CONTRASTING EFFECTS ON HLA AND PD-L1 EXPRESSION THROUGH INHIBITION OF MAPK PATHWAY IN TRIPLE NEGATIVE BREAST CANCER CELLS

By

Andrea N Rasmussen

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Master of Science

Immunology and Infectious Diseases Group, Division of BioMedical Sciences

Faculty of Medicine

Memorial University of Newfoundland

Oct 2016

St. John's Newfoundland and Labrador

ABSTRACT

Expression of human leukocyte antigen (HLA) molecules is essential for anti-tumour immune responses. Interferon gamma (IFN-γ) up-regulates HLA and, paradoxically, programmed cell death ligand-1 (PD-L1), which inhibits immune activation. The mitogen-activated protein kinase pathway (MAPK), overactivated in triple negative breast cancer (TNBC), is implicated in HLA and PD-L1 expression. We hypothesized that its inhibition may modulate HLA and PD-L1 expression in TNBC.

Human TNBC and non-TNBC cell lines were treated with various MEK inhibitors (MEKi) in the presence/absence of IFN-γ. Flow cytometry, immunofluorescence and Western blotting were used to assess MEKi mediated changes in constitutive and inducible HLA and PD-L1 expression.

U0126 antagonized cell surface HLA expression, but not total HLA protein, while the clinically relevant MEKi(s), Trametinib and Selumetinib, generally augmented constitutive and inducible cell surface HLA class I. All MEKi (s) reduced PD-L1 expression. These results have important implications for MEKi in targeting immunotherapy for TNBC.

ACKNOWLEDGMENTS

I would like to thank my supervisor and mentor, Dr. Sheila Drover for her patience and guidance over the course of my degree. Her support and encouragement has allowed me to get the most out of this program and I am ever grateful that she has given me the opportunity to work as her student on this importance research.

I would also like to thank the members of my supervisory committee, Dr. Rodney Russell, who has always been there to listen and give advice, and Dr. Kensuke Hirasawa, who never hesitated to lend resources or a helping hand.

I am grateful for Diane Codner and Ahmed Mostafa, who helped train me and introduce me to the lab, as well as Dr. Sherri Christian who lent us cells, which were pivotal in this project.

I would like to thank my current and previous lab-mates, Kendra Smith, Louisa Wiede as well as Rebecca Quilty and Megan Parker who not only helped me in my work, but who have always been there for support and friendship.

Lastly, I would like to thank the funding agencies, School of Research and Graduate Studies as well as the Canadian Breast Cancer Foundation (Atlantic chapter), without which, I would have been unable to conduct this research.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBRVIATIONS

A/A	Antibiotic antimycotic
AML	Acute myeloid leukemia
AP-1	Activator protein-1
APC	Antigen Presenting Cell
APS	Ammonium Persulfate
ATCC	American Tissue Culture Collection
BC	Breast cancer
BCA	Bicinchoninic acid
BCCL	Breast cancer cell lines
BID	Bi-daily
BSA	Bovine serum albumin
CDFBS	Charcoal/dextran fetal bovine serum
CIITA	Class II Transactivator
CLIP	Class II associated Ii peptide
CM	Complete media
CREB	cAMP response element-binding
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
E	Glutamic acid
E_2	Estradiol
EDTA	Ethylene-diaminetetraaccetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated protein kinases
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ESCC	Esophageal squamous cell carcinoma
FBS	Fetal bovine serum
GAM	Goat anti mouse
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAR	Goat anti rabbit
GDP	Guanine diphosphate
GTP	Guanine triphosphate
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor
HLA	Human leukocyte antigen
Hr	Hour
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma
IFNGR	Interferon gamma receptor

Ii	Invariant chain
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IRF	Interferon regulatory factor
IRF-E	IFN-regulatory factor element
ISRE	Interferon-sensitive response element
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch receptor
JAK	Janus activating kinases
JNK	c-Jun N-terminal kinases
K	Lysine
Kb	Kilobase
kDa	Kilo Dalton
MAP-K2	MAPK-activated protein kinase-2
MAPK	Mitogen-activated protein kinase
MAPKAP	MAPK-activated protein kinase
ME	Mercaptoethanol
MEK 1	MAPK/ERK kinase 1
MEKK	MEK kinase
MEM	Minimum essential medium
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIIC	MHC class II loaded compartments
Min	Minute
MIP-1α	Macrophage inflammatory protein-1 alpha
MKK6	MAPK kinase 6
NAC	Neoadjuvant chemotherapy
NF-Y	Nuclear transcription factor Y
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKT	Natural killer T cells
NSCLC	Non-small-cell lung carcinoma
PAGE	Polyacrylamide gel
PBS	Phosphate buffer saline
pCR	Pathological complete response
PD-L1	Programmed cell death ligand-1
pI, pII, pIII, pIV	CIITA promoters 1-4
PI3-K	Phosphoinositide-3-kinase
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RIPA	Radioimmunoprecipitation assay

