER antibody. TAM and ICI treatments resulted in slightly decreased pHER2 in the absence of IFN-γ, but conversely, increased its expression in IFN-γ-treated cells. Compared to DMSO, Lap treatment abrogated HER2 phosphorylation in IFN-γ-stimulated (or not) cells with concomitant inhibition of pAKT and pERK.

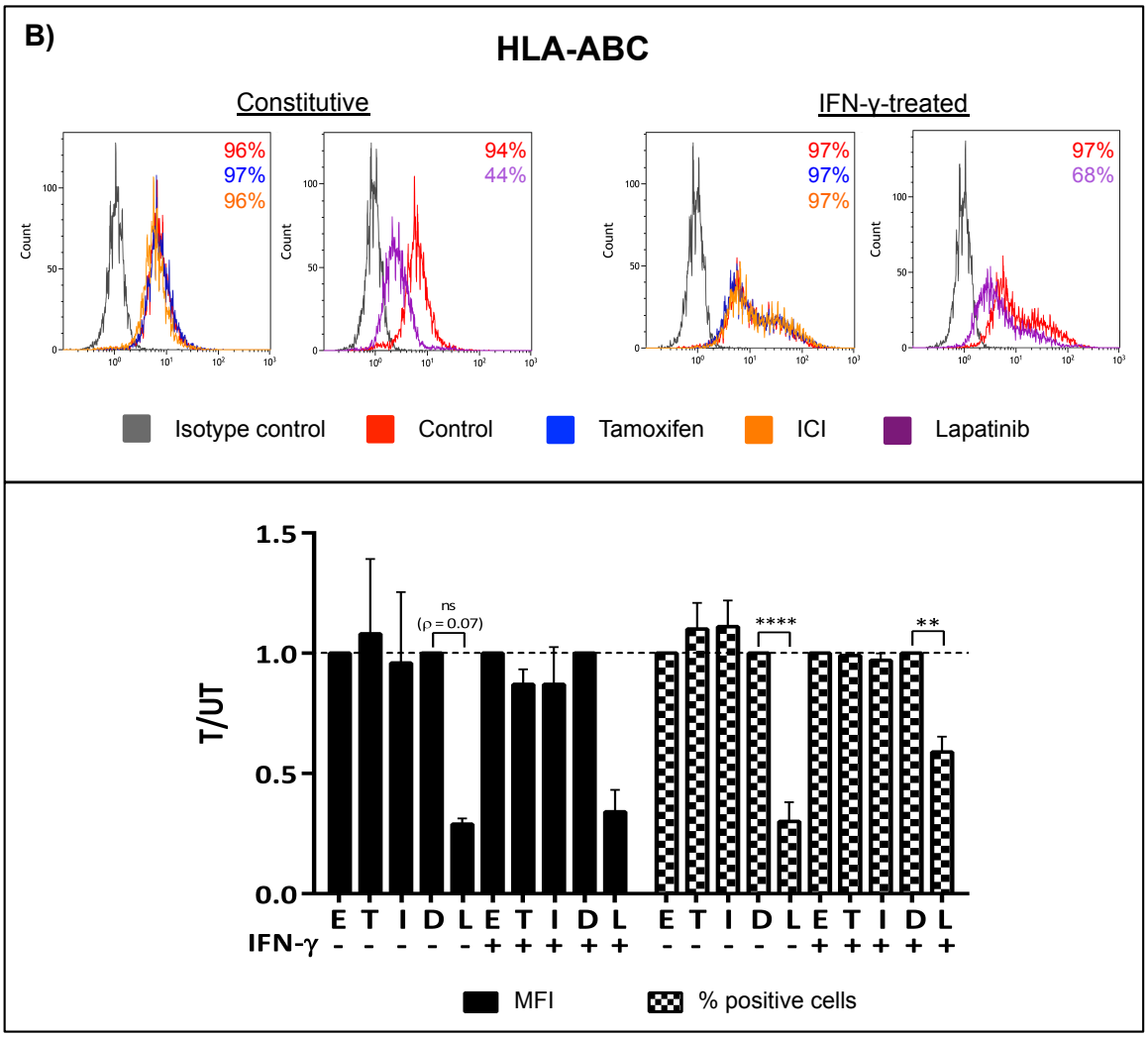
IFN-γ signalling molecules, pSTAT-1 and IRF-1, and HLA-BC heavy (H) chains were not detected constitutively, either in control-treated, anti-estrogen or Lap-treated cells (Figure 3.8A-B). Levels of pSTAT-1, IRF-1 and HLA-BC H-chains, while induced by IFN-γ, were not significantly altered by either targeted treatment. There were, however, notable trends: 1) compared to ethanol control, TAM and ICI treatment increased pSTAT-1 and IRF-1 while HLA-BC H-chain expression was variable; 2) Lap, compared to DMSO-treated cells, increased IRF-1 expression, but markedly decreased HLA-BC; 3) reduced HLA-BC H-chain expression in Lap-treated cells is consistent with significantly Lap-mediated decreased HLA-ABC surface expression (Figure 3.7B).

Taken together, these results suggest successful targeting of ERα and HER2 pathways by TAM and Lap, respectively, while ERα knockdown by ICI was incomplete. However, neither treatment significantly changed expression levels of IFN-γ signalling molecules. Moreover, reduction of pHER2 and ERα via the appropriate drug treatment without IFN-γ stimulation demonstrates the efficacy of the drugs themselves and shows that our findings are not based on insufficient or inactive drug treatments.

Figure 3.7: Effects of anti-estrogens and Lap on cell surface expression of IFNGR1, HLA-ABC and HER2 in BT-474 cell.

Flow cytometric analysis of BT-474 treated with TAM(T) ($10^{\wedge-6}$ M), ICI (I) ($10^{\wedge-6}$ M), Lap (L) (0.5 µM), Ethanol (E) (vehicle control for T and I), or DMSO (D) (vehicle control for L), +/- IFN-γ (100 U/mL) for 24 hours was used to determine constitutive and IFN-γ-stimulated IFNGR1 (A), HLA-ABC (B), and HER2 (C) cell surface expression. Top: Histogram overlays are representative of one experiment. The percentage of positive cells is colour-matched accordingly. Bottom: Bar graphs represent the mean MFI or %+ cells of treated (T, I, or L +/- IFN-γ) over untreated (E or D +/- IFN-γ) with error bars ± SEM and are indicative of three independent experiments. Statistical analysis was done by one-way ANOVA and Tukey HSD test (*ρ ≤0.05, **ρ <0.01, ****ρ <0.0001).


A)
IFNGR1
Constitutive
IFN-γ-treated
13%
19%
25%
18%
2%
15%
18%
17%
17%
1%
Count
Isotype control
Control
Tamoxifen
ICI
Lapatinib
T/UT
2.0
1.5
1.0
0.5
0.0
*
*
*
ns
(ρ = 0.08)
E T I D L E T I D L E T I D L E T I D L
IFN-γ - - - - - + + + + + - - - - - + + + + +
MFI
% positive cells


B)
HLA-ABC
Constitutive
IFN-γ-treated
96%
97%
96%
94%
44%
97%
97%
97%
97%
68%
Count
Isotype control
Control
Tamoxifen
ICI
Lapatinib
T/UT
ns
(p = 0.07)
****
**
E T I D L E T I D L E T I D L E T I D L
IFN-γ - - - - - + + + + + - - - - - + + + + +
MFI
% positive cells

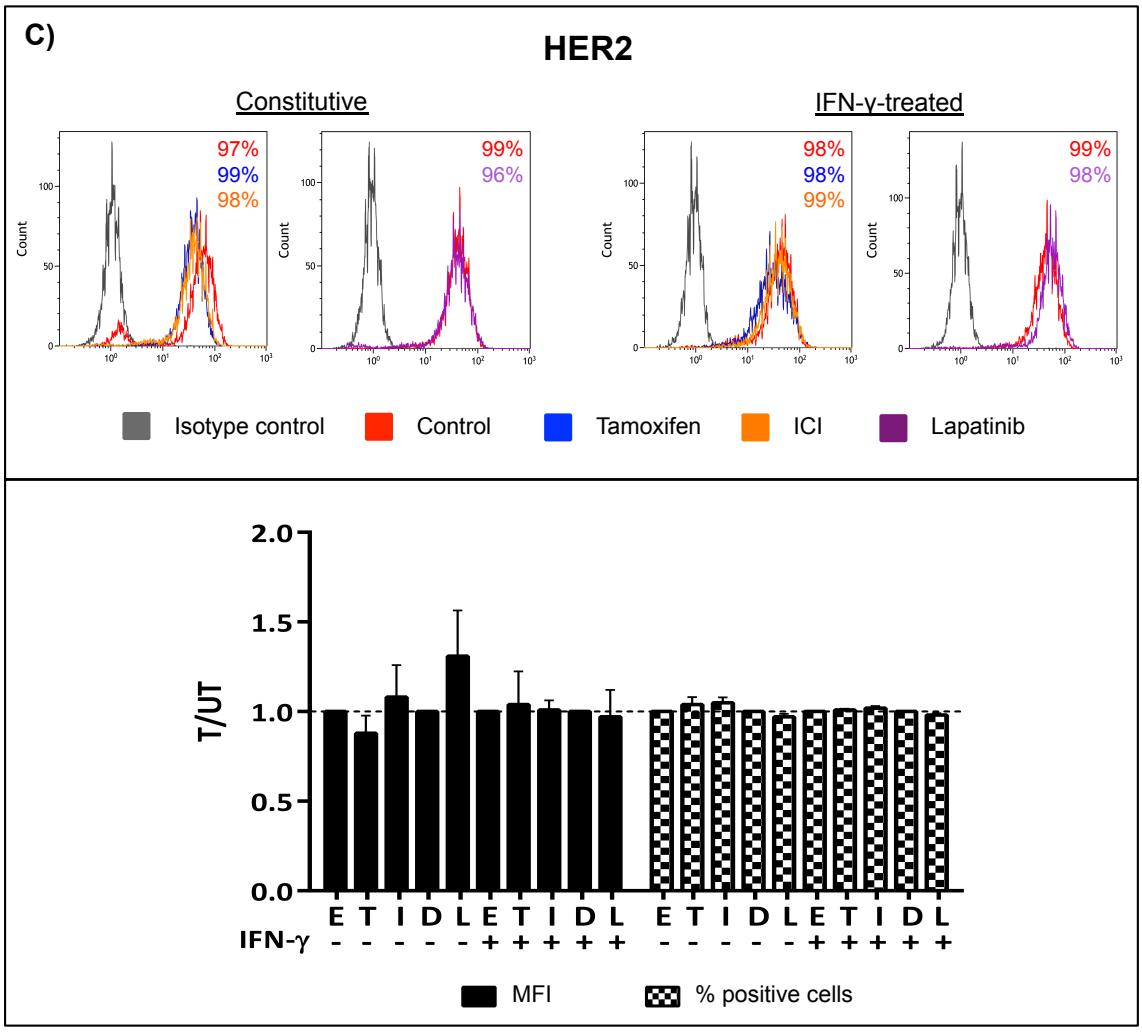
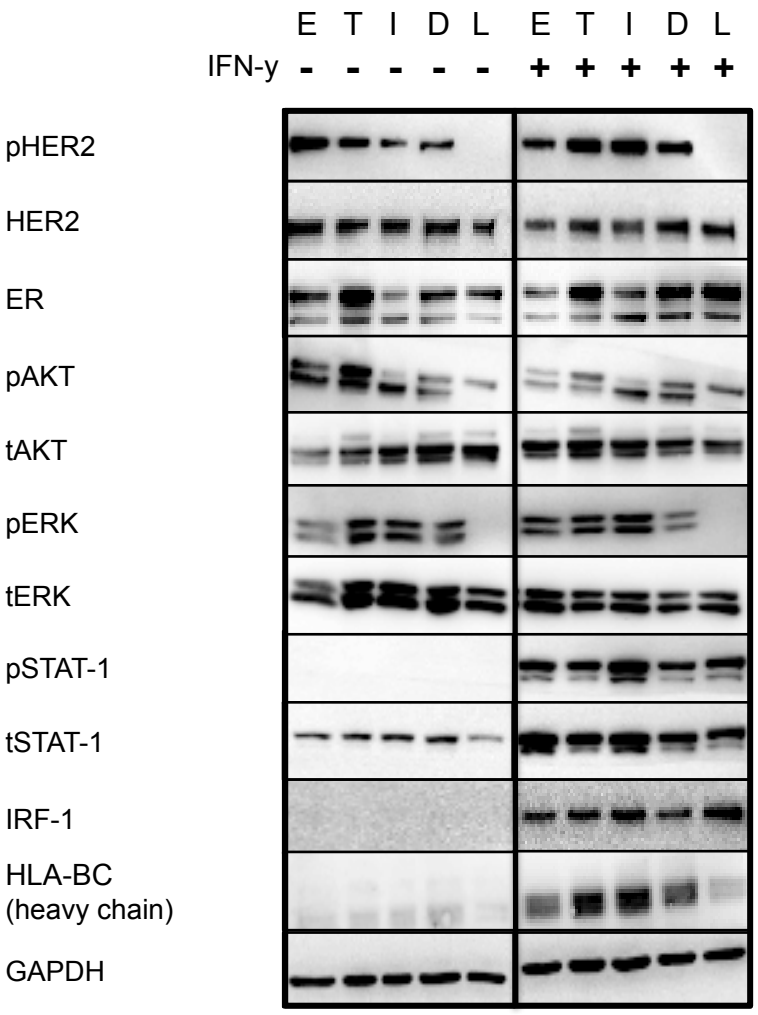

C)
HER2
Constitutive
IFN-γ-treated
97%
99%
98%
99%
96%
98%
98%
99%
99%
98%
Count
Isotype control
Control
Tamoxifen
ICI
Lapatinib
T/UT
2.0
1.5
1.0
0.5
0.0
E T I D L E T I D L E T I D L E T I D L
IFN-γ - - - - - + + + + + - - - - - + + + + +
MFI
% positive cells

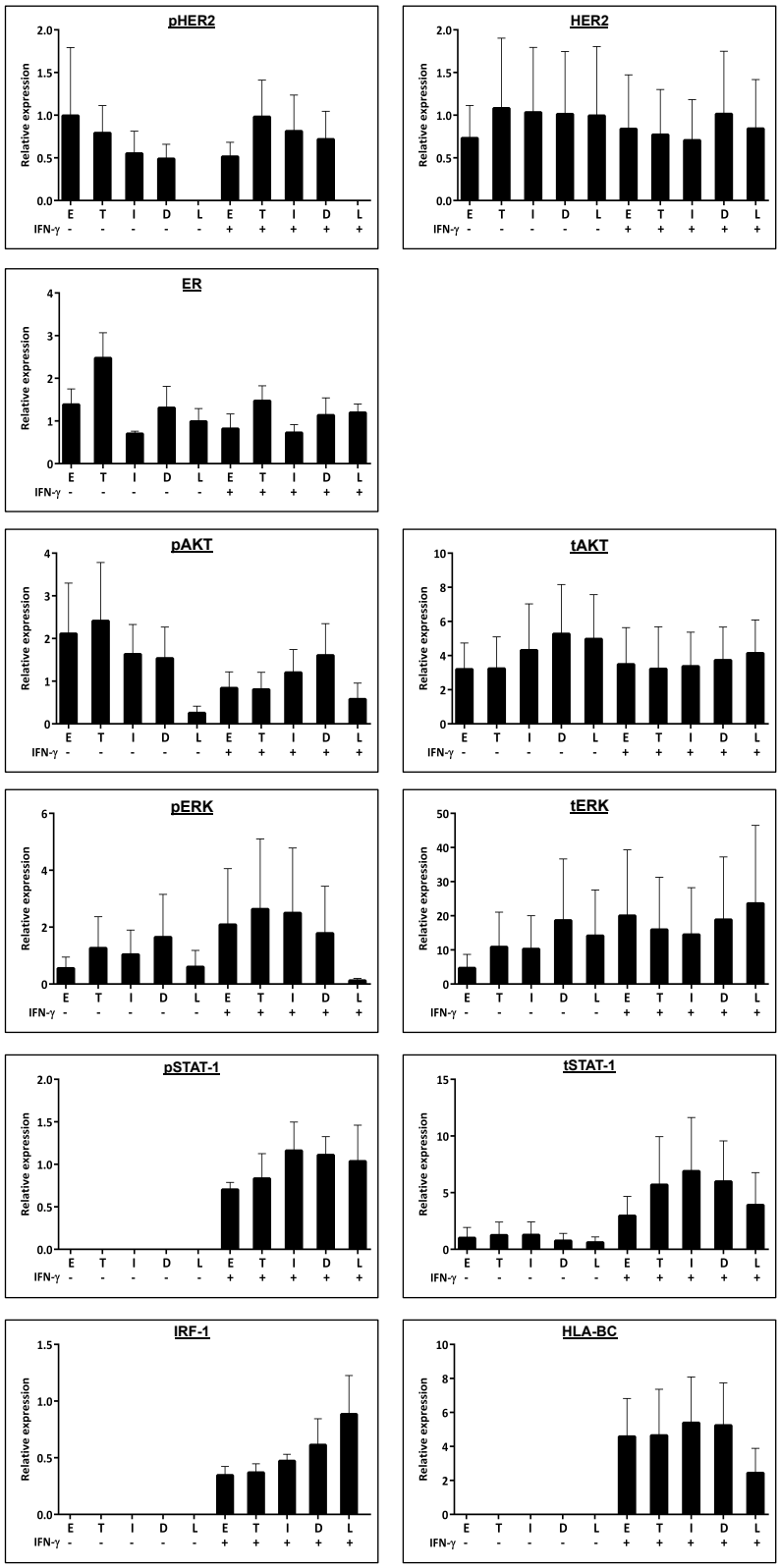
Figure 3.8: Targeting ERα and HER2 in BT-474 differentially modulates the IFN-γ pathway components and HLA-BC heavy chain accumulation.

WCLs prepared from BT-474 cells treated with TAM (T) (10^{-6} M), ICI (I) (10^{-6} M), Lap (L) (0.5 µM), Ethanol (E) (vehicle control for T and I), or DMSO (D) (vehicle control for L), +/- IFN-γ (100 U/mL) for 24 hours, were analyzed by western blotting for the indicated proteins. A) Representative western blot. B) Bar graphs for relative expression show the ratio of band intensity for indicated proteins, normalized to GAPDH band intensity. Graphs represent the mean ± SEM of three independent experiments. Statistical analysis was done by one-way ANOVA and Tukey HSD test.

A)


E T I D L E T I D L
IFN-γ - - - - - + + + + +
pHER2
HER2
ER
pAKT
tAKT
pERK
tERK
pSTAT-1
tSTAT-1
IRF-1
HLA-BC
(heavy chain)
GAPDH

B)


pHER2
HER2
ER
pAKT
tAKT
pERK
tERK
pSTAT-1
tSTAT-1
IRF-1
HLA-BC
Relative expression
E T I D L E T I D L
IFN-γ - - - - - + + + + +

3.2.3 Targeting HER2 and/or ERα with siRNA modulates IFN-γ signalling and HLA Class I expression in BT-474

BT-474 cells were treated with ERBB2-siRNA, scrambled siRNA (SC), or siRNA buffer (Mock) for 24 hours followed by IFN-γ stimulation for an additional 24 hours. Cell surface expression of IFNGR1, HLA-ABC, and HER2 were determined by flow cytometry. Transfection with ERBB2-siRNA successfully knocked down HER2 as its expression was significantly reduced (> 50%) in untreated and IFN-γ-stimulated cells while the %+ HER2 BT-474 cells remained unchanged. Constitutive and IFN-γ-induced IFNGR1 and HLA-ABC surface expression and their respective %+ cells, were slightly, yet not significantly, reduced (Figure 3.9A-C).