RT-PCR	Reverse transcriptase polymerase chain reaction
Sel	Selumetinib
SEM	Standard errors of the mean
SH2	Src homology 2
Shp2	SH2-containg tyrosine phosphatase-2
siRNA	Small interfering RNA
SOS	Son of sevenless
STAT	Signal transducer and activator of transcription
TAP	Transporter associated with antigen processing
TBS	Tris-buffered saline
TCR	T cell receptor
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Th1	T helper type 1
TIL	Tumour infiltrating lymphocytes
TNBC	Triple negative breast cancer cells
TNF-α	Tumour necrosis factor alpha
Tram	Trametinib
V	Valine
WB	Washing buffer
β_2M	Beta-2-micoglobulin

LIST OF APPENDICES

Chapter 1: Introduction

1. Breast Cancer

Breast cancer is the most common malignancy in women worldwide, and despite significant advancements in treatment, the death toll for Canadian women before this year ends will be approximately 5000[1]. Despite better health care, earlier detection and treatments, breast cancer is the second leading cause of cancer-related death in Canadian women[1,2]. This is in large part due to the vast differences between the subsets of breast cancer.

Breast cancer can be sub-divided into three main groups:

1. Luminal A, consists of tumours that are ER-α^+, can be $PR^{+/-}$ and HER-2 negative,
2. Luminal B, consists of tumours that are ER-α^+, PR^+ and $HER\text{-}2^+$ and,
3. Triple negative (TN)/basal like, which are breast tumours that are ER-α^-, PR^-, and $HER2^-$.

Luminal A breast tumours tend to have the best prognosis, whereas the most aggressive subset is triple negative breast cancer (TNBC), aptly named due to its absence of the ER, PR, and lack of HER2 on the tumour surface[3,4]. This cancer tends to affect younger women; has a worse prognosis and is more prominent in African American female populations[5]. Because of the lack of targets for conventional therapies, non-specific treatments such as radiation and the only systemic therapy currently available is chemotherapy[6] and additional treatments are limited. NAC is gaining in popularity and

has a success rate of pCR in 30% of TNBC patients[7]. However, in the remaining 70%, there exists some potential micro-metastases and recurrence[7,8]. TNBC(s) account for approximately 10-17% of all breast cancers. Given its poor prognosis and difficulty to treat, TNBC remains an important research area with a focus on finding new treatment options.

There are ways to predict a patient's response to treatment, including the TMN staging classification system, which takes into account the size of the initial tumor, the nearby affected lymph nodes and if the tumour has metastasized[9], as well as the expression of receptors on the tumour surface. Additionally, the number, location, and types of TILs can also be indicative of a patient's response[10]. TILs are immune cells that, when activated, can proliferate and destroy foreign or harmful material within the body (including tumours) however in order to activate these cells, tumour antigens must be presented to them. This so-called "presentation" is achieved via MHC or HLA; in humans.[11,12]

Among new cancer treatment options, immunotherapy has emerged as a viable option for those who are not eligible for, or resistant to, usual treatments. Ever since it was discovered in 1957 that tumour cells could potentially evoke an immune response, research has been intent upon finding ways to make cancer more recognizable to our immune systems[13].

1.2 Major Histocompatibility Complex

1.2.1 Overview

MHC, or HLA in humans, is located on chromosome 6[14]. There are three HLA regions: class I, class II and class III. HLA class I molecules are expressed on the cell surface of nearly all nucleated cells including tumour cells, and their function is to present peptides of approximately 8-10 amino acids in length that has been digested inside the cell to CD8+ T-cells, whereas HLA class II present peptides to CD4+ T cells, also called helper T-cells[15]. Activation of both CD4 and CD8+ T cells into an effector cell is achieved through the recognition of two signals; the first signal is the interaction of the HLA-loaded molecules with the TCRs and associated proteins. The second signal needed is the interaction of the co-stimulatory molecules such as the B7 proteins (CD80 and CD86) on the APC with CD28 present on the T cell. CD4+ T cells are also important in helping CD8+ T cell activation as well. CD4+ cells can first interact with APCs such as DCs that have taken up tumour antigen and then migrated to the lymph nodes to display this antigenic peptide to naïve T cells. If recognized, then the T cell can differentiate into effector T cells. If these activated cells are CD8+ cytolytic T cells, they can initiate tumour destruction[16]. The HLA class I glycoproteins form heterodimers with another molecule that is coded for by a separate gene, the β2m[17] (Figure 1.1).

Tumour antigens can also be presented on HLA class II molecules, which normally are only present on APCs, such as B cells, Dendritic cells and macrophages[18]. HLA class II consists of the heterodimeric molecules HLA-DR, HLA-DQ, and HLA-DP that consist of two distal and two proximal α1 and β1 domains that are coded for by exon 2 and 3 of the alpha and beta chain genes, whereas HLA-DR is encoded by the *DRA* and *DRB*

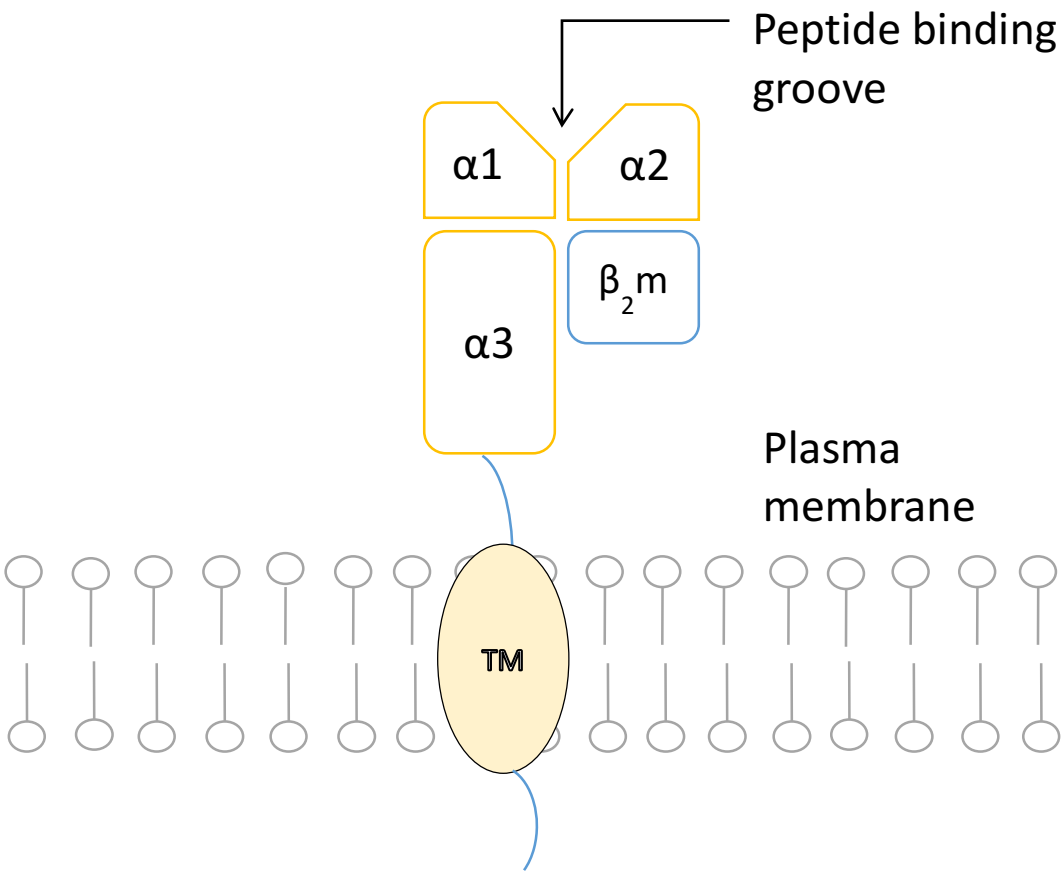

Peptide binding groove
α1
α2
β2m
α3
Plasma membrane
TM

Figure 1.1:Human leukocyte antigen class I molecule structure: The HLA class I glycoproteins (α1, α2, & α3) combine with β2m, forming a heterodimer. HLA class I is anchored in the plasma membrane via transmembrane domains (TM) and a cytoplasmic tail. Adapted from (15).

genes[11]. The peptide binding groove is comprised of two alpha helices above a beta-pleated sheet and is open-ended, unlike the closed-ended groove in class I, in order to bind peptides that are 12-24 amino acids long. HLA class II molecules present peptides derived from endocytosed proteins to CD4+ T cells, which upon activation differentiate into one of several subsets, depending in part on the cytokine milieu[18] (Figure 1.2). Several different CD4 T-cell subsets have been identified in the tumour microenvironment including TH1 (producing IFN-γ), TH2 (IL-4) and regulatory T-cells[19].

The expression of HLA class II can be induced on other cell types through stimulation of certain cytokines, including, but not limited to, IFN-γ. IFN-γ also further increases HLA class I expression. Higher expression of these molecules, especially HLA class I, has been associated with positive outcomes for the patient in some cancer types[8].