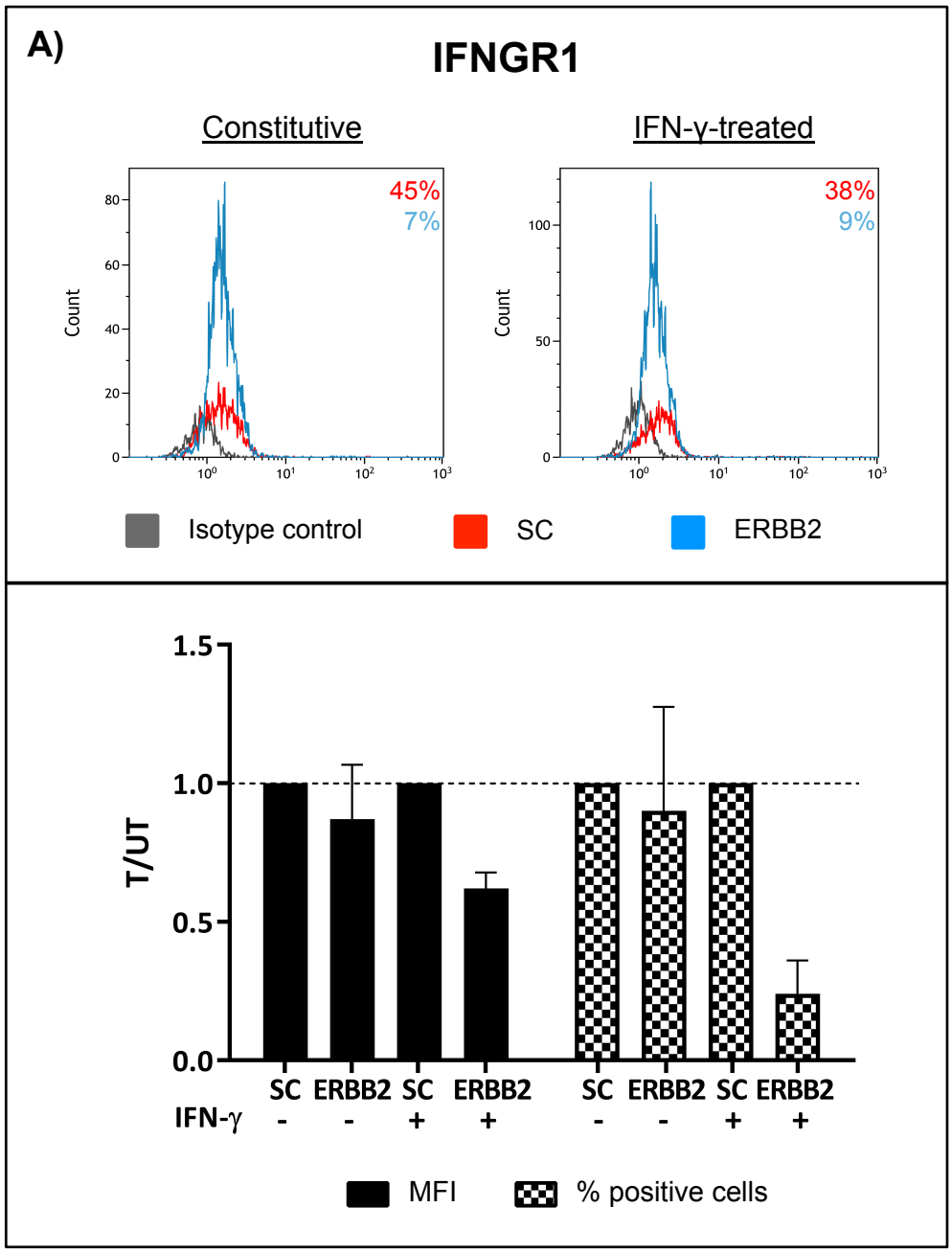
BT-474 cells were next transfected with ERBB2 siRNA and ESR1 siRNA to silence HER2 and ER signalling individually or simultaneously, followed by IFN-γ treatment or not for a further 24 hours. WCLs, prepared and analyzed for relevant proteins by western blotting, showed ERα expression was mostly abolished in cells transfected with ESR1 or co-transfected with ERBB2 siRNA (Figure 3.10B). Phosphorylated HER2 and HER2 were greatly reduced in untreated and IFN-γ-stimulated BT-474 cells, transfected with ERBB2 siRNA alone or combined with ESR1 siRNA. Downstream signalling molecules, pAKT and pERK, were also severely diminished (Figure 3.10B). ER knockdown by ESR1 siRNA led to increased pHER2 and HER2, which is likely a result of well-described bidirectional molecular crosstalk between HER2 and ER signalling [38,51].

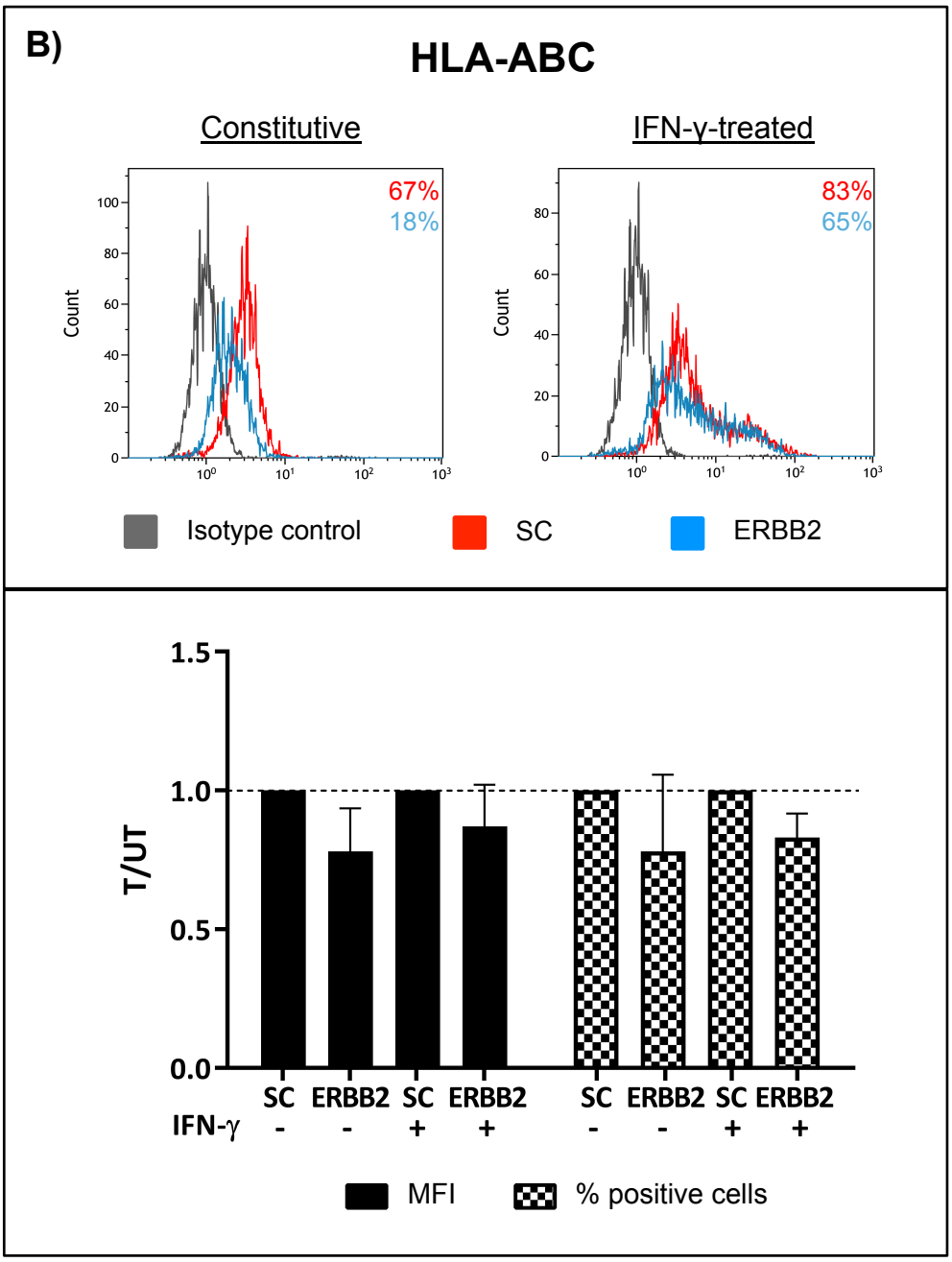
Phosphorylated STAT-1, IRF-1, and HLA-BC H-chains were upregulated in IFN-γ-stimulated BT-474 cells, with very weak constitutive expression in some controls (Figure 3.10A-B). Neither knockdown resulted in statistically significant changes in pSTAT-1, IRF-1 or HLA-BC H-chains in the IFN-γ-treated cells. Notably, HER2 knockdown, alone or combined with ER knockdown, increased pSTAT-1 and IRF-1, yet decreased HLA-BC H-chain, which was similar to observations in Lap-treated cells (Figure 3.8A-B). ER knockdown alone led to slightly increased pSTAT-1, but decreased IRF-1, and HLA-BC levels. However, expression levels of IFN-γ signalling molecules and HLA-BC H-chains were highly variable between experiments as indicated by the high standard errors.

Taken together, ERBB2-siRNA transfection significantly reduced HER2 surface expression and greatly diminished HER2 signalling but did not restore HLA-BC H-chains or HLA-ABC surface levels besides some small increases in pSTAT-1 and IRF-1 expression. This suggests a possible dysregulation of APM components independent from HER2 expression, which will be further discussed in section 3.2.9 and section 4.1.

Figure 3.9: Effect of ERBB2 siRNA knockdown in BT-474 cells on cell surface expression of IFNGR1 and HLA-ABC.

BT-474 cells, treated with either ERBB2 siRNA (5 μM) or SC siRNA (5 μM) for 24 hours, followed by IFN-γ stimulation (100 U/mL) for an additional 24 hours, were analyzed by flow cytometry for cell surface expression of IFNGR1 (A), HLA-ABC (B), and HER2 (C). Top: Histogram overlays are representative of one experiment. The percentage of positive cells is colour-matched accordingly. Bottom: Bar graphs represent the MFI or %+ cells of treated (ERBB2 siRNA +/- IFN-γ) over untreated (SC siRNA +/- IFN-γ) with error bars showing mean ± SEM of three independent assays. Statistical analysis was done by one-way ANOVA and Tukey HSD test (****ρ <0.0001).


A)
IFNGR1
Constitutive
IFN-γ-treated
45%
7%
38%
9%
Count
Isotype control
SC
ERBB2
1.5
1.0
0.5
0.0
T/UT
SC ERBB2 SC ERBB2 SC ERBB2 SC ERBB2
IFN-γ - - + + - - + +
MFI
% positive cells


B)
HLA-ABC
Constitutive
IFN-γ-treated
67%
18%
83%
65%
Count
Isotype control
SC
ERBB2
1.5
1.0
0.5
0.0
T/UT
SC ERBB2 SC ERBB2 SC ERBB2 SC ERBB2
IFN-γ - - + + - - + +
MFI
% positive cells

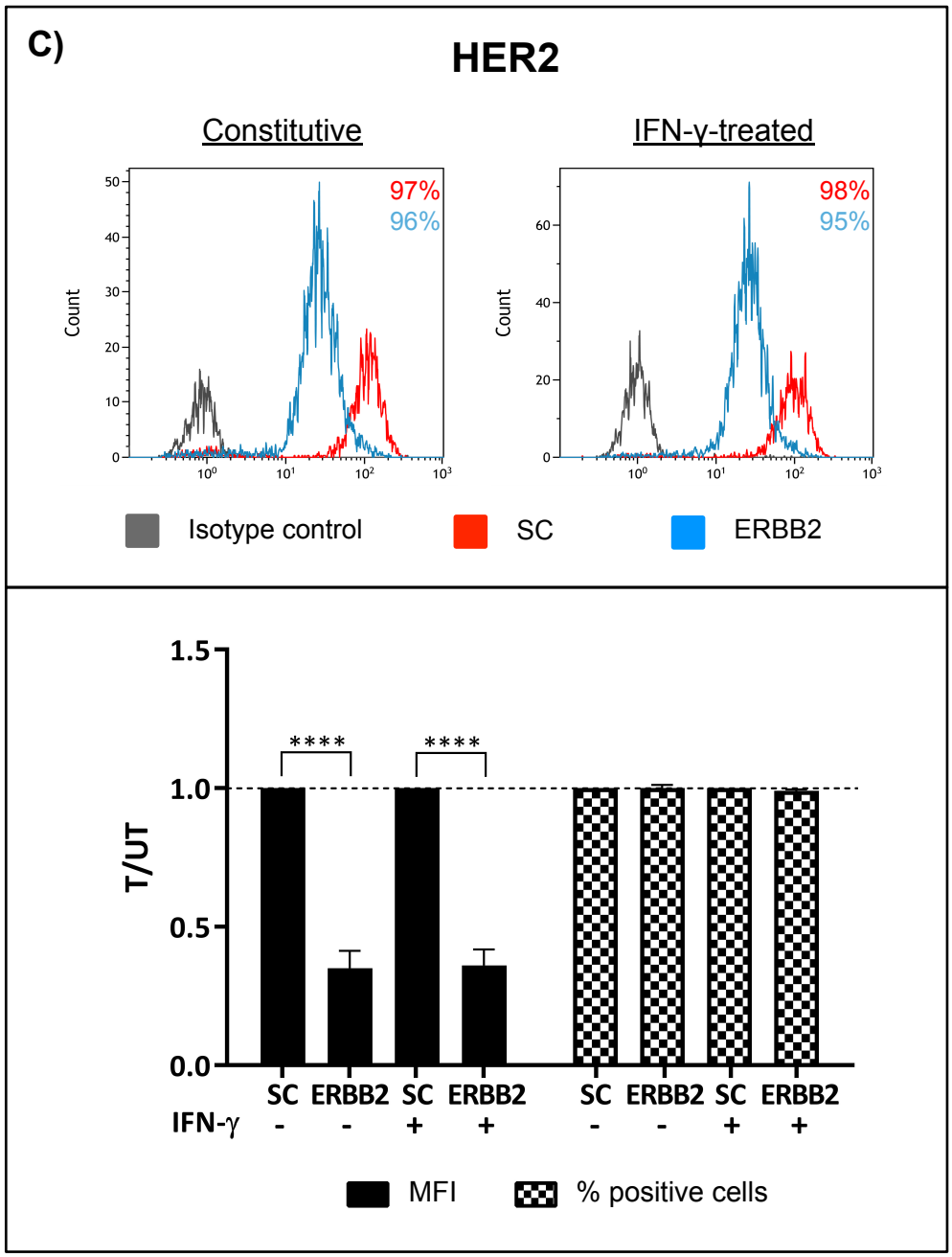
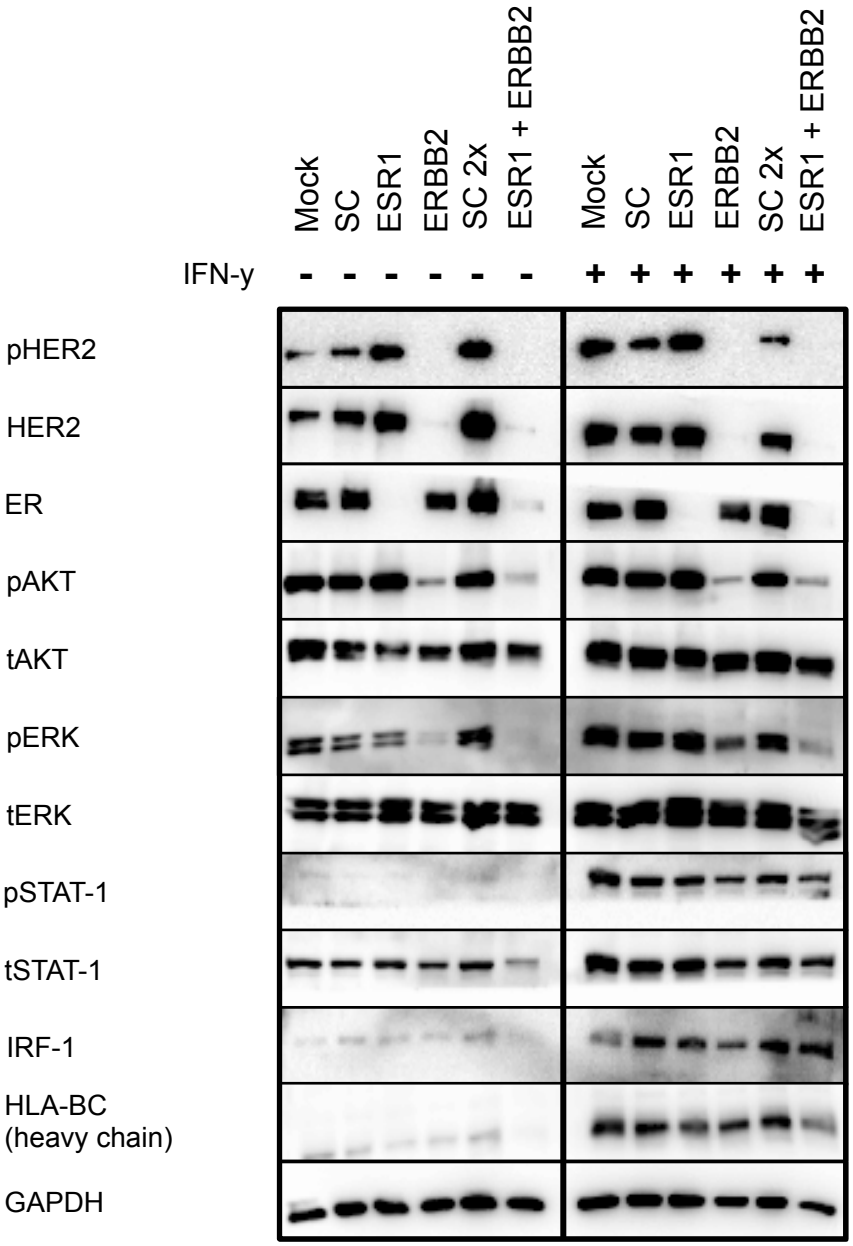

C)
HER2
Constitutive
IFN-γ-treated
97%
96%
98%
95%
Count
Isotype control
SC
ERBB2
T/UT
****
****
SC ERBB2 SC ERBB2 SC ERBB2 SC ERBB2
IFN-γ - - + + - - + +
MFI
% positive cells

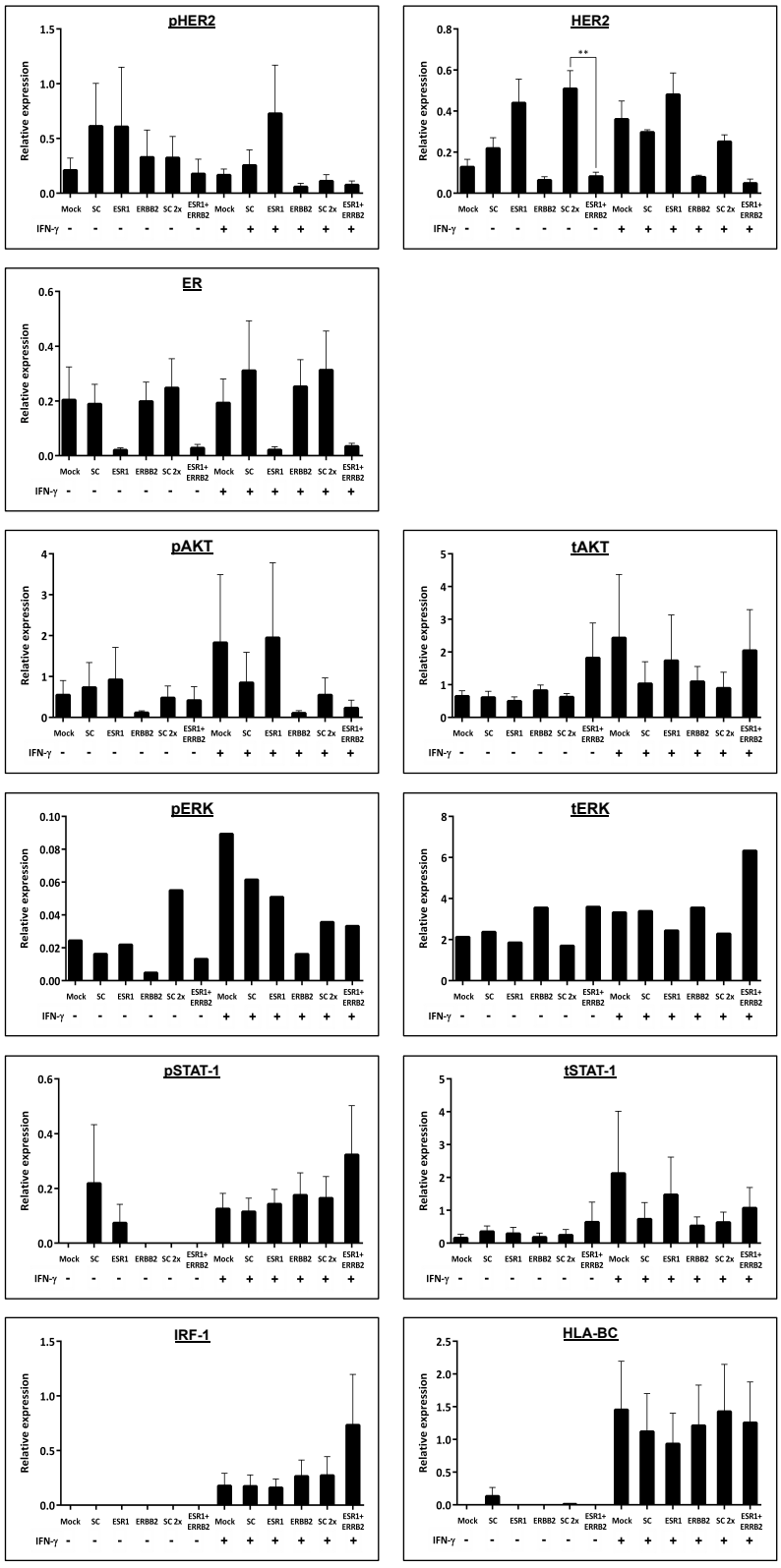
Figure 3.10: Effects of single and double siRNA knockdowns of HER2 and ER in BT-474 cells on IFN-γ-induced proteins.

BT-474 cells were treated singly or combined with ERBB2 siRNA (5 µM), ESR1 siRNA (5 µM) and SC siRNA (5 µM) or siRNA buffer Mock (5 µM) for 24 hours, stimulated or not with IFN-γ (100 U/mL) for an additional 24 hours. WCLs were prepared and analyzed by western blotting using the indicated antibodies. A) Representative western blot. B) Bar graphs for relative expression show the ratio of band intensity for indicated proteins, normalized to GAPDH band intensity. Graphs represent the mean ± SEM of three replicate assays (Exception: Antibodies for pERk and tERK detection were only used on one set of lysates). Statistical analysis was done by one-way ANOVA and Tukey HSD test (**$\rho<0.01$).