1.2.2 Endogenous HLA class I Pathway

Classical HLA class I molecules are assembled in the endoplasmic reticulum (ER), whereas the proteins that are recognized by the cell as foreign are degraded into peptides by the proteasome, an ATP-dependent protease, in the cytosol[17]. The peptides are then translocated to the ER by the TAP proteins. ER chaperones, calnexin and calrecticulin as well as tapasin facilitate the assembly of the β2m with the heterodimers to make the complete HLA molecule. Once the peptide is bound into the peptide-

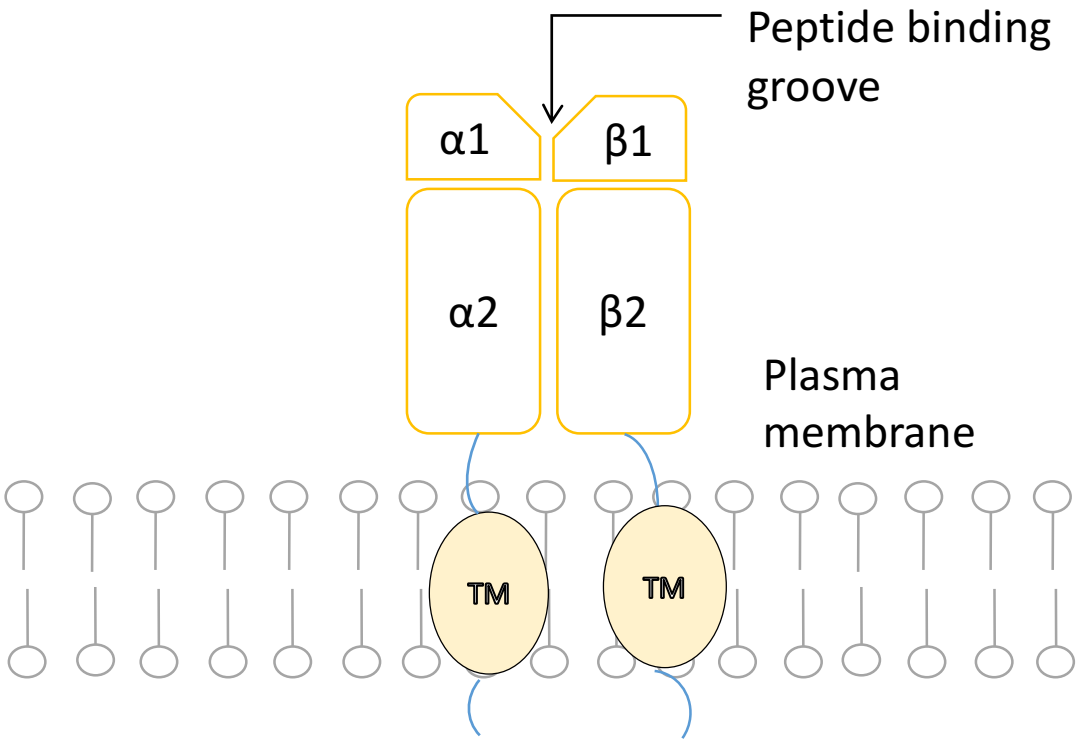

Peptide binding groove
α1
β1
α2
β2
Plasma membrane
TM
TM

Figure 1.2: Human leukocyte antigen class II molecule structure. The HLA class II molecule is comprised of two glycoprotein transmembrane chains, an α and a β chain. HLA class II molecules are anchored in the plasma membrane via the TM and cytoplasmic tails. Adapted from (12)

binding groove, the molecule begins transport to the cell surface. Without peptide binding, the proteasome subsequently degrades the HLA molecule[17] (Figure 1.3).

1.2.3 Exogenous HLA class II Pathway

HLA class II molecules are also assembled in the ER along with the Ii[12]. The Ii has multiple functions: It serves as a co-chaperone in the assembly of HLA class II, ensuring proper folding and stability, also, the Ii binds to the peptide-binding groove of class II and acts as a peptide editor; exchanging itself out for other peptides of the correct size[20,21]. The Ii chain trimer acts to block the peptide-binding cleft in the HLA class II molecule, the two trimers (nonamer) then migrate from the ER to the Golgi apparatus and the endocytic compartment where the lysosomes then fuse with a late-endosome-like compartment containing large amounts of HLA class II molecules, called MIIC. Cathepsin S subsequently degrades the Ii, leaving a small peptide called CLIP[22]. This peptide is then exchanged for a higher affinity peptide as the fully assembled class II molecule makes its way to the surface of the cell[23] (Figure 1.4).