A)


Mock
SC
ESR1
ERBB2
SC 2x
ESR1 + ERBB2
Mock
SC
ESR1
ERBB2
SC 2x
ESR1 + ERBB2
IFN-y
- - - - - - + + + + + +
pHER2
HER2
ER
pAKT
tAKT
pERK
tERK
pSTAT-1
tSTAT-1
IRF-1
HLA-BC
(heavy chain)
GAPDH

B)


pHER2
HER2
ER
pAKT
tAKT
pERK
tERK
pSTAT-1
tSTAT-1
IRF-1
HLA-BC
Relative expression
Mock SC ESR1 ERBB2 SC 2x ESR1+ ERRB2
IFN-γ

3.2.4 Effects of trastuzumab+pertuzumab-mediated inhibition of HER2 pathway on IFN-γ signalling and HLA Class I induction in BT-474

Two HER2-targeting antibodies, trastuzumab and pertuzumab (T/P), which bind different extracellular domains of the HER2 receptor and result in its inactivation [60], were used to treat BT-474 cells simultaneously with and without IFN-γ over a time course of 24, 48, 72, and 96 hours. The diluent, PBS, and PBS+IFN-γ treatments served as controls. Flow cytometric analysis was performed to assess cell surface expression of IFNGR1, HLA-ABC, and HER2. T/P treatment with and without IFN-γ negatively affected expression levels and % IFNGR1+ and HLA-ABC+ cells in a time dependent manner (Figure 3.11A- B) but had little to no effect on surface HER2 expression (Figure 3.11C).

To confirm T/P inactivation of HER2 signalling and to determine whether T/P treatment modulated the IFN-γ pathway, WCLs prepared from T/P-treated cells were analysed by western blotting (Figure 3.12). Analysis showed T/P treatment without IFN-γ (Figure 3.12A, left panel) generally reduced basal levels of pHER2 and HER2 except for a blip at the 48 hour time point. By contrast, basal ERα levels increased following T/P treatment peaking at 48-72 hours. Aside from reduced pAKT at 24 and 96 hours and slightly reduced pERK at 96 hours, HER2 downstream signalling was not significantly altered by T/P treatment alone. Similar to previous experiments, pSTAT-1, IRF-1 or HLA-HC chains were not detected in the absence of IFN-γ.

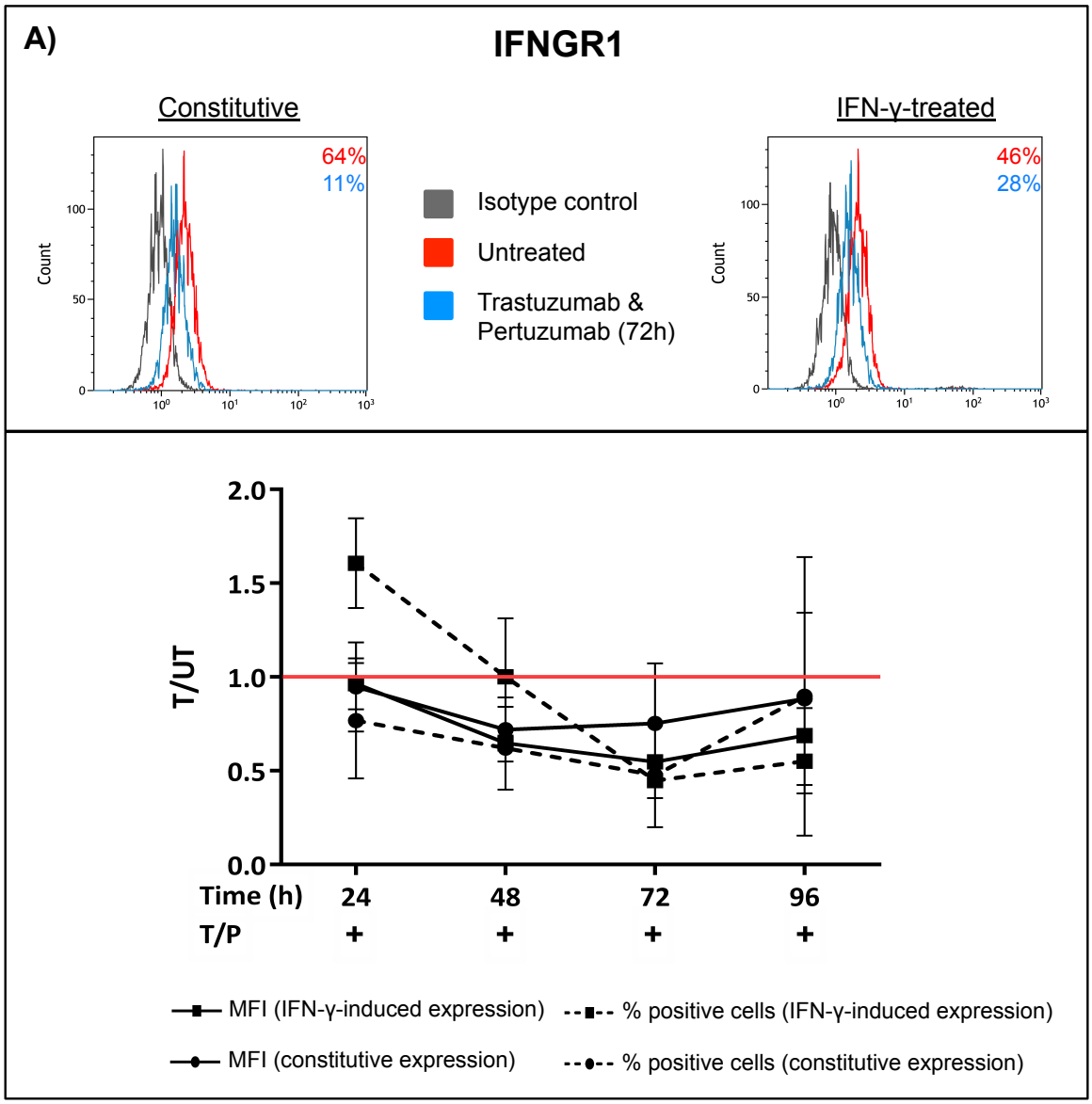
Comparing the effects of T/P treatment in IFN-γ-stimulated cells to IFN-γ-stimulated cells alone (Figure 3.12A, right and middle panels, respectively and Figure

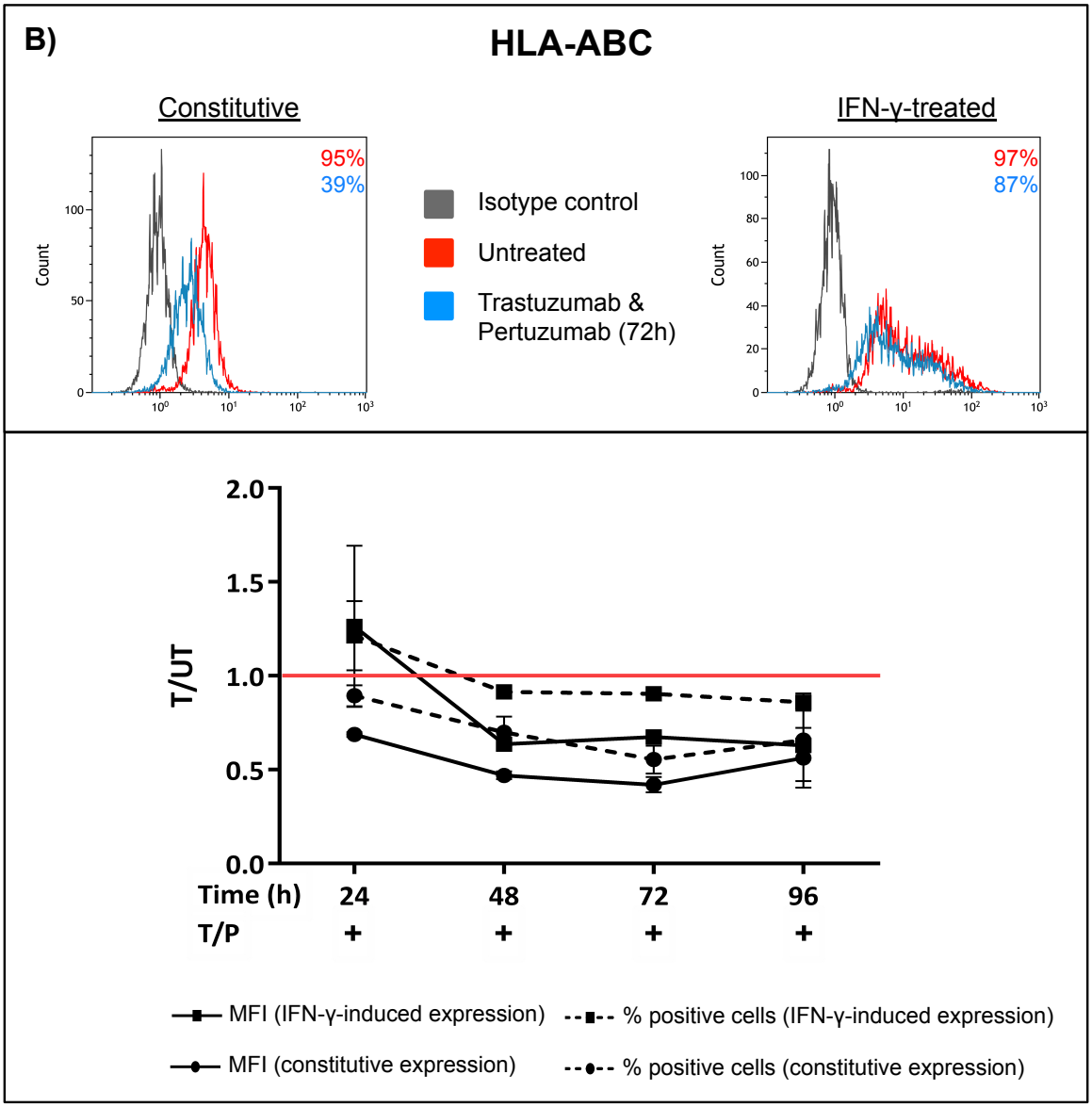
3.12B), evidently T/P greatly reduced pHER2, HER2, pAKT and pERK. Interestingly, ERα levels also declined in a time-dependent manner in T/P plus IFN-γ-treated BT-474 cells. Expression of both pSTAT-1 and IRF-1 decreased over time in PBS+IFN-γ-treated cells while HLA-BC levels gradually increased, peaking at 96 hours. Levels of pSTAT-1 and IRF-1 levels were clearly higher at all time points in T/P+IFN-γ-treated BT-474 as compared to treated PBS+IFN-γ. Similarly, HLA-BC chains in the absence and presence of T/P accumulated in a time-dependent manner, but more rapidly in the T/P-treated cells. Thus, over time T/P treatment abolished HER2 signalling, which correlated with increased IFN-γ signalling in BT-474 cells.

In summary, increased levels of pSTAT-1, IRF-1, and HLA-BC H-chains in T/P+IFN-γ-treated BT-474 over time did not correspond with increased levels of surface HLA-ABC suggesting dysfunctional APM proteins may be involved in the lack of surface HLA-ABC upregulation. However, western blotting has only been performed once, limiting our interpretation and more experiments are needed to confirm these results.

Figure 3.11: Treatment with trastuzumab+pertuzumab modulates cell surface IFNGR1 and HLA-ABC levels in BT-474 cells.

BT-474 cells treated with T/P (10 μg/mL each), stimulated or not with IFN-γ (100 U/mL), at the indicated time points. Flow cytometric analysis was used to assess cell surface expression levels of IFNGR1 (A), HLA-ABC (B), and HER2 (C). Top: Histogram overlays are representative of one experiment. Percentage of positive cells is colour-matched accordingly. Bottom: Line graphs represent the mean MFI or %+ cells of treated (T and P +/- IFN-γ) over untreated (PBS +/- IFN-γ) with error bars ± SEM of three replicate experiments. Statistical analysis was done by two-way ANOVA and Tukey HSD test.


A)
IFNGR1
Constitutive
IFN-γ-treated
64%
11%
46%
28%
Count
Isotype control
Untreated
Trastuzumab & Pertuzumab (72h)
T/UT
Time (h)
T/P
24
48
72
96
MFI (IFN-γ-induced expression)
% positive cells (IFN-γ-induced expression)
MFI (constitutive expression)
% positive cells (constitutive expression)


B)
HLA-ABC
Constitutive
IFN-γ-treated
95%
39%
97%
87%
Count
Isotype control
Untreated
Trastuzumab & Pertuzumab (72h)
T/UT
Time (h)
T/P
24
48
72
96
MFI (IFN-γ-induced expression)
% positive cells (IFN-γ-induced expression)
MFI (constitutive expression)
% positive cells (constitutive expression)

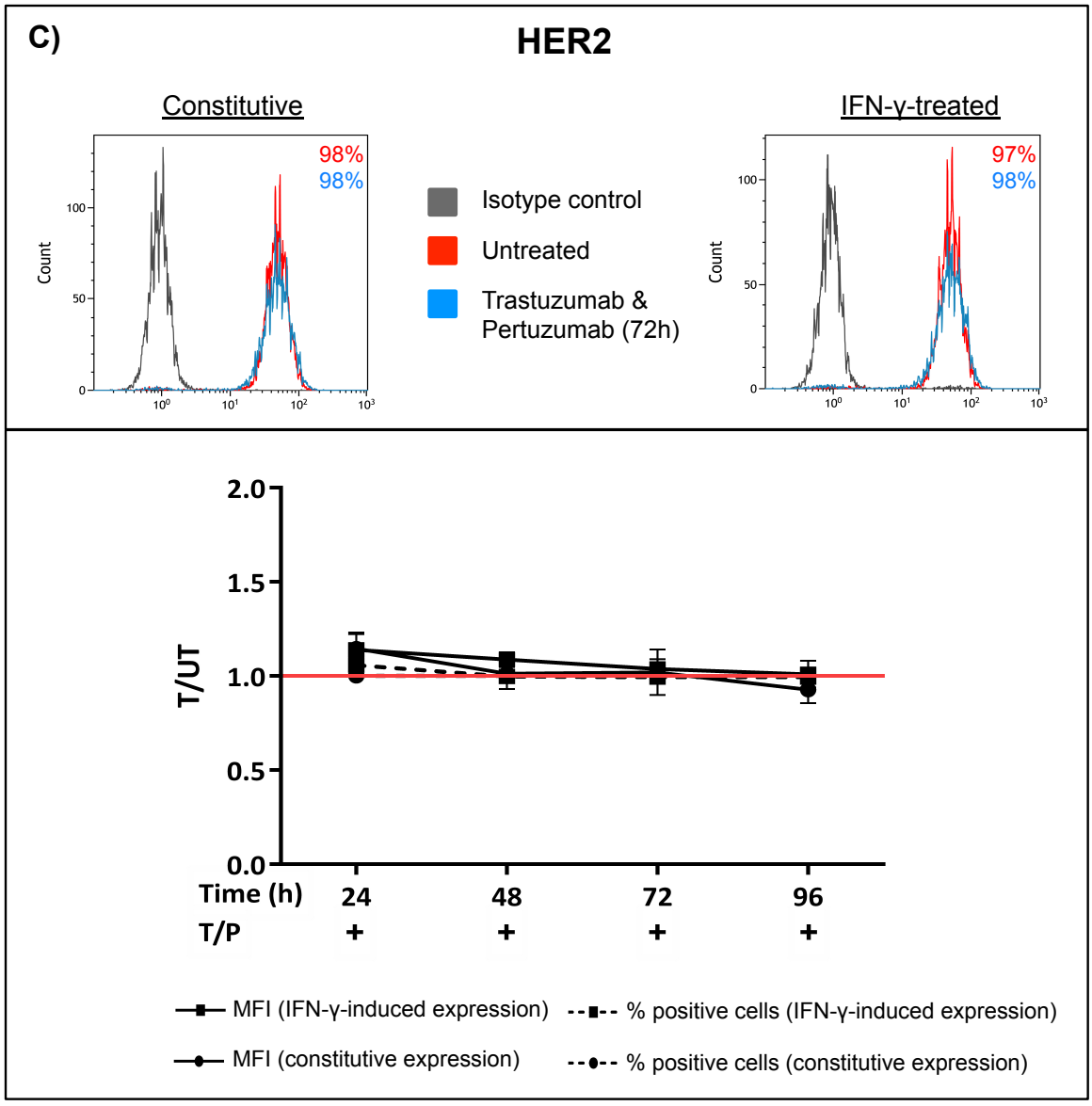
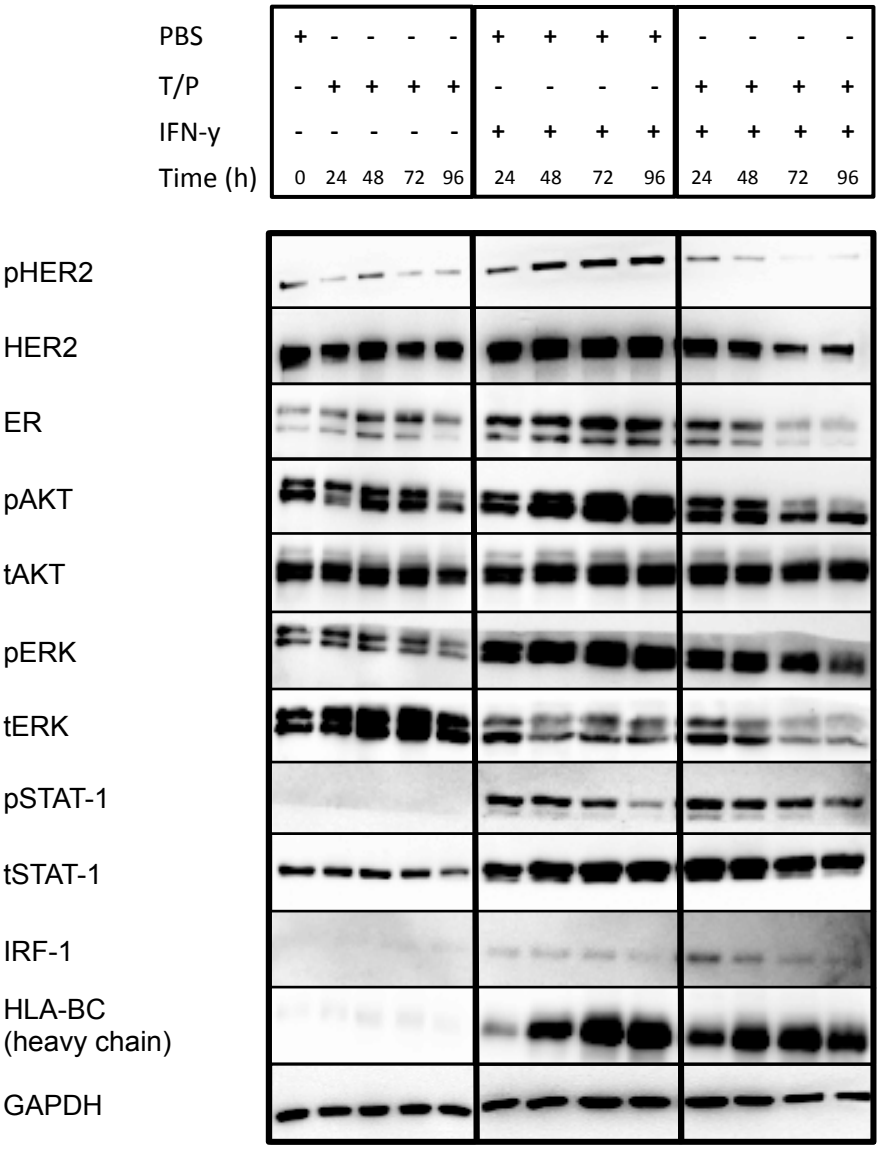

C)
HER2
Constitutive
IFN-γ-treated
98%
98%
97%
98%
Count
Isotype control
Untreated
Trastuzumab &
Pertuzumab (72h)
T/UT
Time (h)
T/P
24
48
72
96
MFI (IFN-γ-induced expression)
% positive cells (IFN-γ-induced expression)
MFI (constitutive expression)
% positive cells (constitutive expression)

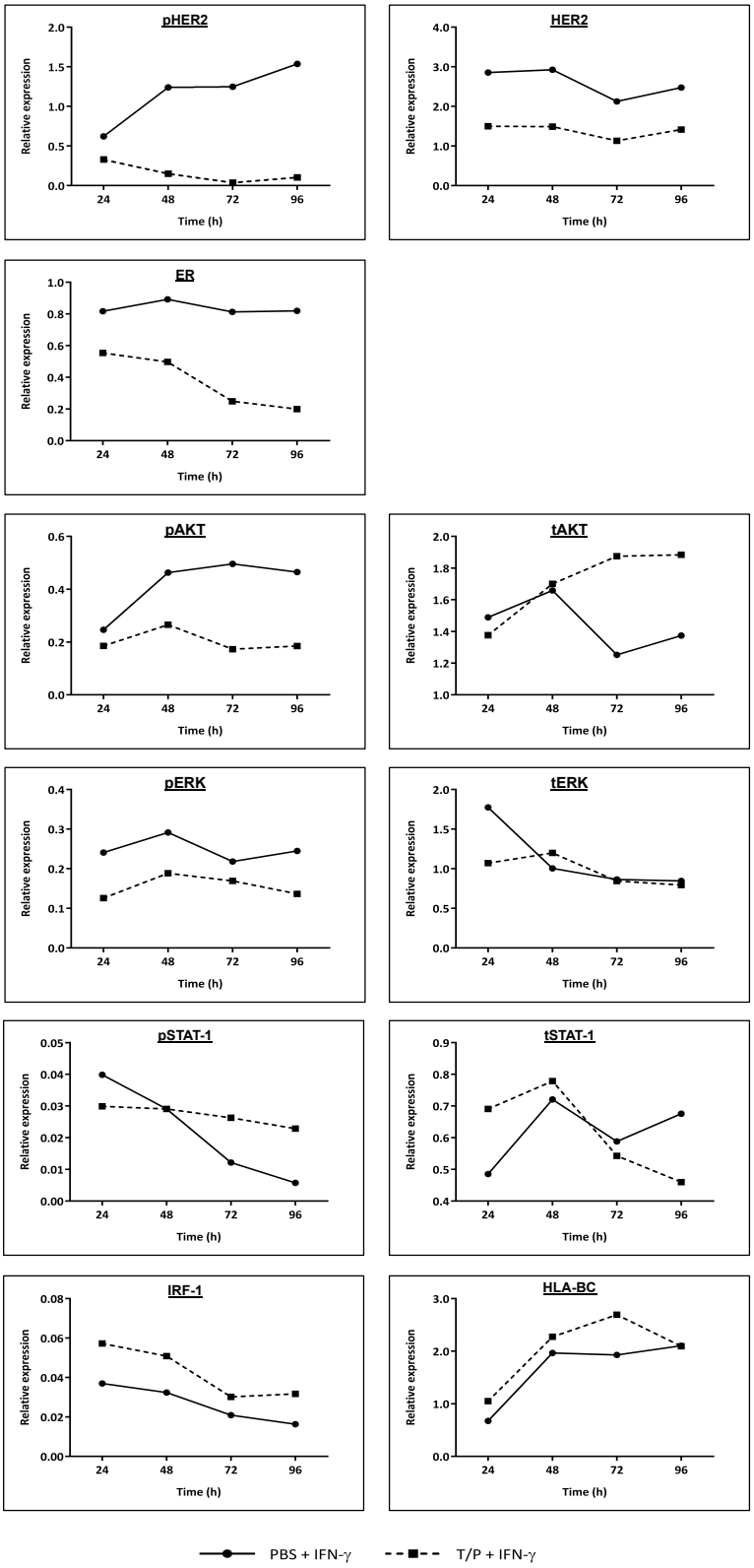
Figure 3.12: Trastuzumab+pertuzumab-mediated inhibition of HER2 signalling in BT-474 resulted in time-dependent alterations of IFN-γ proteins and HLA-BC induction.

BT-474 cells were treated with T/P (10 μg/mL each) or PBS (vehicle control), +/- IFN-γ (100 U/mL), at the indicated time points. Western blotting from WCLs was used to determine expression levels of indicated proteins. A) Representative western blot. B) Bar graphs for relative expression show the ratio of band intensity for indicated proteins, normalized to GAPDH band intensity. Graphs represent one experiment.

A)


PBS + - - - - + + + + - - - -
T/P - + + + + - - - - + + + +
IFN-y - - - - - + + + + + + + +
Time (h) 0 24 48 72 96 24 48 72 96 24 48 72 96
pHER2
HER2
ER
pAKT
tAKT
pERK
tERK
pSTAT-1
tSTAT-1
IRF-1
HLA-BC (heavy chain)
GAPDH

B)


pHER2
HER2
ER
pAKT
tAKT
pERK
tERK
pSTAT-1
tSTAT-1
IRF-1
HLA-BC
Relative expression
Time (h)
PBS + IFN-γ
T/P + IFN-γ

3.2.5 Effects of HER2 silencing on IFN-γ signalling in HER2-overexpressing cell line SKBR3

To further explore the relationship between HER2 activation and IFN-γ signalling without the potentially confounding effect of ERα, we inhibited HER2 signalling in SKBR3 as described in Section 3.2. This was followed by analysis of IFN-γ pathway proteins and HLA Class I induction using flow cytometry and western blotting.

3.2.6 Lap-mediated HER2 inhibition alters IFN-γ signalling and HLA Class I expression in SKBR3

SKBR3 cells, treated with Lap (0.5μM) or DMSO (control) with and without IFN-γ for 24 hours, were analyzed by flow cytometry to determine cell surface expression of IFNGR1, HLA-ABC, and HER2. Similar to the results observed for BT-474 (Section 3.2.2), Lap treatment significantly reduced IFNGR1 and HLA-ABC expression as well as % IFNGR1+ in untreated and IFN-γ-treated SKBR3 cells (Figure 3.13A-B). HER2 expression was not significantly affected by Lap (Figure 3.13C).

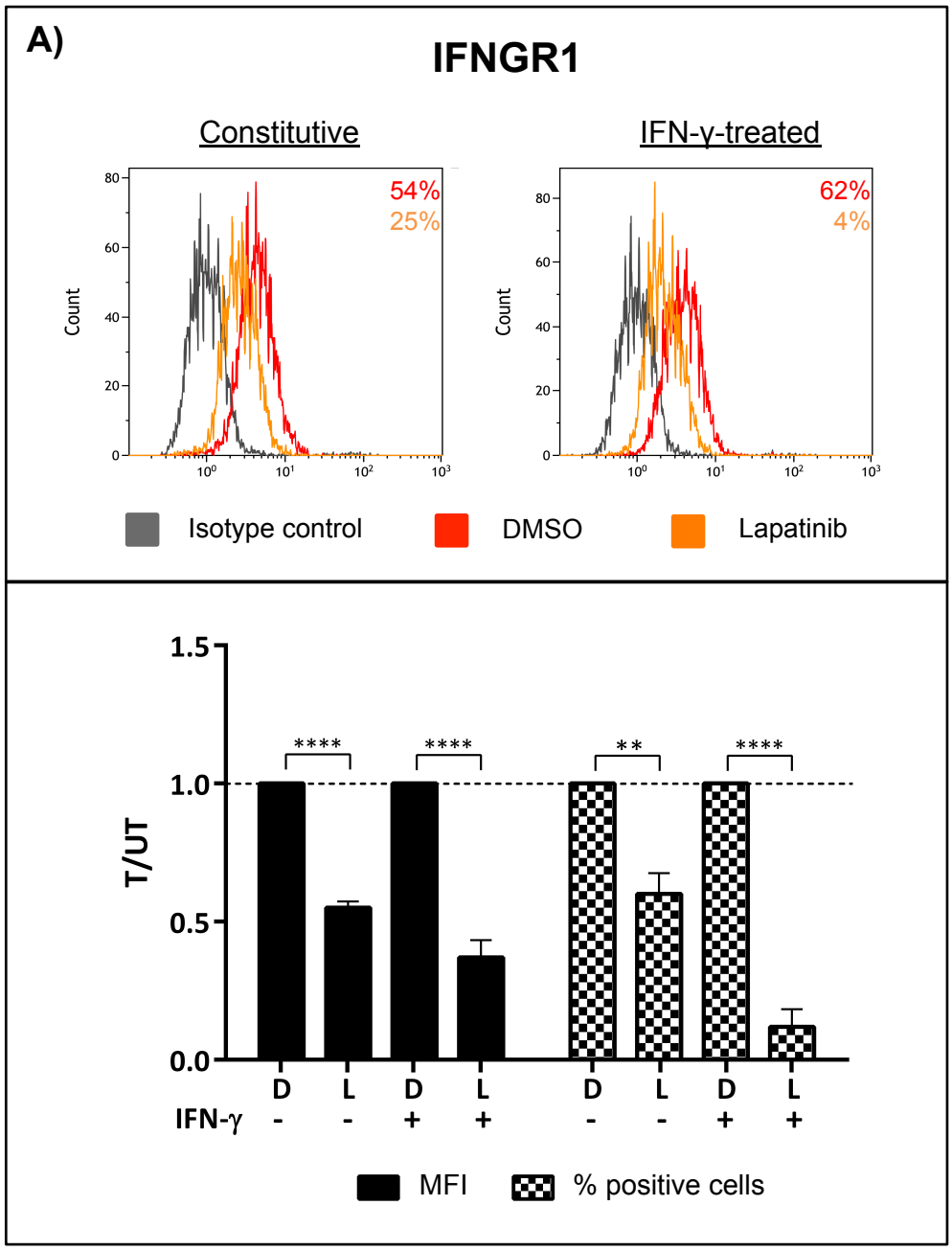
To confirm the efficacy of Lap, western blotting was performed to ensure inactivation of HER2 signalling and to examine whether HER2 inhibition modulated IFN-γ signalling in the SKBR3 cells. WCLs prepared from SKBR3 cells, treated as described above, were analyzed for the indicated proteins (Figure 3.14A-B). Lap significantly diminished pHER2 levels in untreated and IFN-γ-stimulated SKBR3 cells together with strongly reduced pAKT and pERK, confirming Lap efficacy. Basal expression of pSTAT-1,

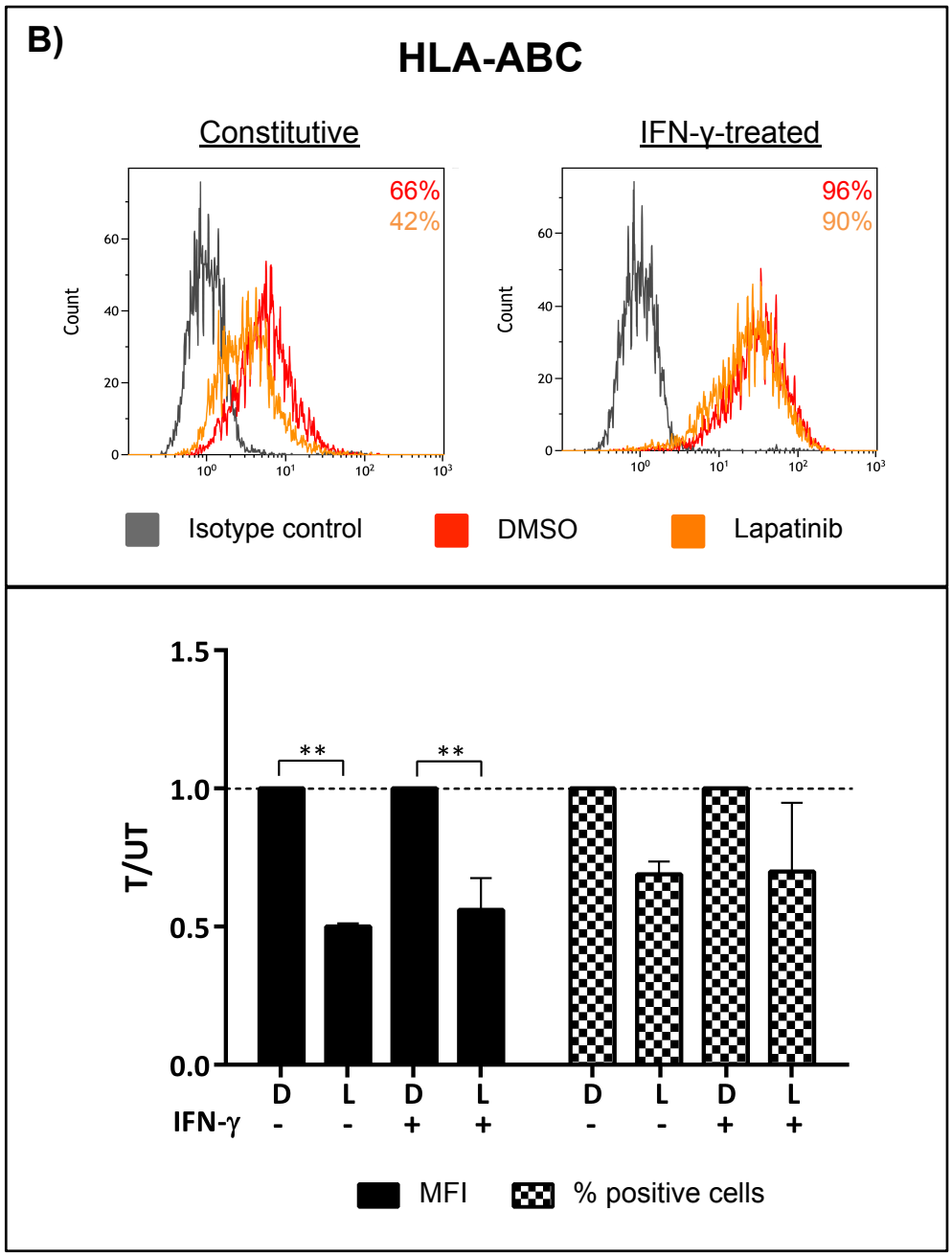
IRF-1, and HLA-BC H-chains was not detected, but all were clearly upregulated in IFN-γ-treated cells. Lap, compared to DMSO treatment, significantly reduced pSTAT-1 levels. Unfortunately, IRF-1 and HLA-BC levels were variable as indicated by the high standard errors, which limits the evaluation of these findings (Figure 3.14B).

Taken together, successful inhibition of HER2 signalling by Lap did not lead to increased surface HLA-ABC. In fact, constitutive and IFN-γ-induced HLA-ABC along with IFNGR1 were significantly reduced when SKBR3 cells were treated with Lap. This suggests that Lap-mediated downregulation of HER2 may inhibit HLA-ABC expression independent of IFN-γ stimulation.

Figure 3.13: Lap significantly modulates IFNGR1 and HLA-ABC on the cell surface of SKBR3.

Flow cytometric analysis of SKBR3, treated with Lap (L) (0.5 μM) or DMSO (D) (vehicle control), +/- IFN-γ (100 U/mL) for 24 hours, was used to determine constitutive and IFN-γ-stimulated IFNGR1 (A), HLA-ABC (B), and HER2 (C) cell surface expression. Top: Histogram overlays are representative of one experiment. The percentage of positive cells is colour-matched accordingly. Bottom: Bar graphs represent the mean MFI or %+ cells of treated (L +/- IFN-γ) over untreated (D +/- IFN-γ) with error bars ± SEM of three independent experiments. Statistical analysis was done by one-way ANOVA and Tukey HSD test (**$\rho<0.01$, **** $\rho<0.0001$).


A)
IFNGR1
Constitutive
IFN-γ-treated
54%
25%
62%
4%
Count
Isotype control
DMSO
Lapatinib
T/UT
1.5
1.0
0.5
0.0
****
****
**
****
D L D L D L D L
IFN-γ - - + + - - + +
MFI
% positive cells


B)
HLA-ABC
Constitutive
IFN-γ-treated
66%
42%
96%
90%
Count
Isotype control
DMSO
Lapatinib
T/UT
1.5
1.0
0.5
0.0
**
**
D L D L D L D L
IFN-γ - - + + - - + +
MFI
% positive cells

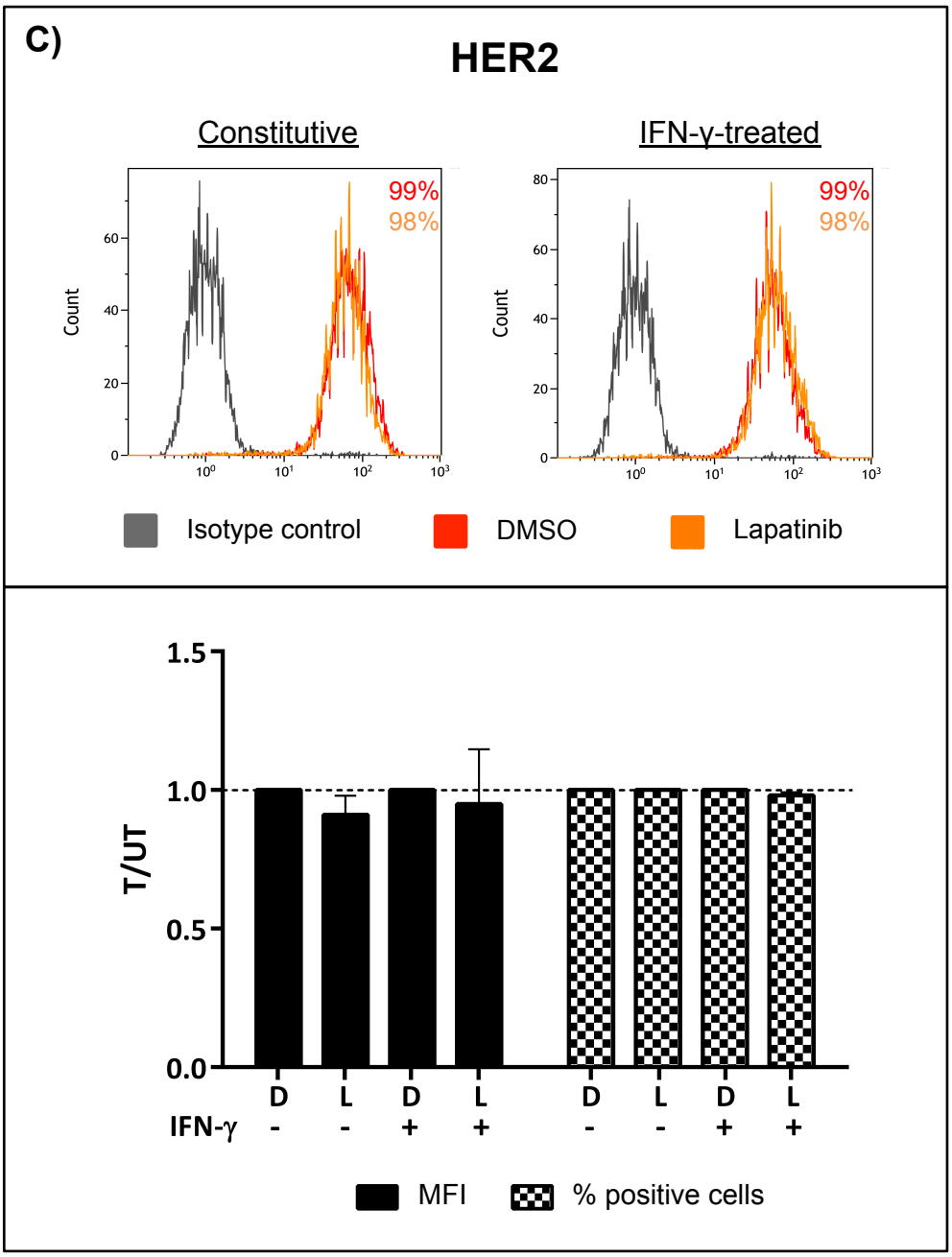
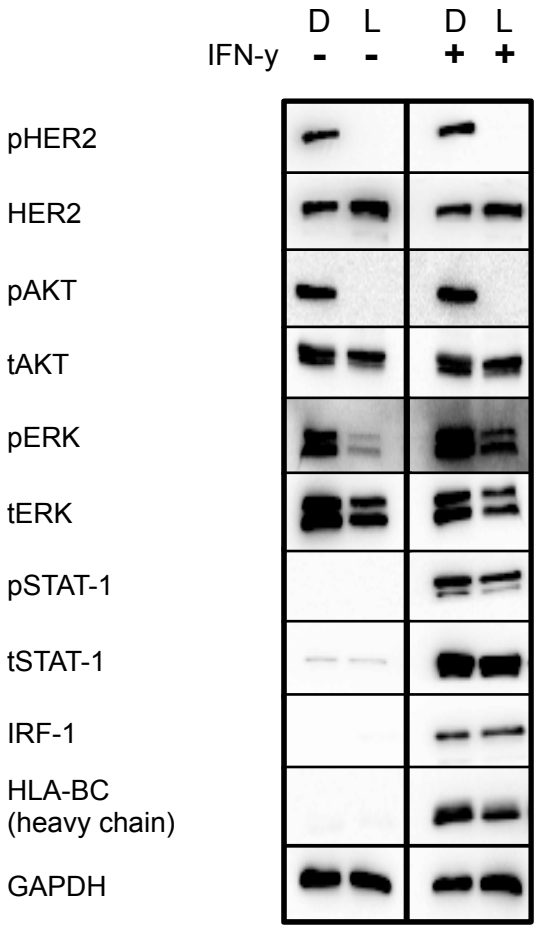

C)
HER2
Constitutive
IFN-γ-treated
99%
98%
99%
98%
Count
Count
Isotype control
DMSO
Lapatinib
T/UT
1.5
1.0
0.5
0.0
D L D L D L D L
IFN-γ - - + + - - + +
MFI
% positive cells

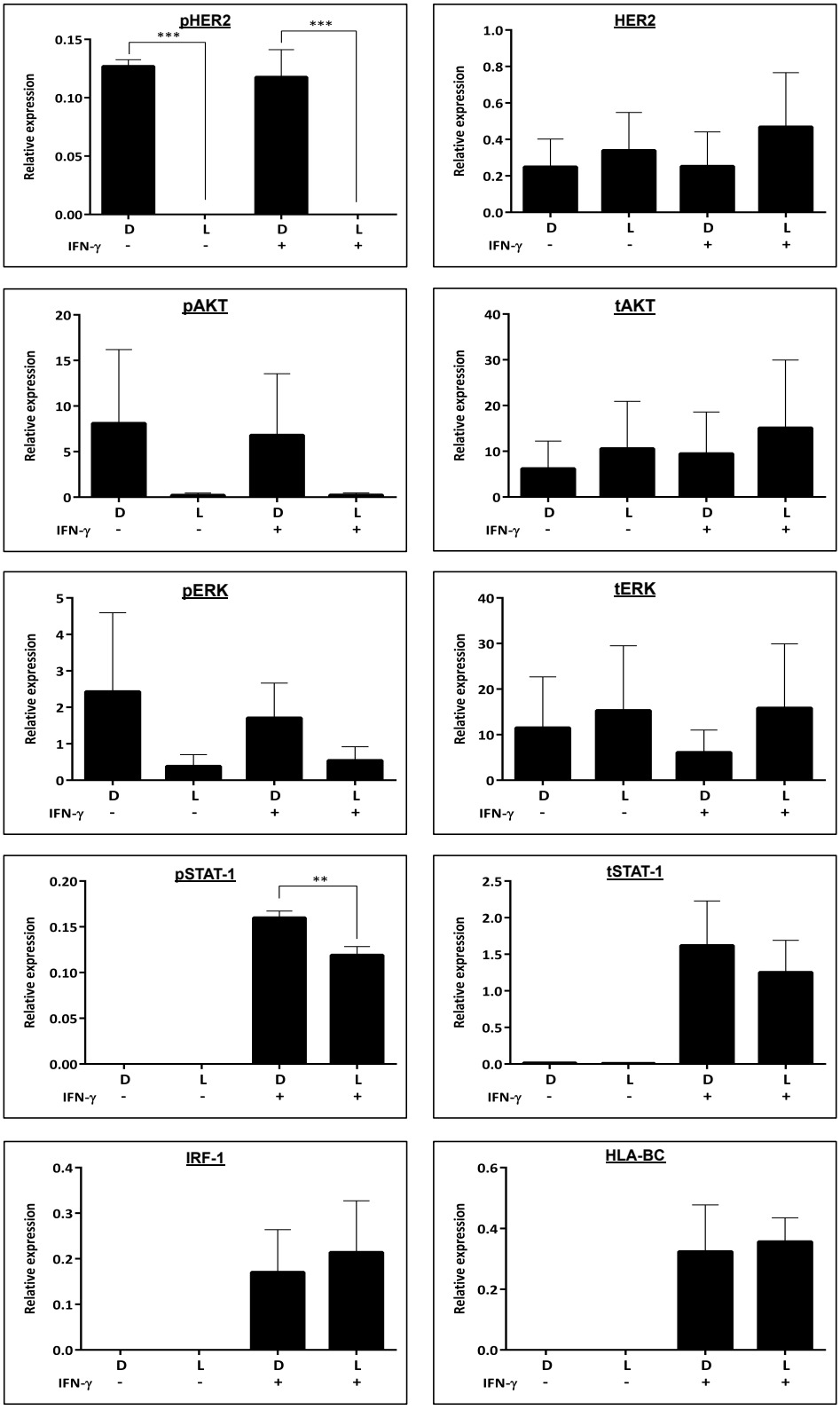
Figure 3.14: Lap-induced inhibition of HER2 signalling differently modulates IFN-γ pathway components in SKBR3.