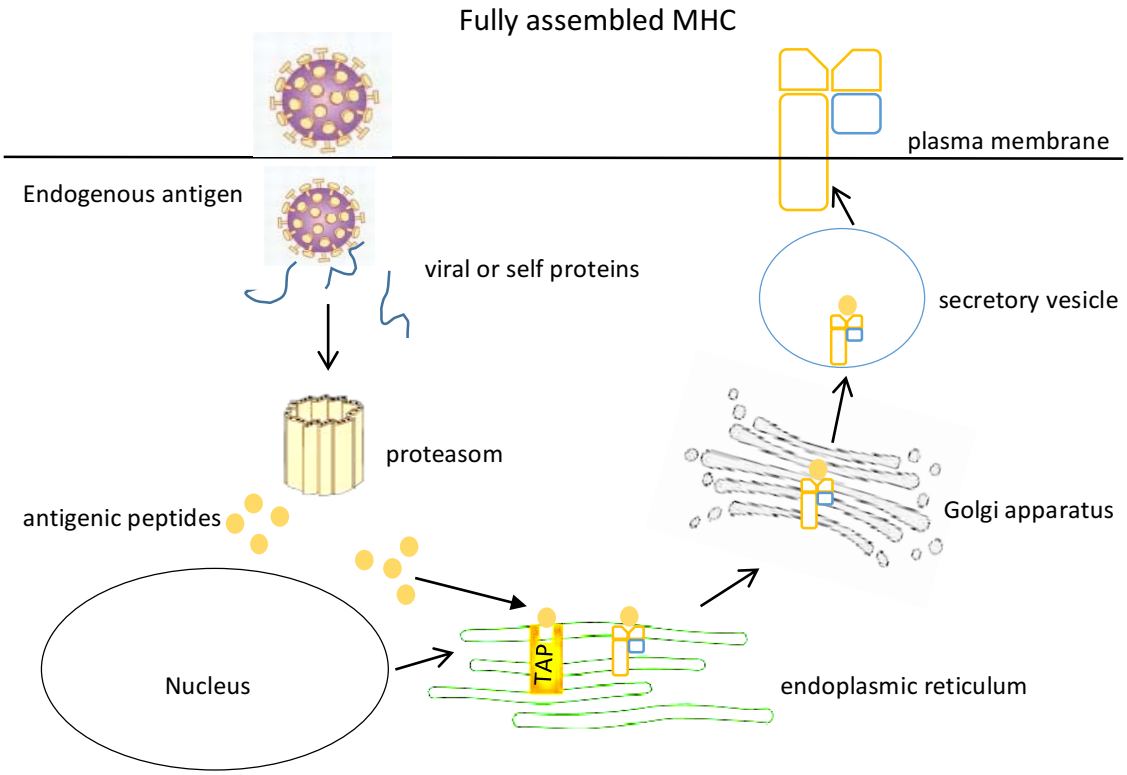

Fully assembled MHC
plasma membrane
Endogenous antigen
viral or self proteins
secretory vesicle
proteasom
antigenic peptides
Golgi apparatus
Nucleus
TAP
endoplasmic reticulum

Figure 1.3: Endogenous human leukocyte antigen class I pathway.

HLA class I proteins are synthesized in the endoplasmic reticulum (ER) and antigenic proteins are degraded by the proteasome in the cytosol. TAP molecules translocate the peptides to the ER. ER chaperones, calnexin and calrecticulin as well as tapasin facilitate the assembly of the β2m with the heterodimers to make the complete HLA molecule. Once the peptide is stably bound into the peptide-binding groove, the molecule begins transport to the cell surface. Adapted from (17).