SKBR3 cells were treated with Lap (L) (0.5 μM) or DMSO (D) (vehicle control), stimulated or not with IFN-γ (100 U/mL) for 24 hours and analyzed via western blotting of WCLs for the indicated proteins. A) Representative western blot. B) Bar graphs for relative expression show the ratio of band intensity for indicated proteins, normalized to GAPDH band intensity. Graphs represent the mean ± SEM of three replicate experiments (Exception: Antibodies for pERk and tERK detection were only used on one set of lysates). Statistical analysis was done by one-way ANOVA and Tukey HSD test (**$\rho<0.01$, ***$\rho<0.001$).

A)


D L D L
IFN-γ - - + +
pHER2
HER2
pAKT
tAKT
pERK
tERK
pSTAT-1
tSTAT-1
IRF-1
HLA-BC
(heavy chain)
GAPDH

B)


pHER2
***
***
HER2
pAKT
tAKT
pERK
tERK
pSTAT-1
**
tSTAT-1
IRF-1
HLA-BC
Relative expression
D L D L
IFN-γ - - + +

3.2.7 Effects of ERBB2 siRNA silencing on IFN-γ signalling and HLA Class I expression in SKBR3

SKBR3 cells, treated with ERBB2 siRNA, SC siRNA or Mock for 24 hours, then stimulated or not with IFN-γ for an additional 24 hours, were analyzed by flow cytometry as described in the previous section. Compared to control siRNA, ERBB2 silencing downregulated IFNGR1 in untreated and IFN-γ-treated SKBR3 (Figure 3.15A). Constitutive, but not IFN-γ-inducible % of HLA-ABC+ cells was significantly reduced in ERBB2 siRNA-transfected cells (Figure 3.15B). A similar trend was observed for HLA-ABC expression (MFI). HER2 levels were significantly decreased in SKRB3 cells following ERBB2 siRNA transfection with or without IFN-γ (Figure 3.15C).

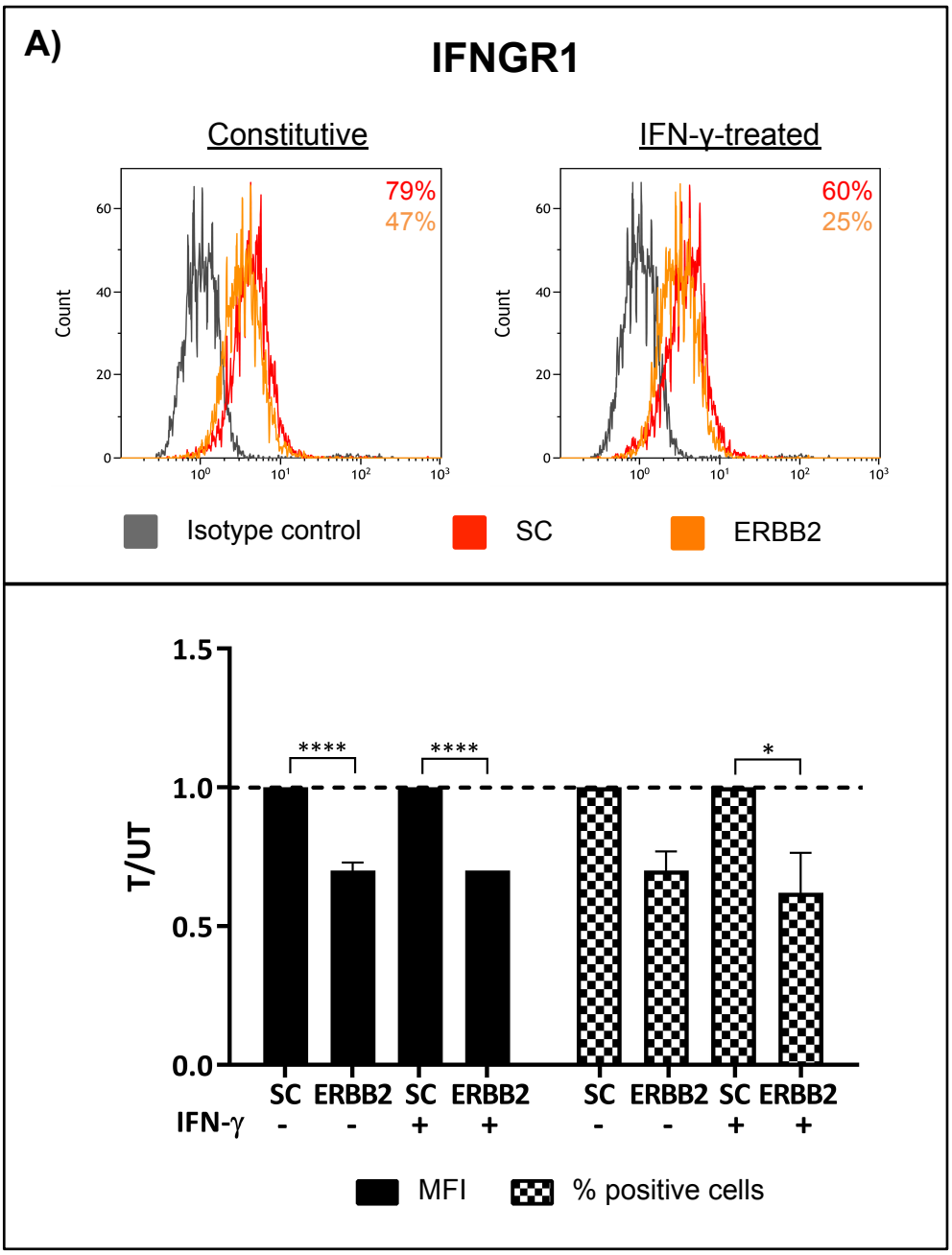
Western blot analysis of WLC prepared from transfected SKBR3 cells, treated and analyzed for proteins as indicated (Figure 3.16A-B), showed ERBB2 siRNA abolished pHER2 and HER2 expression and reduced pAKT and pERK in IFN-γ-treated (or not) cells. Very low levels of constitutive pSTAT-1, observed in some experiments (Figure 3.16A) and higher levels of pSTAT-1, IRF-1, and HLA-BC H-chains detected in IFN-γ-stimulated cells (Figure 3.16A-B), were marginally affected by ERBB2 siRNA silencing. For example, pSTAT-1 was decreased, IRF-1 somewhat upregulated and HLA-BC expression slightly reduced in ERBB2-siRNA transfected as compared to SC siRNA control (Figure 3.16A-B).

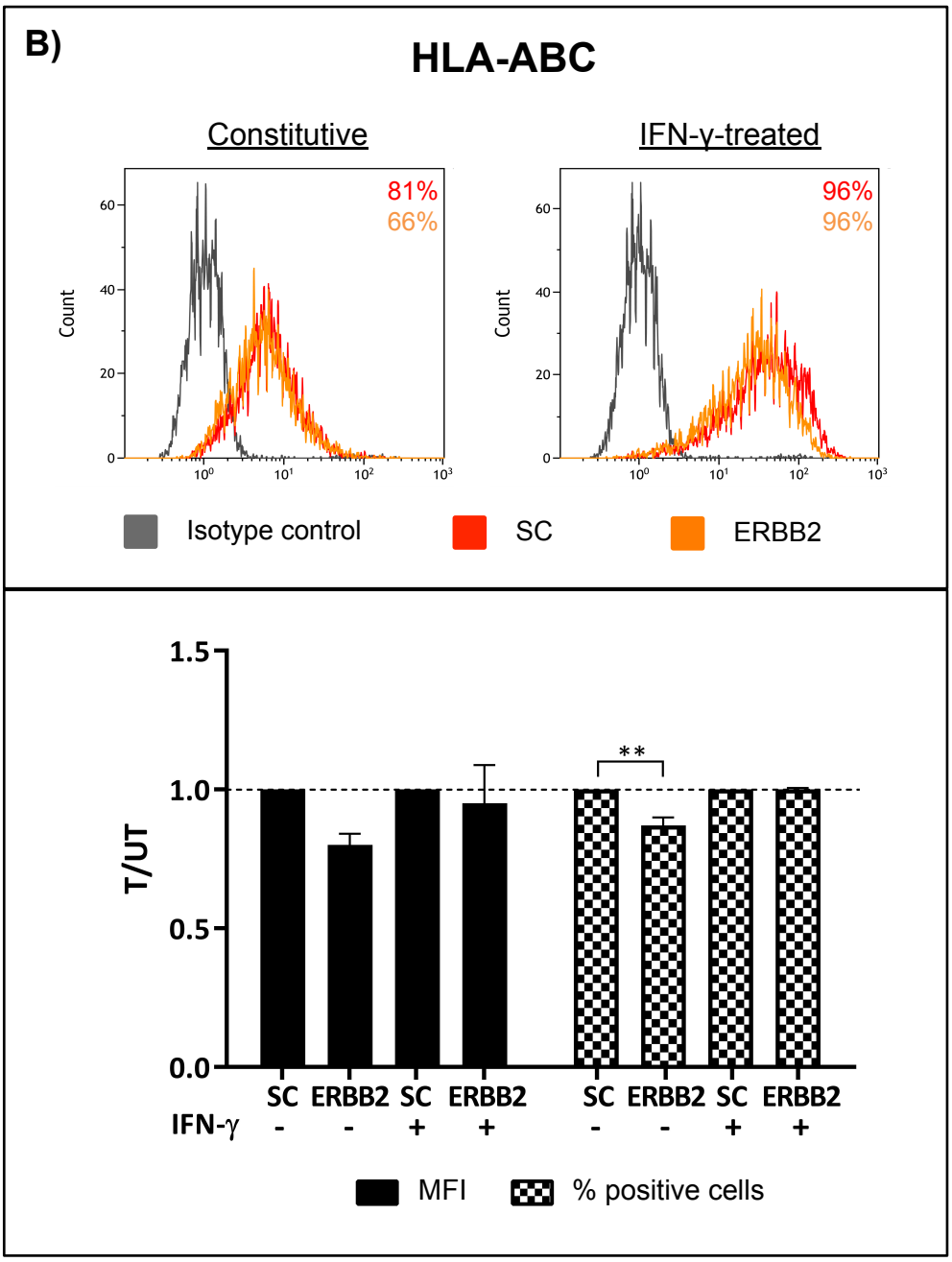
In summary, silencing of HER2 signalling by ERBB2-siRNA was accompanied by significantly reduced surface IFNGR1 suggesting a possible correlation between HER2

and IFNGR1 expression in SKBR3 while HLA-BC H-chain levels were not affected. However, we did observe a significant decrease in constitutive % of HLA-ABC+ cells.

Figure 3.15: siRNA ERBB2-mediated knockdown of HER2 alters cell surface expression of IFNGR1 and HLA-ABC in SKBR3.

SKBR3 cells were treated with either ERBB2 siRNA (5 µM) or SC siRNA (5 µM) for 24 hours followed by IFN-γ (100 U/mL) stimulation for an additional 24 hours. Expression levels of IFNGR1 (A), HLA-ABC (B), and HER2 (C) were analyzed by cell surface flow cytometry. Top: Histogram overlays are representative of one experiment. The percentage of positive cells is colour-matched accordingly. Bottom: Bar graphs represent the MFI or %+ cells of treated (ERBB2 siRNA +/- IFN-γ) over untreated (SC siRNA +/- IFN-γ) with error bars showing mean ± SEM of three replicate assays. Statistical analysis was done by one-way ANOVA and Tukey HSD test (*ρ<0.05, **ρ<0.01, ***ρ<0.001, ****ρ<0.0001).


A)
IFNGR1
Constitutive
IFN-γ-treated
79%
47%
60%
25%
Count
Isotype control
SC
ERBB2
T/UT
****
****
*
SC ERBB2 SC ERBB2 SC ERBB2 SC ERBB2
IFN-γ - - + + - - + +
MFI
% positive cells


B)
HLA-ABC
Constitutive
IFN-γ-treated
81%
66%
96%
96%
Count
Isotype control
SC
ERBB2
1.5
1.0
0.5
0.0
T/UT
**
SC ERBB2 SC ERBB2 SC ERBB2 SC ERBB2
IFN-γ - - + + - - + +
MFI
% positive cells

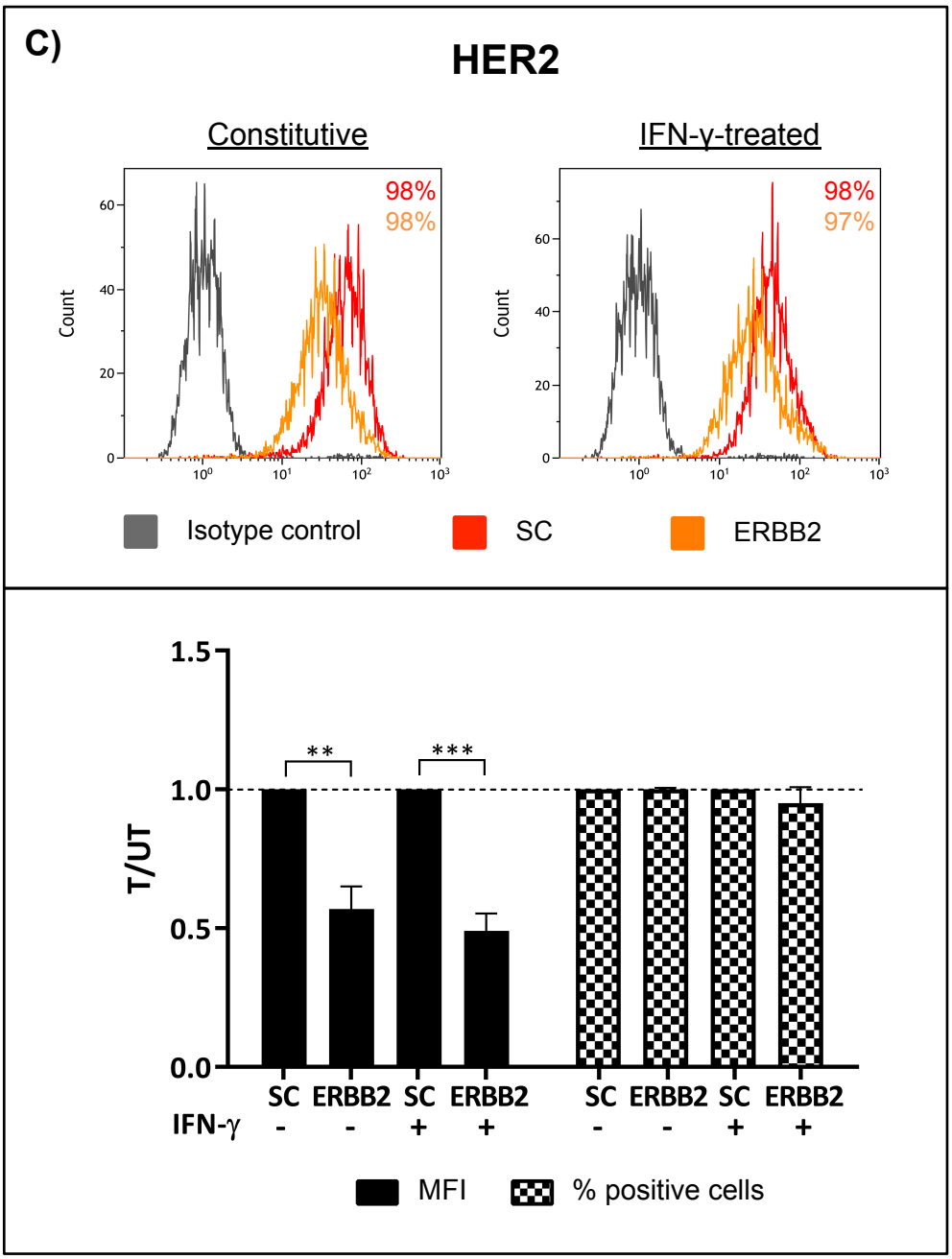
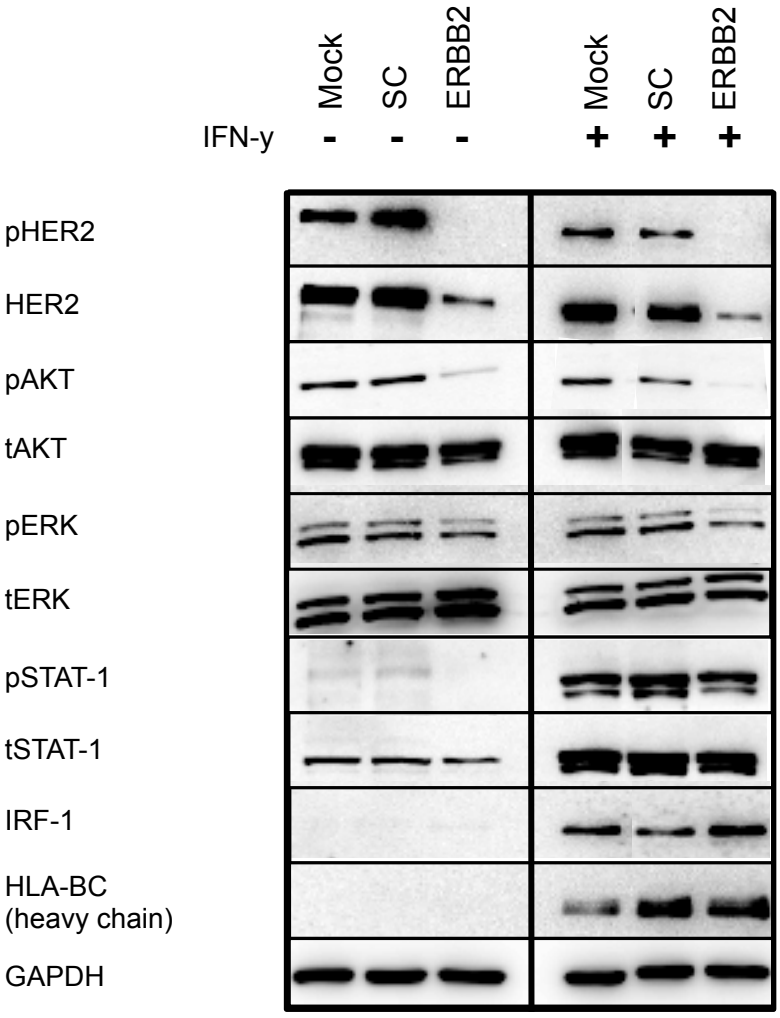

C)
HER2
Constitutive
IFN-γ-treated
98%
98%
98%
97%
Count
Isotype control
SC
ERBB2
T/UT
1.5
1.0
0.5
0.0
**
***
SC ERBB2 SC ERBB2 SC ERBB2 SC ERBB2
IFN-γ - - + + - - + +
MFI
% positive cells

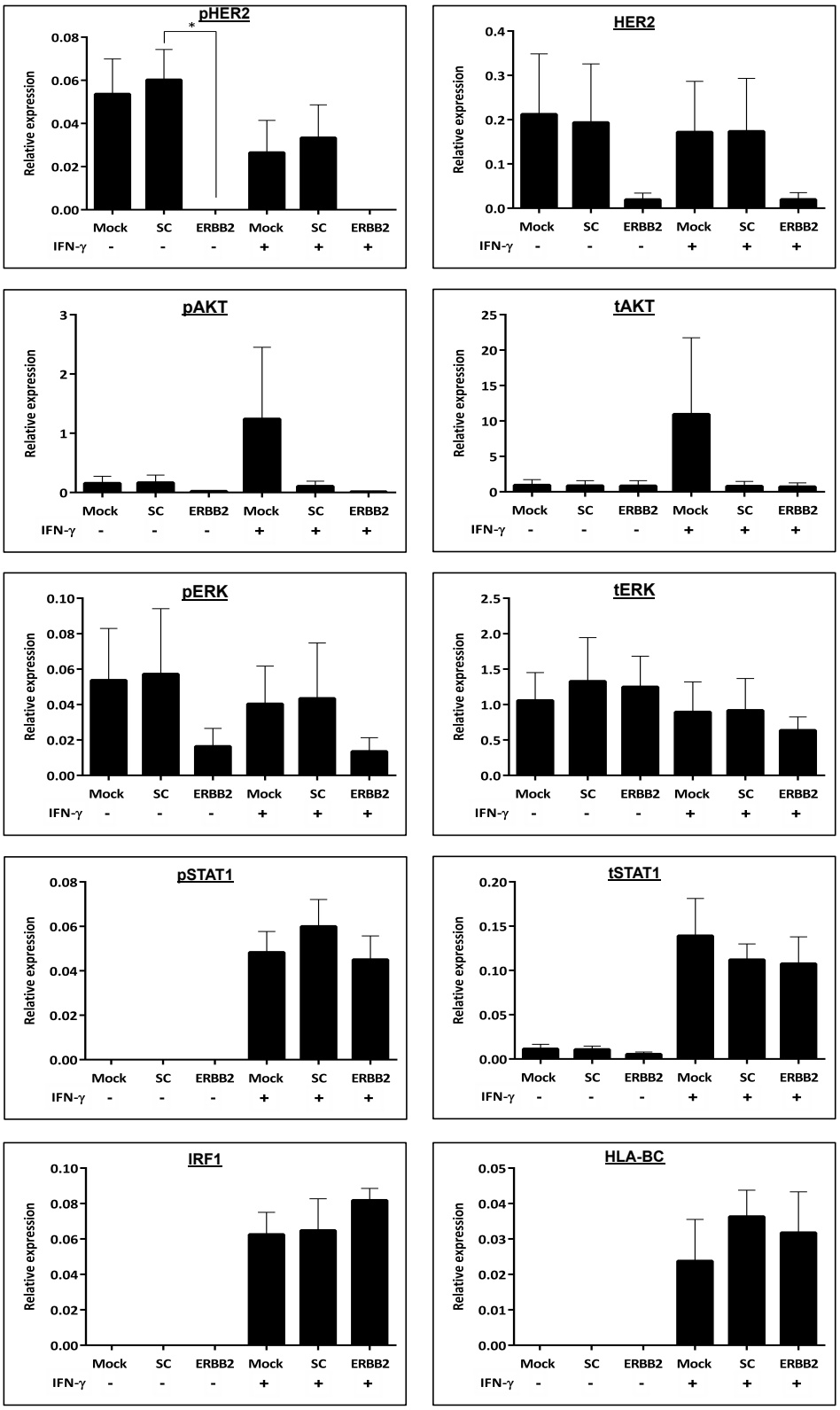
Figure 3.16: Inhibition of HER2 signalling by ERBB2-siRNA differentially affects IFN-γ pathway components in SKBR3.

SKBR3 cells were treated with ERBB2 siRNA (5 μM), SC siRNA (5 μM), or Mock (5 μM) for 24 hours followed by IFN-γ stimulation (100 U/mL) for another 24 hours. Expression levels of indicated proteins were assessed by western blotting of WCLs. A) Representative western blot. B) Bar graphs for relative expression show the ratio of band intensity for indicated proteins, normalized to GAPDH band intensity. Graphs represent the mean ± SEM of three replicate experiments. Statistical analysis was done by one-way ANOVA and Tukey HSD test (*$\rho<0.05$).

A)


Mock
SC
ERBB2
Mock
SC
ERBB2
IFN-y
-
-
-
+
+
+
pHER2
HER2
pAKT
tAKT
pERK
tERK
pSTAT-1
tSTAT-1
IRF-1
HLA-BC
(heavy chain)
GAPDH

B)


pHER2
HER2
pAKT
tAKT
pERK
tERK
pSTAT1
tSTAT1
IRF1
HLA-BC
Relative expression
Mock
SC
ERBB2
IFN-γ

3.2.8 Trastuzumab+pertuzumab-induced inhibition of HER2 pathway modulates IFN-γ signalling and HLA Class I induction in SKBR3

The experiments described in the previous two sections (Section 3.2.6 and 3.2.7) were done at t=24 hours post IFN-γ-stimulation. In the following experiments, SKBR3 cells were simultaneously treated with HER2-targeting antibodies T/P, with and without IFN-γ over 24, 48, 72, and 96 hours. PBS and PBS+IFN-γ treatments served as controls. Flow cytometry analysis (Figure 3.17) revealed IFNGR1 expression levels were not significantly altered by T/P in IFN-γ- or not stimulated cells, even at the 24 hour time point. Interestingly, decreased %+ IFNGR1 cells were observed in T/P-treated cells without IFN-γ (Figure 3.17A), compared to increased %+ cells treated with both T/P and IFN-γ up to the 96 hour time point. HLA-ABC expression, stimulated or not with IFN-γ, was slightly upregulated by T/P over time compared to appropriate PBS controls while %+ cells remained unchanged (Figure 3.17B). Similarly, T/P reduced HER2 expression in a time-dependent manner in IFN-γ-treated and untreated cells but had no effect on %+ HER2 cells (Figure 3.17C). Thus, in these experiments, reduced surface HER2 expression inversely correlates with increased HLA Class I expression.

To explore T/P effects on IFN-γ pathway components and HER2 downstream signalling, we performed a single western blot analysis of WCLs from SKBR3, treated as indicated in Figure 3.18A-B. In cells treated with T/P alone (Figure 3.18A, left panel) or with T/P+IFN-γ (Figure 3.18A right panel), levels of pHER2 and HER2 had barely decreased at 24 hours, whereas in the previous set of experiments, both were nearly

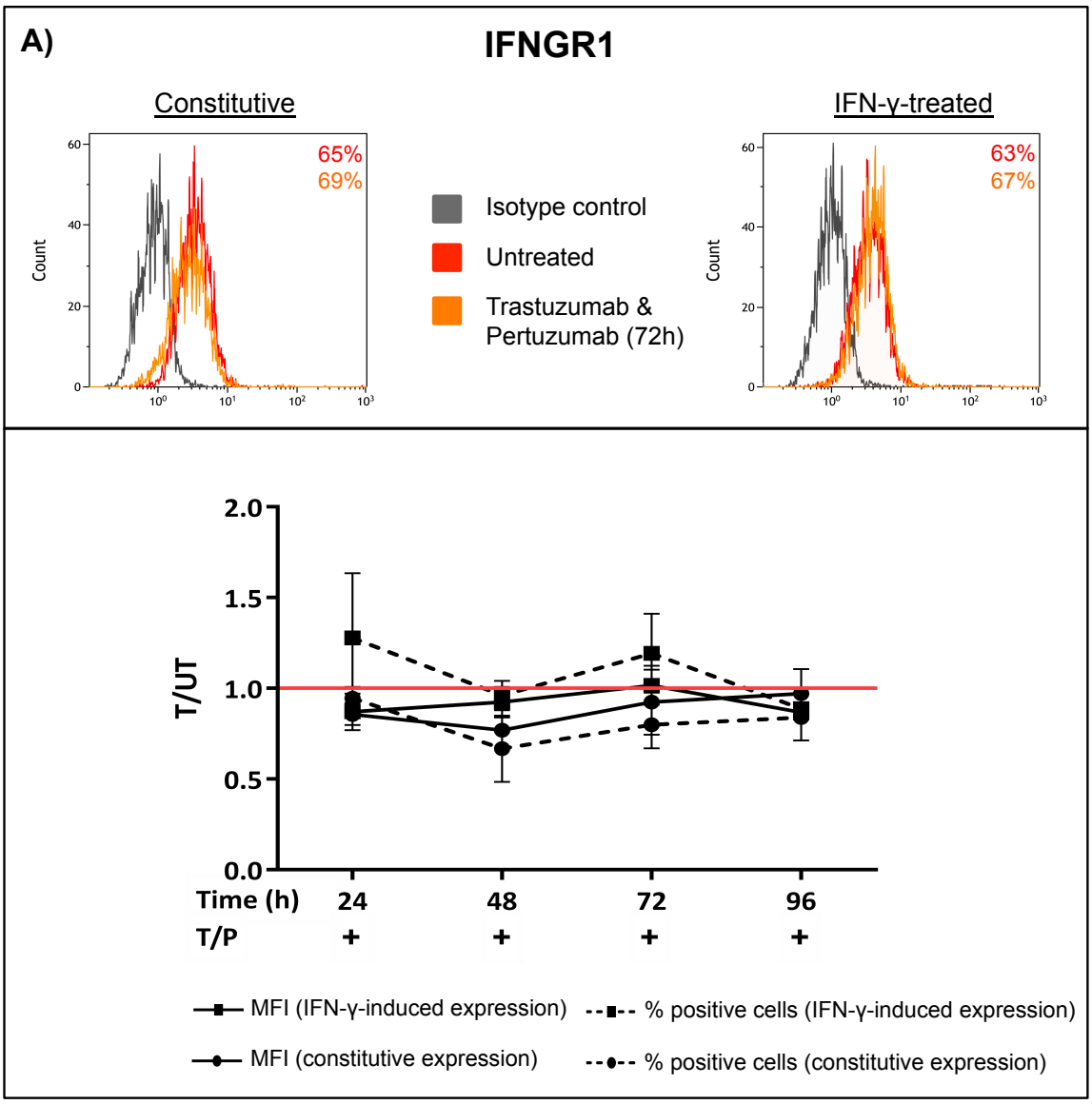
abolished in ERBB2 siRNA-treated cells (Figure 3.15C and Figure 3.16A-B) at that time point. However, levels declined over time following T/P treatment reaching the lowest expression at 72 hours without IFN-γ (Figure 3.18A, left panel) and 96 hours with IFN-γ (Figure 3.18A, right panel). These changes were generally reflected in downstream pAKT and pERK levels.

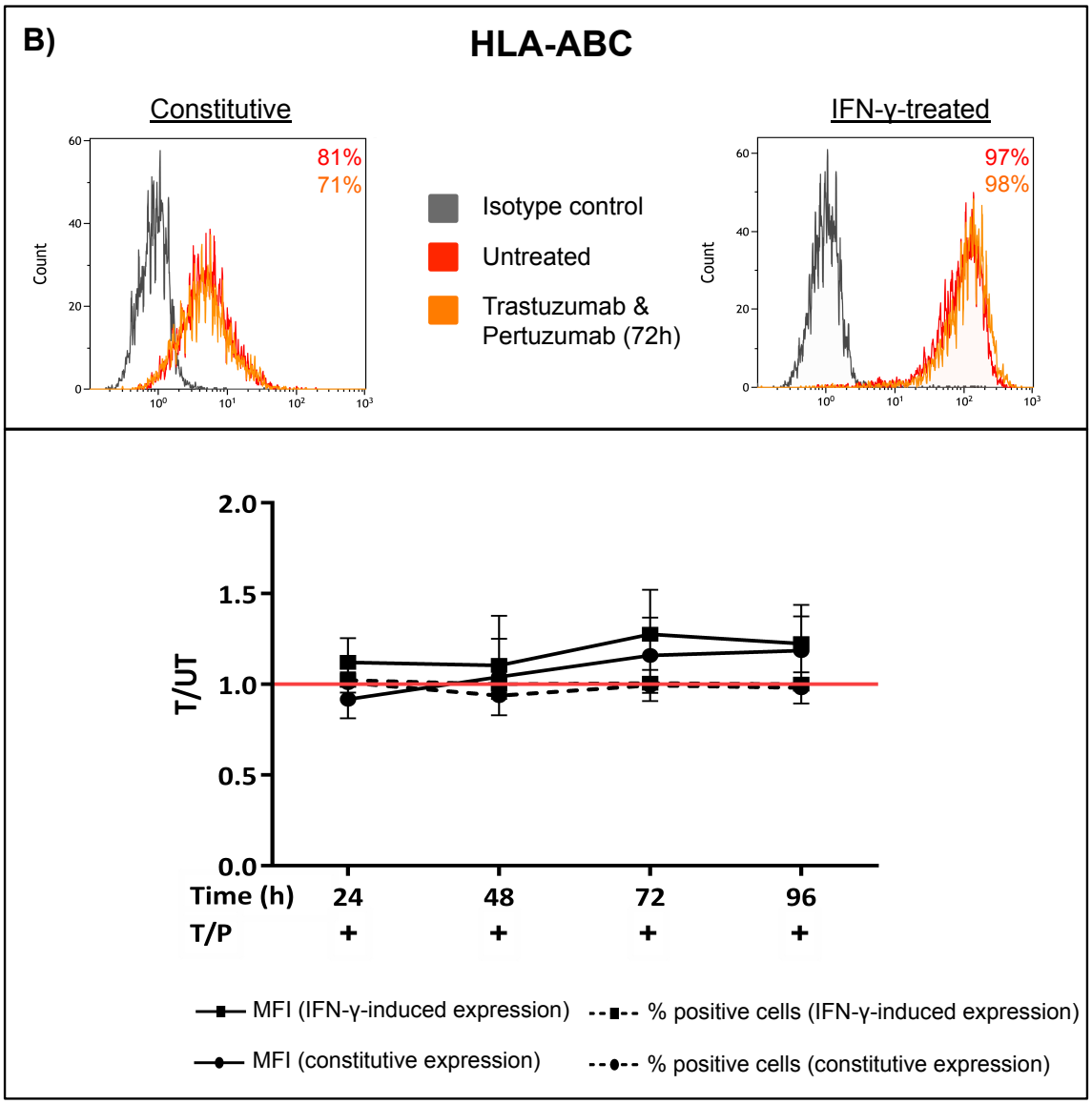
Phosphorylated STAT-1, IRF-1, and HLA-BC H-chains were not detect in untreated SKBR3 cells (Figure 3.18A left panel) but were strongly upregulated in IFN-γ-treated cells (Figure 3.18A middle panel). Levels of pSTAT-1 did not change over time, IRF-1 levels declined and HLA-BC H-chains increased in a time dependent manner. Compared to IFN-γ alone, cells treated with both, T/P and IFN-γ (Figure 3.18A right panel), increased pSTAT-1 and IRF-1 in a time-dependent manner and showed even higher levels of HLA-BC H-chains, reaching highest expression at 96 hours (Figure 3.18A-B)

Intracellular IRF-1 and HLA-BC H-chains increased over time when SKBR3 cells were treated with T/P and IFN-γ, which correlated with a marginal yet not significant upregulation of surface HLA-ABC. This indicates that APM and subsequent trafficking of HLA Class I molecules to the cell surface may be dysregulated. Notably, western blotting was only performed once limiting the evaluation of these results and more experiments are needed to confirm our findings.

Figure 3.17: Treatment with trastuzumab+pertuzumab has diverse effects on IFNGR1, HLA-ABC, and HER2 expression in SKBR3 over time.

SKBR3 cells were treated with T/P (10 μg/mL each), stimulated or not with IFN-γ (100 U/mL), at the indicated time points. Flow cytometric analysis was used to determine IFNGR1 (A), HLA-ABC (B), and HER2 (C) cell surface expression. Top: Histogram overlays are representative of one experiment. Percentage of positive cells is colour-matched accordingly. Bottom: Line graphs represent the mean MFI or %+ cells of treated (T and P +/- IFN-γ) over untreated (PBS +/- IFN-γ) with error bars ± SEM of three independent experiments. Statistical analysis was done by two-way ANOVA and Tukey HSD test.


A)
IFNGR1
Constitutive
IFN-γ-treated
65%
69%
63%
67%
Count
Isotype control
Untreated
Trastuzumab &
Pertuzumab (72h)
T/UT
Time (h)
T/P
24
48
72
96
MFI (IFN-γ-induced expression)
% positive cells (IFN-γ-induced expression)
MFI (constitutive expression)
% positive cells (constitutive expression)


B)
HLA-ABC
Constitutive
IFN-γ-treated
81%
71%
97%
98%
Count
Isotype control
Untreated
Trastuzumab & Pertuzumab (72h)
T/UT
Time (h)
T/P
24
48
72
96
MFI (IFN-γ-induced expression)
% positive cells (IFN-γ-induced expression)
MFI (constitutive expression)
% positive cells (constitutive expression)

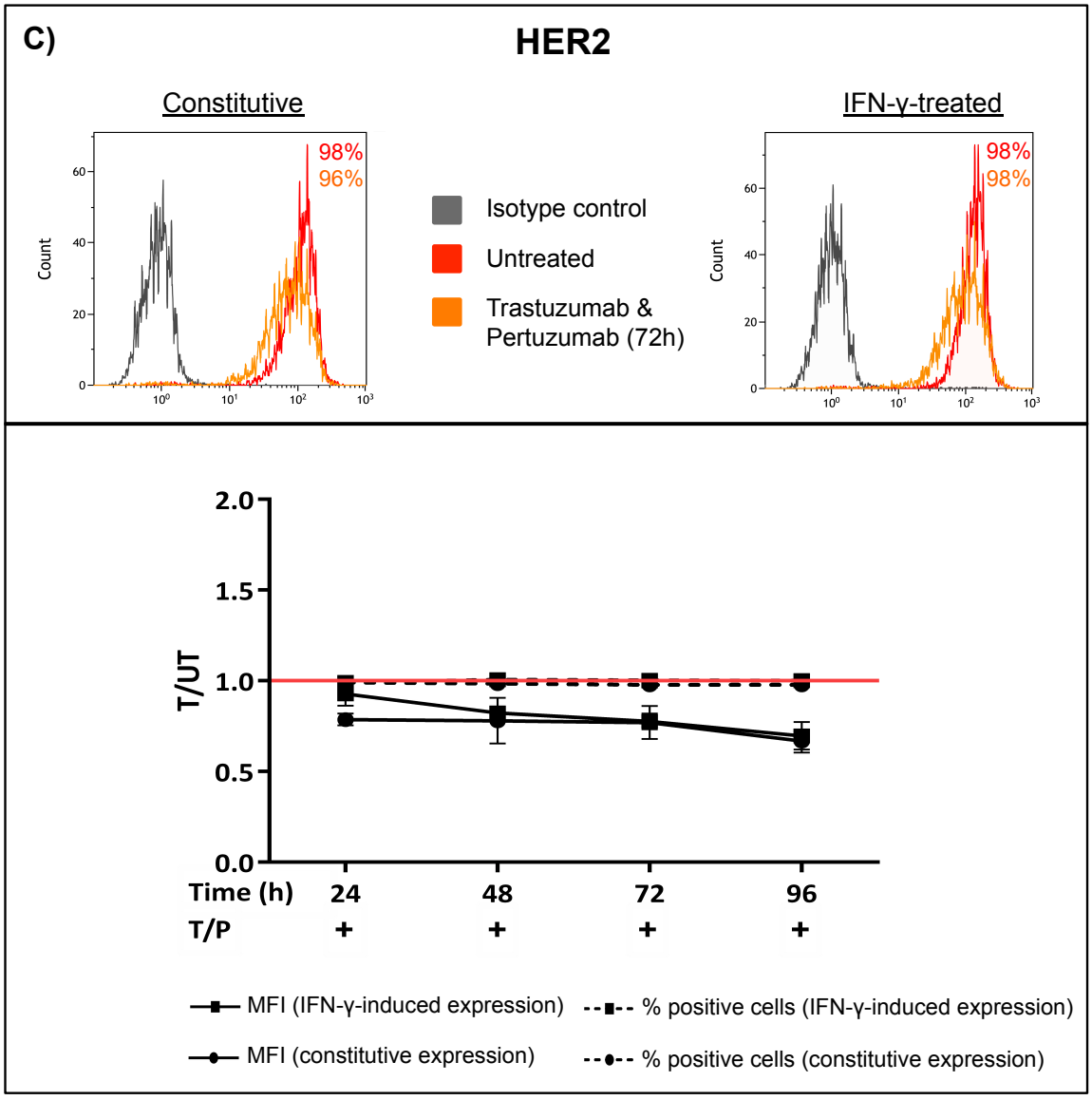
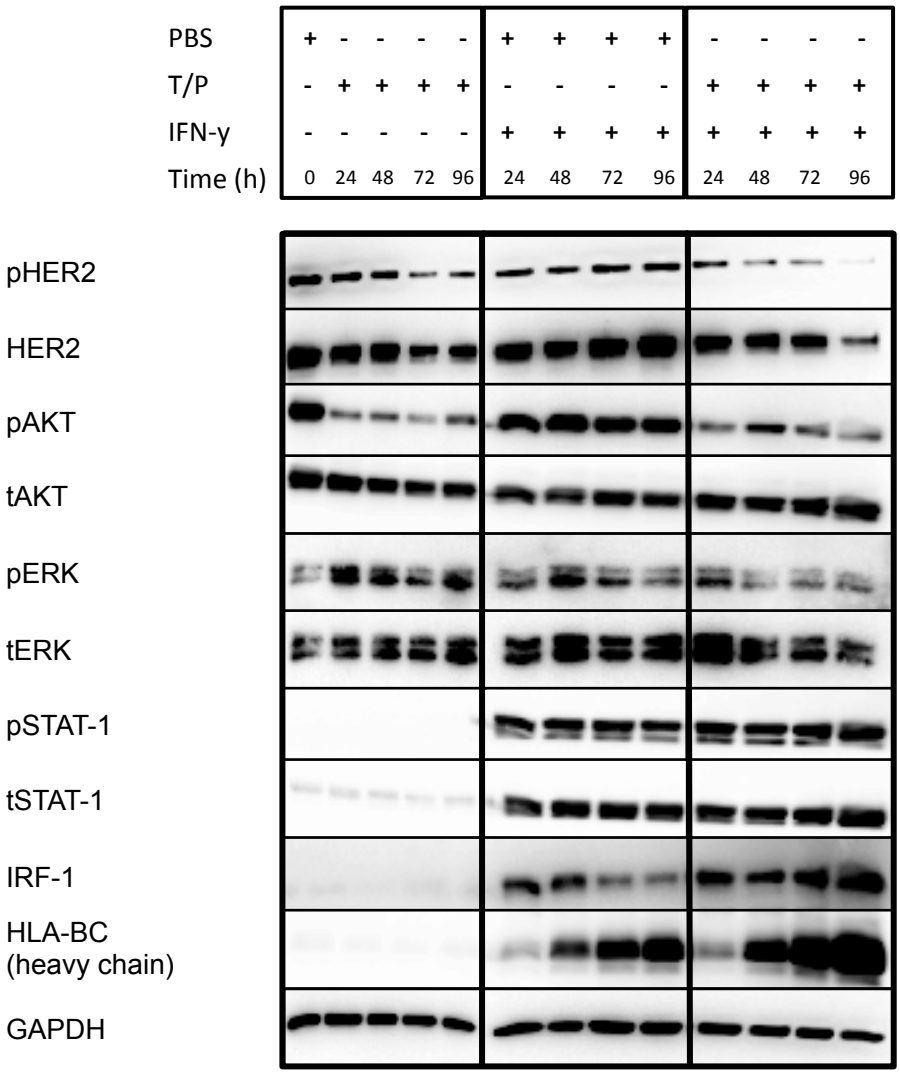