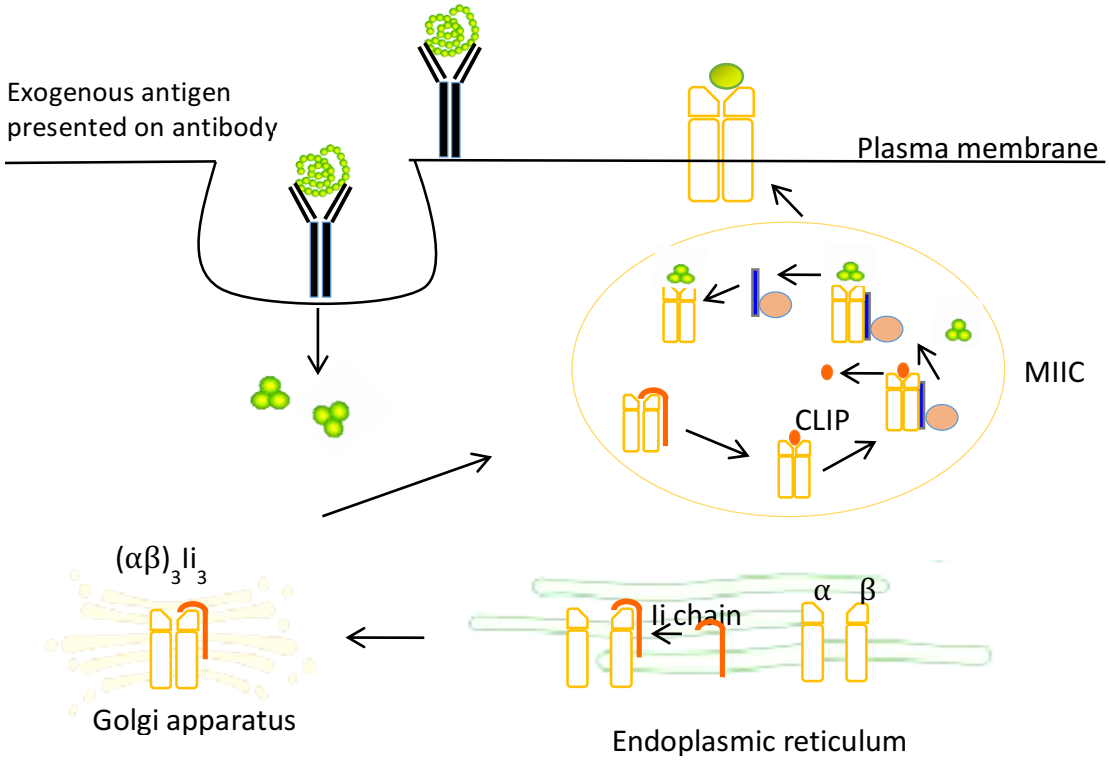

Exogenous antigen
presented on antibody
Plasma membrane
MIIC
CLIP
$(\alpha\beta)_3 Ii_3$
Golgi apparatus
Ii chain
α
β
Endoplasmic reticulum

Figure 1.4: Exogenous human leukocyte antigen class II pathway.

HLA class II heterodimers are synthesized in the endoplasmic reticulum along with the Ii, which acts as a peptide editor that sits in the peptide binding groove. The HLA class II molecule and the Ii combine to form the $(\alpha\beta)_3Ii_3$ complex, which then moves into the MIIC. Cathepsin S degrades the Ii, leaving behind the CLIP fragment. HLA-DM, associated with HLA-DO, acts to switch out the CLIP fragment in exchange for a high affinity peptide. HLA-DM and DO dissociates from the HLA complex, and the HLA complex, loaded with the peptide is transported to the cell surface. Adapted from (20).

1.2.4 Class II Transactivator and Function

The CIITA regulates expression of the class II molecule. CIITA is a non-DNA binding protein that acts as a transcriptional integrator. Its expression directly correlates with the expression of class II genes[24]. A large, regulatory protein drives the transcriptional activity of CIITA. This gene contains four promoter regions: pI, pII, pIII, & pIV. With the exception of pII, with its function unknown, pI, pIII and pIV are all highly conserved between mice and humans and their functions are as follows: pI is a myeloid-specific cell promoter and drives CIITA in macrophages that are activated by IFN-γ as well as DCs. pIII is lymphoid cell-specific and is responsible for driving CIITA in B-cells, DCs and activated T-cells. pIV drives CIITA expression in non-bone marrow derived cells through IFN-γ stimulation[24] (Figure 1.5).

1.2.5 Expression of Major Histocompatibility Complexes(s) I & II in Breast Cancer

Expression of HLA class I is required to activate an immune response by CD8+ T-cells, and in cases of cancer, the activation of anti-tumour CTLs is dependent upon the recognition of epitopes that are presented on the HLA class I molecule on the tumour cell[25]. Therefore, the loss of these surface molecules can lead to reduced activation of CTLs and reduced killing of tumour cells leading to increased malignancy. The down-regulation of HLA class I is believed to be one of the main mechanisms by which tumours evade the immune system and the loss of such molecules has been shown to increase tumour aggressiveness and invasiveness[10,26]. Specifically, in breast cancer, approximately two thirds of tumours have shown HLA class I loss[27,28].