C)
HER2
Constitutive
IFN-γ-treated
98%
96%
98%
98%
Count
Isotype control
Untreated
Trastuzumab &
Pertuzumab (72h)
T/UT
2.0
1.5
1.0
0.5
0.0
Time (h)
24
48
72
96
T/P
+
+
+
+
MFI (IFN-γ-induced expression)
% positive cells (IFN-γ-induced expression)
MFI (constitutive expression)
% positive cells (constitutive expression)

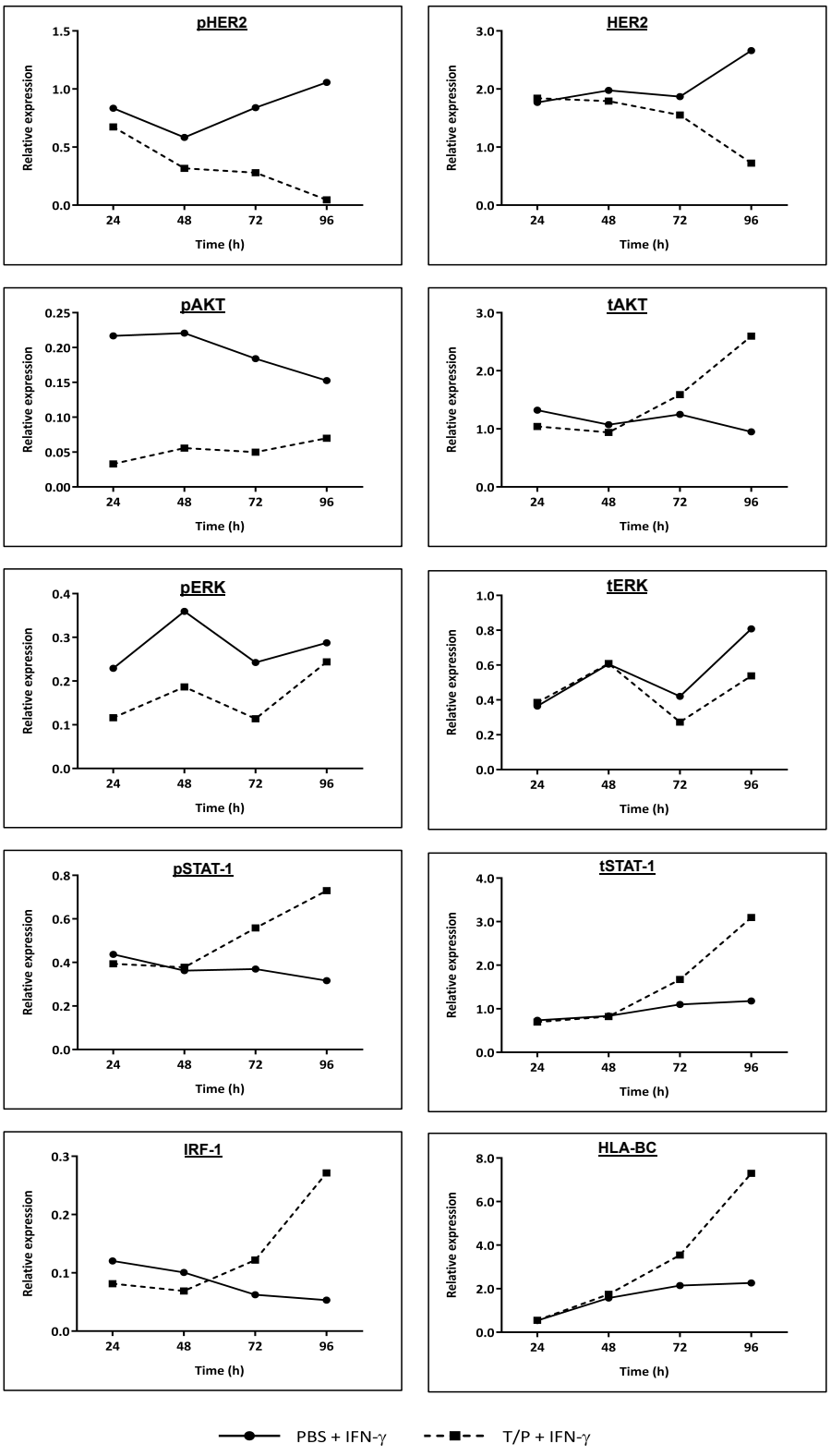
Figure 3.18: Inhibition of HER2 signalling by trastuzumab+pertuzumab in SKBR3 resulted in time-dependent changes of IFN-γ proteins and HLA-BC accumulation.

SKBR3 cells were treated with T/P (10 μg/mL each) or PBS (vehicle control), +/- IFN-γ (100 U/mL) at the indicated time points. Cells were analyzed by western blotting from WCLs and probed with indicated antibodies. A) Representative western blot. B) Bar graphs for relative expression show the ratio of band intensity for indicated proteins, normalized to GAPDH band intensity. Graphs represent one experiment.

A)


PBS + - - - - + + + + - - - -
T/P - + + + + - - - - + + + +
IFN-y - - - - - + + + + + + + +
Time (h) 0 24 48 72 96 24 48 72 96 24 48 72 96
pHER2
HER2
pAKT
tAKT
pERK
tERK
pSTAT-1
tSTAT-1
IRF-1
HLA-BC (heavy chain)
GAPDH

B)


pHER2
HER2
pAKT
tAKT
pERK
tERK
pSTAT-1
tSTAT-1
IRF-1
HLA-BC
Relative expression
Time (h)
24
48
72
96
PBS + IFN-γ
T/P + IFN-γ

3.2.9 Discussion

3.2.9.1 Result summary

An important finding from the previous study (Section 3.1) was BT-474 (ER+HER2+) lacked the capacity to upregulate surface HLA-ABC in response to IFN-γ whereas it was able to do so in response to IFN-α and TNF-α. This combined with low levels of IFNGR1 suggested dysregulation of the IFN-γ pathway via IFNGR1 and JAK1 signalling. By contrast SKBR3 (HER2+) upregulated HLA-ABC in response to all three cytokines. We also showed HER2 levels in MCF-7/neo and its transfectant MCF-7/HER218 were not inversely associated with constitutive or IFN-γ inducible HLA-ABC. The effect of HER2 and ER signalling on IFN-γ activation and HLA Class I expression is unclear with conflicting reports in the literature [19,69,70,79,80,91,92,98].

In this follow-up study using appropriately targeted drugs, siRNAs and the mAb T/P, to inhibit ERα and/or HER2 signalling, we performed flow cytometry to determine their effects on cell surface expression of IFNGR1 and HLA-ABC in the presence and absence of IFN-γ. Western blotting of lysates from untreated and treated cells was also performed to ascertain IFN-γ activation via pSTAT-1 and IRF-1 and accumulation of HLA-H chains in BT-474 and SKBR3. Key findings include: 1) Cell surface expression of HLA-ABC and IFNGR1 on BT-474 were slightly increased by targeting ERα with TAM and ICI, whereas their expression was severely decreased by knocking down HER2 with Lap and ERBB2 siRNA. Furthermore, their expression also decreased over time in a 96-hr time

course using T/P. Similar results were observed for HER2 knockdowns in SKBR3, with the exception that T/P increased HLA I expression over the 96 hr time; 2) pSTAT-1, IRF-1 and HLA-H chains were detected in the presence of IFN-γ even in BT-474 despite its low levels of IFNGR1 and poorly inducible cell surface HLA-ABC; 3) targeting ERα in BT-474 with endocrine inhibitors TAM and ICI did not significantly alter IFN-γ signalling components, while targeting with ESR1 siRNA alone or combined with ERBB2 siRNAs consistently but insignificantly increased pSTAT-1 and IRF-1 with no clear effect on accumulation of HLA-BC H-chains; 4) Lap and siRNA ERBB2 knockdown of HER2 increased IRF-1 in both cells lines, significantly decreased pSTAT-1 only in SKBR3, and had variable effects on HLA-BC H-chains accumulation; 5) T/P monoclonal antibodies, directed against HER2 signalling over a 96-hr period, decreased pSTAT-1 and IRF-1 but increased accumulation of HLA-BC H-chains in BT-474. By contrast T/P treatment of SKBR3 increased pSTAT-1, IRF-1 and HLA-BC H-chains, which is congruent with surface HLA mediated by T/P-treatment.

3.2.9.2 Limitation of endocrine therapy and result interpretation

Targeting ERα signalling in BT-474 cells with TAM and ICI may not have been ideal due to interfering substances in the culture medium. Cells were grown in IMDM, which contains high levels of phenol-red known to have estrogenic like activity [99,100] and 10% FBS which contains variable amounts of bovine estrogens. Estradiol has been reported to modulate IRF-1 and HLA-DR expression [68,101]; thus, the experimental

conditions may have interfered with the activities of TAM and ICI, limiting our interpretation. While the knockdown of ER by ICI, an ER antagonist, was incomplete, ERα was not detected in BT-474 lysates from cells treated with ESRI siRNA and ESRI+ERBB2 siRNAs. However, as we used WCLs and an antibody not specific for pERα, it is possible to have missed residual nuclear pERα . Although we did not monitor IFNGR1 and HLA expression in this set of knock downs in BT-474 via western blotting, a single experiment by an Honors student (Matthew Young, unpublished data) in the Drover lab showed ESRI siRNA increased IFNGR1 and HLA expression. This result, together with our findings of increased pSTAT-1 and IRF-1, albeit not significant, in response to ICI and ESR1 knockdowns, is congruent with a report by Lee et al. [102] who showed a negative association between HLA Class I and ERα expression. The same article also showed IFNGRs were higher on HER2+ and basal breast carcinomas as compared to luminal breast cancers, which fits with the very low levels observed on BT-474, a luminal B BCCL [102].

3.2.9.3 Correlation between HER2 overexpression and HLA Class I upregulation remains controversial

Contrary to our hypothesis, inhibition of HER2 pathway in BT-474 and SKBR3 by any of the treatments demonstrated a correlation between reduced HER2 signalling and decreased surface expression of IFNGR1 and HLA-ABC except for T/P-treated SKBR3 cells. Similarly, studies done on breast cancer tissues [19] and BCCL [92], including BT-474 and SKBR3, did not detect a significant association between HER2 and HLA Class I

expression. Furthermore, Chaganty and colleagues observed no change in HLA Class I expression following ERBB2 siRNA or Lap treatment in BCCLs despite the observed reduced expression in HER2 and pHER2 demonstrating a successful HER2 pathway inhibition [92]. In fact, interruption of HER2 signalling led to decreased HLA Class I in BT-474 and SKBR3, which is consistent with our findings. Conversely, several other studies showed an upregulation of HLA Class I expression following treatments with ERBB2 siRNA in ESCC and BCCLs, respectively [69,70,98]. Higher HLA Class I levels were also found by Herrmann and colleagues in tetracycline-controlled HER-2/neu cells (tet-HER-2/neu+) generated from murine NIH3T3 cells after the addition of anhydrotetracycline hydrochloride, thereby leading to downregulation of HER2 (tet-HER-2/neu-) [91]. Interestingly, when BT-474 and SKBR3 were treated with trastuzumab no change in HLA Class I was detected upon co-culturing with peripheral blood mononuclear cells (PBMCs) [92,98]. Furthermore, Chaganty and colleagues demonstrated IFN-γ, released by immune cells within the PBMCs as a result of trastuzumab treatment, was responsible for upregulated HLA Class I in all BCCLs tested including BT-474 and SKBR3 [92]. These findings are partially consistent with our study as only SKBR3, not BT-474, slightly upregulated surface HLA-ABC after T/P treatment despite the increase in HLA-BC H-chains in both cell lines over time. Possible reasons for these observed differences are discussed below:

Differences in mode of action of anti-HER2 treatments

A small study by Lowenfeld and colleagues showed a drastic rise in HLA Class I in HER2-expressing BCCLs following combined treatment of T/P and IFN-γ/TNF-α compared to T/P or cytokine treatment alone [103]. These findings are comparable with our study showing increased HLA-BC H-chains in BT-474 and SKBR3 following T/P treatment with IFN-γ over time. This indicates that the mode of action of either treatment or combination of therapeutic agents might be a crucial factor for cancer cells to either increase or decrease HLA Class I molecules with and without IFN-γ.

T/P treatment involves two monoclonal antibodies targeting two different extracellular domains of HER2 and subsequently leading to apoptosis [62–67,104]. Dual blockage of HER2 can overcome anti-HER2 resistance and enhance anti-trophic effects of trastuzumab when combined with pertuzumab [63,65–67,104], thereby possibly helping to shape IFN-γ response leading to increased HLA Class I. We observed a significant reduction of surface IFNGR1 and HLA-ABC following Lap treatment in BT-474 and SKBR3. Similar results were shown in a study by Chaganty et al, which suggested a Lap-induced inhibition of STAT-1 phosphorylation causing the decrease in HLA-ABC [92]. Lap was also found to stimulate tumour-infiltrating T cells through a STAT-1-dependent pathway, thus reducing its therapeutic efficacy when STAT-1 is deficient [105]. Although phosphorylated STAT-1 plays a crucial role in IFN-γ signalling [17,34,35], its proposed inhibition by Lap does not explain the Lap-mediated reduction of IFNGR1.

HER2 inhibition via ERBB2 siRNA is achieved by formation of siRNA bound RNA-induced silencing complex (RISC) leading to cleavage and degradation of target mRNA [106]. Off-target effects of siRNAs are common and remain to be one of the biggest challenges with this gene silencing approach [106].

However, these limitations can only partially explain the observed differences in HLA Class I expression between T/P and Lap or ERBB2 siRNA treatment as western blot data indicate intact STAT-1 in SKBR3.

Downregulation of APM proteins

Increased levels of pSTAT-1 and IRF-1 following treatment with Lap or ERBB2 siRNA show that BT-474 does respond, albeit weak to IFN-γ stimulation. Since this did not lead to increased intracellular HLA-BC or surface HLA-ABC, it suggests the antigen presentation pathway might also be dysregulated in BT-474. Downregulation of APM is a commonly known mechanism by which cancer cells, including HER2+ BC, reduce HLA Class I to avoid immune detection [71,87,107]. Analyzing expression levels of APM components such as TAP1, TAP2, LMP2, LMP10, PA28 and TPN in CMV-HER-2/neu^{+} Herrmann and colleagues found downregulation of HLA Class I was accompanied by APM deficiencies [91]. This is comparable to our preliminary result from western blot analysis showing decreased TAP1 levels in BT-474 compared to SKBR3 and MCF-7 when cells were stimulated with IFN-γ for 15min (Appendix 5), which likely contributes to the

reduced HLA Class I expression found in BT-474 compared to SKBR3, MCF-7/neo and MCF-7/HER218 (Figure 3.4C and 3.5C). However, more experiments need to be performed to confirm this finding. Additionally, studies done by Vertuani et al. and Maruyama et al. also showed decreased expression of LMP7 as well as PA28α and β, CRT and calnexin due to high HER2 levels in MC-2/HER2 transfectants and ESCC cell line TE4 (HER2 high), respectively [69,80]. IFN-γ treatment restored expression levels of HLA Class I and APM components in MC-2/HER2 cells [80]. Maruyama and colleagues also assessed the effect of ERBB2 siRNA-mediated HER2 knockdown on APM components LMP2, LMP7, TAP1 and TPN, but did not find significant differences between siRNA treatment and control transfectant in ESCC cell lines TE1 (HER2 low) and TE4 (HER2 high). Moreover, siRNA treatment of TE4 cells did not increase expression levels of LMP7, TAP1 and TPN but led to a rise in HLA Class I [69].

Alternative causes of dysregulated IFN-γ signalling and subsequent poor HLA Class I induction

Multiple studies also brought attention to the dual role of IFN-γ, STAT-1, and NLRC5 in cancers subsequently affecting HLA Class I expression. These pathways have been found to exhibit, both tumour-promoting and tumour-suppressing properties, but more research needs to be done to determine the deciding factors driving these pathways to cancer progression or inhibition, which is likely context dependent [87,108–111].

Loss of gene expression either due to mutations or deletion is common among many cancers as they progress. Genetic alterations and deletions have been identified in a number of APM and IFN-γ pathway components such as TAP, TPN, immunoproteosome subunits, and ERAP1 as well as JAK1, JAK2, and STAT-1 thereby decreasing constitutive and IFN-γ-induced HLA Class I levels [71]. Moreover, epigenetic silencing and transcriptional regulations were found to affect expression of IFNGR1, IRF-1, NLRC5, TAP1/2, TPN, immunoproteosome, and ERAP [71,84,87]. Numerous immune evasion strategies have been identified in a variety of cancers including breast cancer, however, discussing them all would be beyond the scope of this study.

Nevertheless, these studies not only highlight the complexity of mechanisms by which cancer cells avoid immune recognition but also show the vital prognostic and therapeutic role immunity plays in cancer. It provides a better understanding of the underlying mechanism the immune system utilizes to help shape its response to cancer treatments [105] potentially discovering novel therapy targets and strategies.

3.2.9.4 Conclusion

As we were unable to restore HLA Class I via IFN-γ stimulation by knocking down HER2 using three different methods, the role of HER2 signalling in downregulation or upregulation of APM components and HLA Class I remains unresolved. We are confident in the validity of our results and cell lines used in this study as all BCCLs haven been authenticated via short tandem repeat (STR) profiling at the Centre of Applied Genomics

(The Hospital for Sick Children, Toronto, ON). Differences in experimental design (e.g., antibody clone, siRNA sequence, in vivo compared to in vitro) and tumour types contribute but do not fully explain these contradictory results and more studies need to be done to further investigate the relationship of HER2, IFN-γ signalling and HLA Class I in human cancers. It is important to note, most of the studies focussed on HER2 expression, but did not investigate possible co-expression of other receptors such as estrogen receptor (ER) and their effects on HLA Class I. Both, preclinical and clinical data show a complex molecular bidirectional crosstalk between the HER2 and ER pathway in luminal B breast cancer (ER^+HER2^+). Moreover, this crosstalk is also known to play a key role in drug resistance in this breast cancer subtype and has become a basis of interest for new therapy strategies [50,51].

4 Discussion

4.1 Overall conclusions

This study further strengthens our hypothesis that IFN-γ signalling is impaired in BT-474 leading to insufficient HLA Class I upregulation and expression on the cell surface. However, against our expectation, our results indicate HER2 overexpression is not involved in dysregulation of IFN-γ signalling in the luminal B cell line BT-474 as successful HER2 pathway inhibition did not lead to increased IFN-γ signalling and subsequent HLA Class I levels. Interestingly, Lap and ERBB2 knockdowns of HER2 in BT-474 resulted in decreased HLA-BC H-chains and surface HLA-ABC, whereas T/P treatment increased HLA-BC H-chain levels over time without increasing HLA-ABC surface expression. Both, HER2-overexpressing SKBR3 and HER2-transfected cell line MCF-7/HER128 exhibit higher levels of surface HER2 than BT-474 but still respond to IFN-γ stimulation with increased HLA-ABC over time. This suggests HER2 does not sterically hinder IFN-γ binding to its receptors, IFNGR1 and IFNGR2. Additionally, SKBR3 also decreased HLA-BC H-chains and surface HLA-ABC expression when HER2 signalling was abrogated by Lap or ERBB2 siRNA but not T/P treatment. Based on our study as well as conflicting results reported by others, [19,69,70,79,80,91,92] it remains controversial whether or not HER2 expression inversely correlates with HLA Class I induction in breast cancer. As the downregulation of HLA Class I and APM components by HER2 overexpression has been reported in different types of human cancers and human cancer cell lines [69,70,79] as well as in mouse model derived tumour cells, [80,91] it

appears to be independent of tissue origin. Several studies argue that a critical 'threshold' of HER2 is required in order to affect HLA Class I levels [69,79].

Taken together, we conclude that expression levels and/or function of IFN-γ pathway and/or APM components may be dysregulated and are therefore responsible for the weak IFN-γ signalling in BT-474 and subsequent poor upregulation of HLA Class I. Whether this dysregulation is connected to HER2 overexpression remains unknown. Nevertheless, the consistent downregulation of surface IFNGR1 and HLA-ABC following some anti-HER2 treatments in BT-474 and SKBR3 suggests a possible crosstalk between these pathways.

4.2 Future direction

Despite considerable effort we were unable to detect wildtype JAK1, JAK2, and surface IFNGR2 by flow cytometry and western blotting using our antibodies. Therefore, more sensitive antibodies are needed to investigate expression of these early proteins in IFN-γ signalling.

SKBR3 and MCF-7, unlike BT-474, express HLA-A2 based on TRON database (Table 2.6–Table 2.8) and Mimura et al. found a correlation between HLA-A2 reduction and HER2 expression [79]. Therefore, assessing HLA Class I transcription via quantitative PCR (qPCR), in BCCLs, especially BT-474, would help to further elucidate the relation between HER2 and HLA Class I variants. Further analysis would include an extended time course as well as the use of antibodies that detect individual HLA Class I variants.

We also showed consistent downregulation of surface HLA Class I by Lap in both, BT-474 and SKBR3. If Lap, currently used in the treatment of metastatic BC [58], causes a similar effect in vivo, it would be expected to have a negative effect on CD8+ T cell restricted killing of tumor cells. Alternatively, tumor cells that have downregulated HLA Class I could be targets for NK cell killing. To test the immunological relevance of downregulated HLA class I, NK lysis assays could be performed on Lap-treated BCCLs as decreasing levels of surface HLA Class I typically initiates increased NK cell activation and cytotoxicity [87]. However, loss of surface HLA Class I is not sufficient to induce full NK cell activation [112] while cancer cells also have been reported to evade NK cell-mediated cytotoxicity via different mechanisms [87]. This must be considered in future experiments.

Extending our study design to include other treatments of HER2+ BC would help gain further understanding of the interplay between anti-HER2 treatment and IFN-γ signalling and potentially explain the observed differences in IFN-γ response, evaluated by HLA Class I status, to the three treatments tested. Women with high-risk disease are currently treated with a combination of T/P plus chemotherapy such as docetaxel while trastuzumab emtansine (T-DM1) is being used in patients with residual disease [7,113,114]. ER+HER2+ BC is now commonly treated with a combination of chemotherapy and trastuzumab before administering endocrine therapy [7]. Treatment options for HER2+ BC widely expanded with the development of new therapeutic agents including TKIs (e.g., Neratinib, Tucatinib), cyclin-dependent kinase 4 and 6 (CDK4/6)

inhibitors (e.g ribociclib, palbociclib) triggering cell cycle arrest and checkpoint inhibitors targeting programmed cell death protein 1 (PD-1) or PD-L1 (e.g. Pembrolizumab, Atezolizumab, respectively). These therapeutic agents are currently tested in various clinical trials either alone or, for the majority, in combination with other new or current anti-HER2 drug treatments and current endocrine therapy (e.g ICI) for the treatment of metastatic and locally advanced ER+HER2+ BC [7,105,113,114].

Additionally, determining expression of APM and IFN-γ pathway components on the gene and protein level via qPCR and western blotting, respectively, as well as gene sequencing to assess potential mutations in these components could help elucidate the mechanism responsible for the lack of HLA Class I upregulation in response to IFN-γ in BT-474.

4.3 Significance

Although the cause of Lap-mediated downregulation of constitutive and IFN-γ-induced HLA Class I on the cell surface remains unknown, it may be an important tool to gain further insight into Lap resistance in HER2 overexpressing BCCLs. Additionally, downregulation of surface IFNGR1 following some anti-HER2 treatments suggests a possible crosstalk between HER2 signalling and IFN-γ-induced HLA Class I upregulation but we were unable to establish a clear correlation between these pathways. Therefore, we cannot exclude the possibility of a HER2 independent dysregulation of IFN-γ in BT-474.

Nevertheless, this study contributes to emerging research investigating the complex role of IFN-γ in BC affecting IFNGR1 levels and IFN-γ-induced APM components, which results in poor HLA Class I upregulation and surface expression and subsequently contributes to tumour immune evasion. Assessing IFN-γ signalling and expression of IFN-γ-regulated genes and proteins in various BCs could be crucial for not only determining the immunological state of breast tumours but it may also reveal potential biomarkers and novel treatment targets that could help develop more effective treatment plans.

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6 Appendices

Appendix 1: List of Materials, Chemicals, Buffers, Media, and Supplements used in this study.

Appendix 1.1: **List of Materials, Chemicals, Buffers, Media, and Supplements used in this study.**

Materials, Chemicals	**Company**	**Catalogue number**
0.25%-Trypsin	Life technologies	25200-114
0.5 M Tris-HCl Buffer pH 6.8	BioRad	161-0799
0.6mL tubes	Corning	MCT-060-C-C
1.5 M Tris-HCl Buffer pH 8.8	BioRad	161-0798
1.5mL tubes	Fisher Scientific	05-408-129
15mL tubes	ProGene	71-1500-B
24-well plate	Corning	353047
50mL tubes	Corning	430829
6-well plate	Corning	3335
Acryl Bis	BioRad	161-0156
Antibiotic/antimycotic (A/A)	Life technologies	15240-112
Aprotinin	Sigma	A4529
Ammonium persulfate (APS)	BioRad	161-0700
BCA assay kit	ThermoScientific Pierce	23227
BCA Protein Assay Kit	ThermoScientific	23225

Appendix 1.2: **List of Materials, Chemicals, Buffers, Media, and Supplements used in this study. Continued.**

Material/Chemical	Company	Catalogue number
BLUeye prestained protein ladder	FroggaBio	PM007-0500
Bovine serum albumin (BSA)	ThermoScientific	23209
Bromphenol blue	J.T. Baker Chemical	D293
Cell Culture dish	Falcon	353003
Cell scraper	Falcon	353085
Clarity Western ECL	BioRad	170-5060
Control siRNA	Santa Cruz Biotechnologies	SC-37007
DharmaFECT 1	ThermoScientific	T-2001-02
Dharmafect Cell Culture Reagent (DCCR)	Dharmacon	B-004500-100
Dimethyl Sulfoxide (DMSO)	Sigma	D4540
FACS tubes	Falcon	352008
Fetal bovine serum (FBS)	Life technologies	12600-044
Fulvestrant (ICI)	Sigma	I4409
Goat serum	Life technologies	16210-064
Halt Phosphatase Inhibitor cocktail	ThermoScientific	78420

Appendix 1.3: List of Materials, Chemicals, Buffers, Media, and Supplements used in this study. Continued.

Material/Chemical	Company	Catalogue number
Human Interferon alpha	BioRad	PHP107Z
Human Interferon gamma	BD Biosciences	554617
Iscove's Modified Dulbecco's Medium (IMDM)	Life technologies	12440-061
L-glutamine	Life technologies	25030-164
Lapatinib Ditosylate	Selleck chemicals	S1028
Leupeptin	Sigma	L2023
Methanol	ACP	M-3640
Nitrocellulose membrane	BioRad	162-0115
NP-40	Sigma	110F-39211
ON-TARGET plus Non-targeting Pool	Dharmacon	D-001810-10-05
ON-TARGET plus SMART pool siRNA for ERBB2	ThermoScientific	L-003126-00
ON-TARGET plus SMART pool siRNA for ESR1	ThermoScientific	L-003401-00
Paraformaldehyde	Sigma Aldrich	P6148
Pepstanin A	Sigma	P5318

Appendix 1.4: List of Materials, Chemicals, Buffers, Media, and Supplements used in this study. Continued.

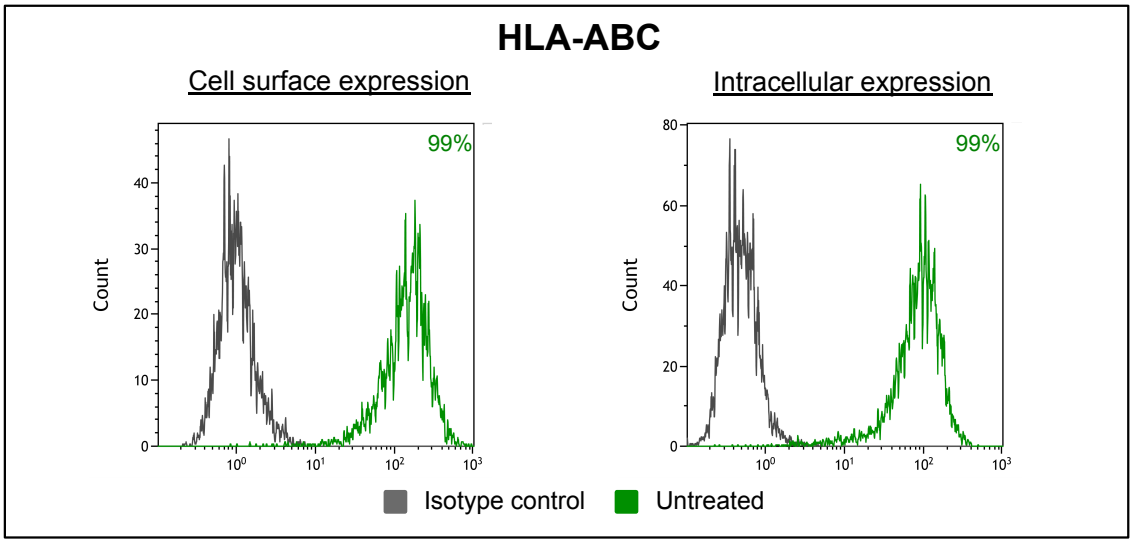
Material/Chemical	Company	Catalogue number
Pertuzumab	Roche	DIN 02405016
PMSF	Sigma	P7626
Potassium Chloride	Fisher Scientific	P-217
Potassium Phosphate	J.T. Baker Chemical	3246
SDS	BioRad	161-0301
siRNA buffer	ThermoScientific	B-00300-WB-100
Sodium azide	Sigma	S2002-100G
Sodium chloride (NaCl)	Sigma	5586
Sodium deoxycholate	Biochemical	43035
Sodium hydroxide	APC	1310-73-2
Sodium Phosphate	Bio Basic INC.	7558-79-4
Substrate Immobilin Western Chemiluminescent HRP Substrate	Millipore	WBKLS0100
Tamoxifen citrate	Calbiochem	579000
Tetramethylethylenediamine (TEMED)	BioRad	161-0801
Thin Blot Paper	BioRad	162-0118

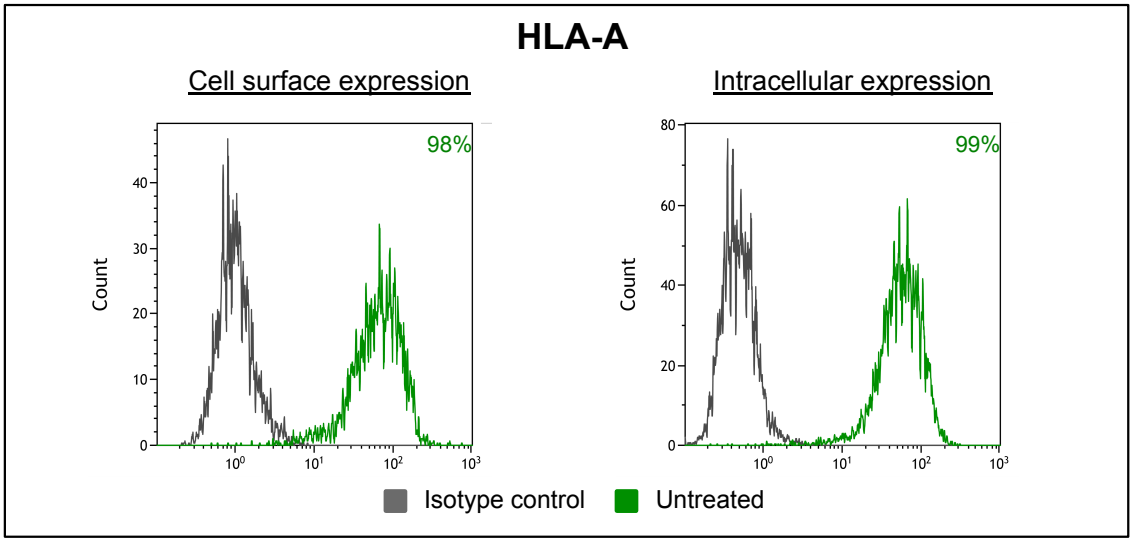
Appendix 1.5: List of Materials, Chemicals, Buffers, Media, and Supplements used in this study. Continued.

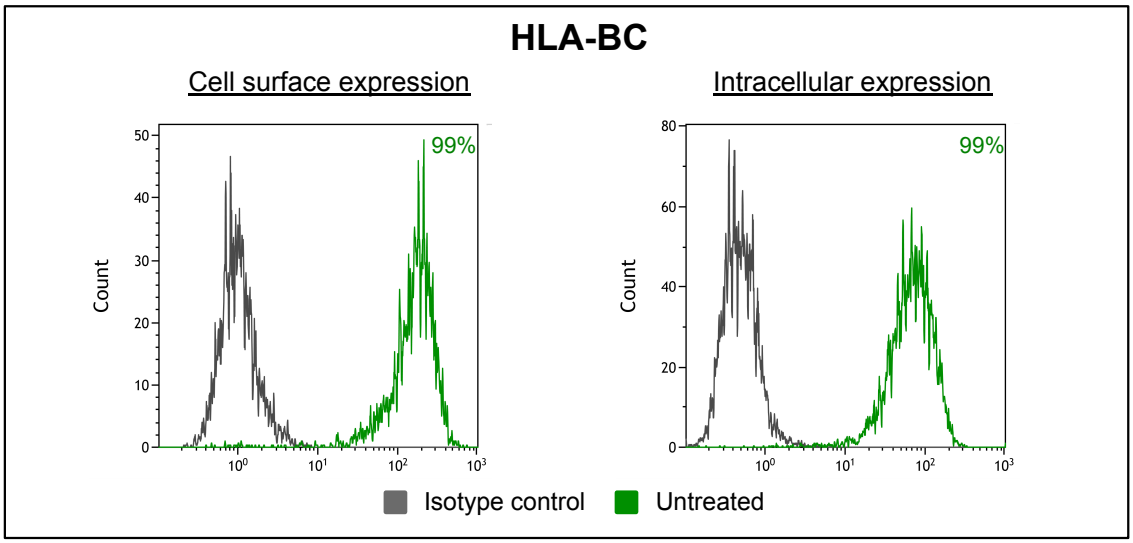
Material/Chemical	Company	Catalogue number
Trastuzumab	Roche	DIN 02240692
Tris HCL	Sigma	T3253
Tween 20	Sigma	P5927
UltraPure glycine	Invitrogen	15527-013
UltraPure Tris	Invitrogen	15504-020

Appendix 2: **Cell surface expression of HLA Class I on B-cell line SAVC.**

Extracellular and intracellular flow cytometric analysis to assess constitutive expression of HLA-ABC (A), HLA-A (B), and HLA-BC (C). Histogram overlays are representative of one experiment.

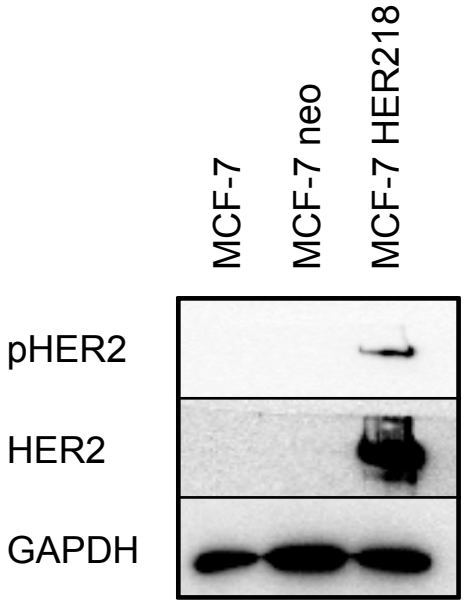

HLA-ABC
Cell surface expression
Intracellular expression
99%
99%
Count
Isotype control
Untreated


HLA-A
Cell surface expression
Intracellular expression
98%
99%
Count
Isotype control
Untreated


HLA-BC
Cell surface expression
Intracellular expression
99%
99%
Count
Isotype control
Untreated

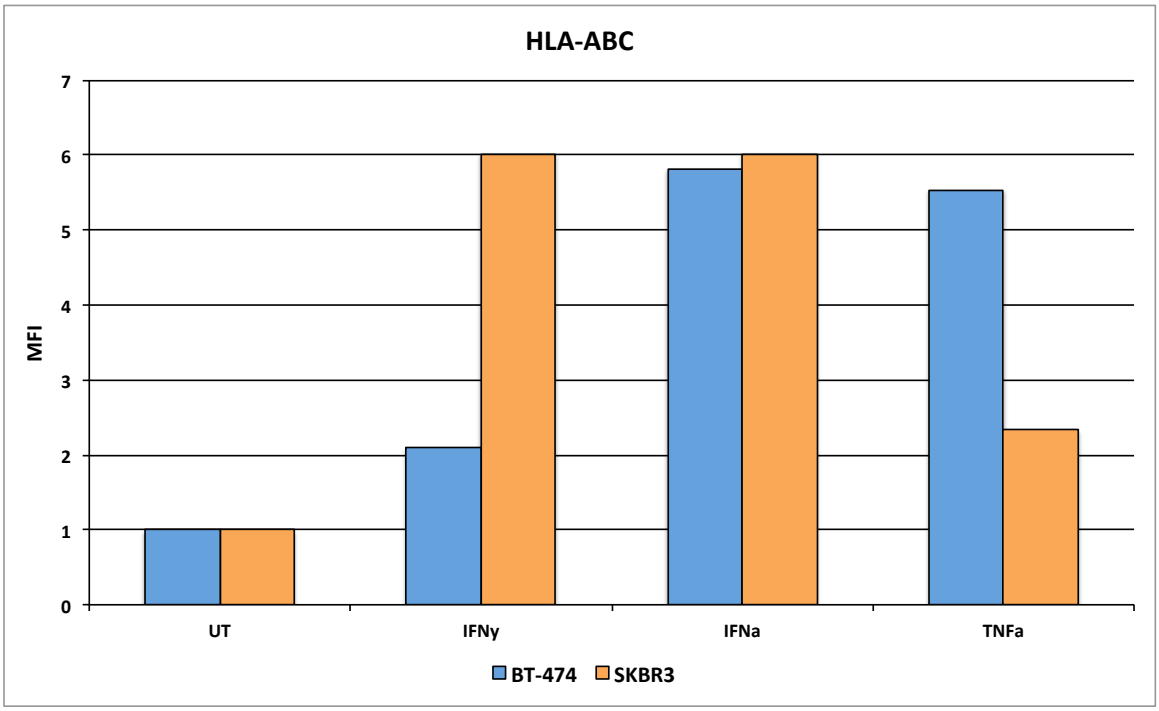
Appendix 3: HER2 transfectant MCF-7/HER128 exhibits functional HER2 signalling.

Comparing pHER2 and HER2 expression in MCF-7, vector control MCF-7/neo, and HER2 transfectant MCF-7/HER218. Western blot is representative of one experiment.


MCF-7
MCF-7 neo
MCF-7 HER218
pHER2
HER2
GAPDH

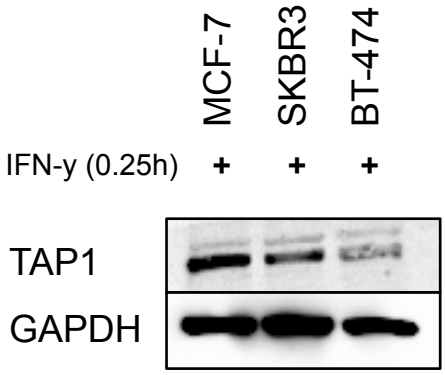
Appendix 4: Cell surface HLA-ABC is higher in BT-474 than SKBR3 in response to TNF-α stimulation.

BT-474 and SKBR3 cells were treated with IFN-γ (100 U/mL), IFN-α (100 U/mL) or TNF-α (100 U/mL) for 24 hours and subjected to flow cytometric analysis to assess HLA-ABC surface expression levels. Bar graphs represent the mean MFI of one experiment.


HLA-ABC
MFI
7
6
5
4
3
2
1
0
UT
IFNy
IFNa
TNFa
BT-474
SKBR3

Appendix 5: TAP1 expression is reduced in BT-474 compared to SKBR3 and MCF-7.

MCF-7, SKBR3, and BT-474 cells were treated with IFN-γ (100 U/mL) for 0.25 hours and then analyzed for IFN-γ-induced TAP1 expression by western blotting. Western blot is representative of one experiment.


MCF-7
SKBR3
BT-474
IFN-y (0.25h)
+
+
+
TAP1
GAPDH