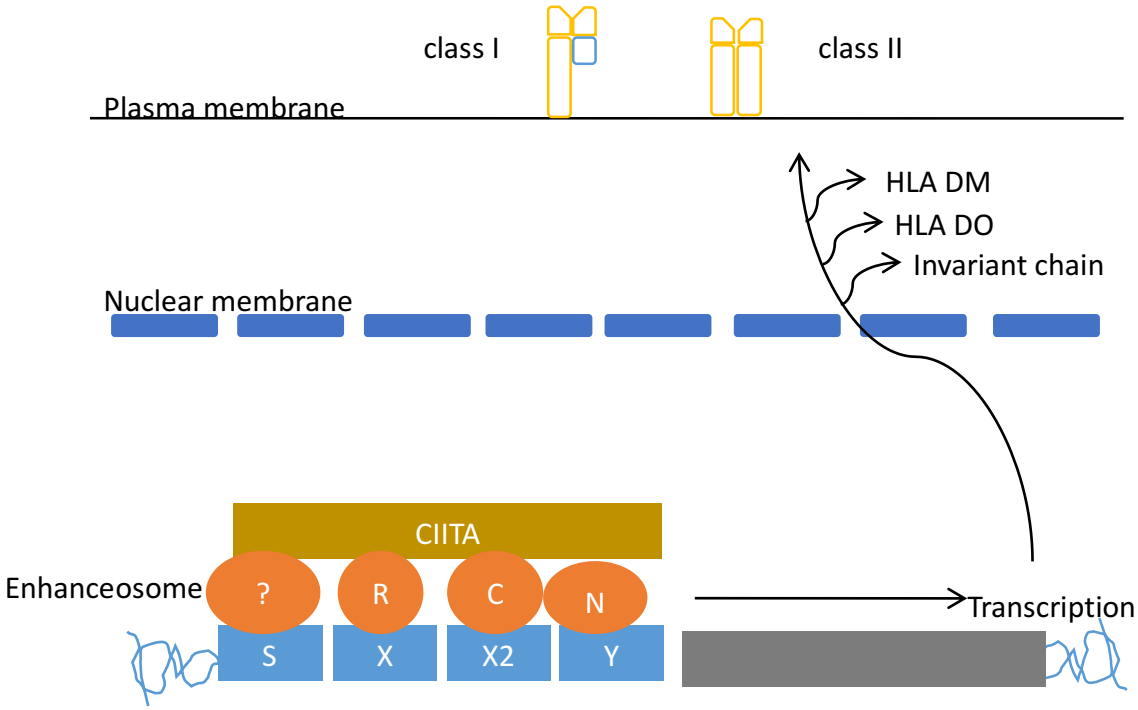

class I
class II
Plasma membrane
HLA DM
HLA DO
Invariant chain
Nuclear membrane
CIITA
Enhanceosome
?
R
C
N
S
X
X2
Y
Transcription

Figure 1.5: Regulation of human leukocyte antigen class II expression.

The regulation of HLA class II is performed at the transcriptional level by binding regulatory factor X (RFX), CREB, and the NF-Y for the S,X,Y module that is in the promoter of HLA class II genes. This allows the CIITA to bind and recruit transcription machinery. Adapted from (21).

The consequences of such loss is significant for nodal metastasis, lymphatic invasion and venous invasion[27]. The study by Kaneko and colleages (2011) also showed that patients with HLA class I-positive tumours have significantly longer disease-free survival (DFS) than those with HLA class I-deficient tumours, emphasizing the importance of having available HLA class I molecules on the tumour surface to present antigenic peptides to surrounding lymphocytes. Although there are many HLA aberrations across the various cancers, the loss of HLA class I seems to have universally negative clinical implications[10,26].

As previously mentioned, HLA class II molecules are only constitutively expressed on APCs; regular breast epithelial cells do not express HLA class II except during lactation[29]. However, patients with breast cancer express HLA class II in approximately 50% of cases, begging the question of the clinical implication(s) of HLA class II expression on breast tumour cells[19]. HLA-DR is the most commonly expressed HLA class II molecule on breast tumours, followed by HLA-DP and DQ[30]. HLA class II can be induced through stimulation of certain cytokines, most notably IFN-γ, as well as interleukin alpha (IL-α), interleukin beta (IL-β), tumour necrosis factor alpha (TNF-α), interleukin-4 (IL-4) and estradiol (E_2)[25].

There is yet no consensus regarding the prognostic implication of expressing HLA class II on breast cancer cells, as the results have been varied and contradictory. In some studies, HLA class II up-regulation through stimulation of the cytokine milieu in the tumour environment has been shown to induce an anti-tumour T-cell response suggesting a benefit of HLA class II expression[31–33]. However, this association is not clear;

with some studies showing that HLA-DR expression is associated with a favorable prognosis, and good differentiation[34], whereas others have found no relationship between the two[35,36].

Increased HLA-DR expression has been observed in ERα- tumours, suggesting that ER has a role in down-regulating HLA-DR[37]. The relationship between HLA class II and prognosis may not be unambiguous, as it has been shown that outcomes differ depending on the co-chaperones present, including HLA-DM and the Ii[19]. In a study by Oldford and colleagues (2006), it was shown that discordant expression of these may negatively impact peptide presentation[19].

1.3 Interferons

Interferons are cytokines that play various roles in the immune system. They can interfere with virus replication, cause inflammation and are secreted by some of the first-line defenders of the immune system itself: NK cells and NKT cells. There are three classes of interferons: class I has IFN-α and IFN-β, class II consists of IFN-γ, and class III contains IFN-λ[38]. The remainder of this introduction will focus on the class II Interferon, IFN-γ, and its classical signal-transduction pathway (Figure 1.6).

1.3.1 IFN-γ Pathway and Function

When released, IFN-γ binds to its cell-surface IFNGR. This receptor consists of two IFNGR1, and two IFNGR2 chains that are responsible for ligand binding and signal

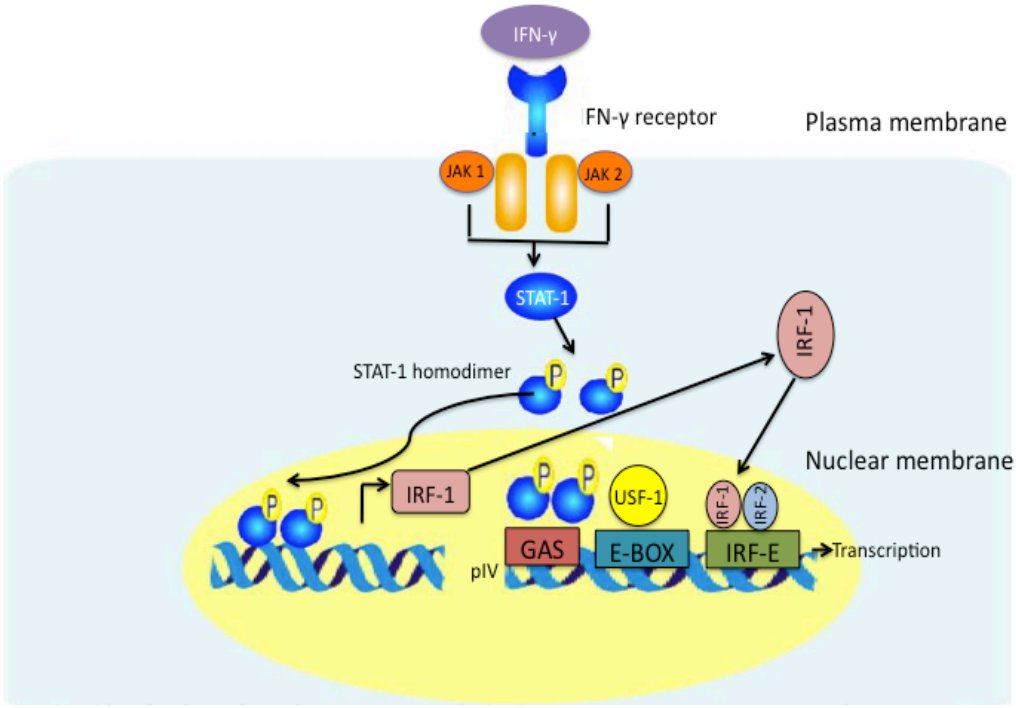

IFN-γ
FN-γ receptor
Plasma membrane
JAK 1
JAK 2
STAT-1
STAT-1 homodimer
P
P
IRF-1
Nuclear membrane
P
P
IRF-1
P
P
USF-1
IRF-1
IRF-2
GAS
E-BOX
IRF-E
Transcription
pIV

Figure 1.6: Interferon gamma signaling pathway.

IFN-γ binds to the surface interferon gamma receptor(s) 1 and 2 and the signal is sent down to activate the JAK I & 2, leading to the phosphorylation of STAT-1. The resulting dimer then translocates to the nucleus and then activates its target promoters. Adapted from (21).

transduction, respectively. Once this binding occurs, the signal is sent down through the receptor to activate the receptor-associated protein tyrosine kinases JAK1 & JAK2. This leads to the phosphorylation of Signal Transducer and Activators of Transcription-1 (STAT-1), which then dimerizes thereby allowing the homodimer to translocate to the nucleus and activate its target promoters including pIV and Interferon Regulator Factor-1 (IRF-1)[24]. STAT-1 homodimer activates gene transcription by binding to the GAS site located in the promoter region of genes. This whole process of early gene transcription takes approximately 15-30 minutes[38].

IFN-γ signaling can also occur independently from JAK/STAT-1 in the non-classical pathway and it has been shown that 30% of genes stimulated by IFN-γ signaling occur in the absence of STAT-1[39]. There are other signaling pathways that work parallel to JAK/STAT-1 signaling including the PI3-K/AKT, PKC and the MAPK pathway, all of which have been shown to phosphorylate STAT-1[39]. In addition to the activation of the MAPK pathway, IFN-γ can also cause the up-regulation of certain cell-surface molecules such as HLA-DR and PD-L1[40].

1.4 Programmed Cell Death Ligand-1 Structure and Function

PD -L1 or B7-H1 is a cell-surface protein belonging to the B7 family[41] (Figure 1.7). The ligand is expressed on APCs and, like HLA class II molecules, can be induced by cytokines such as IFN-γ[40]. PD-L1 interacts with the PD-1 receptor expressed on B-cells, natural killer cells, and activated CD8+ and CD4+ T-lymphocytes causing inhibition

and/or apoptosis[2].Due to its inhibitive nature, the PD-1/PD-L1 interaction is a critical component of immune regulation. Because of their roles in immunoregulation, the presence of PD-L1 on tumour cells has been suggested to help protect the cells from immune surveillance[42].

1.4.1 Programmed Cell Death Ligand-1 expression and MAPK pathway

Because IFN-γ is the best-known regulator of PD-L1, pathways that respond to IFN-γ stimulation, such as the JAK/STAT pathway, PI3K and MAPK pathways have been shown to induce IFN-γ-mediated PD-L1 expression on tumour cells[43]. This was demonstrated in a study by Cheng and colleagues (2007) that treatment of multiple myeloma plasma cells with the pan MEK inhibitor U0126 and IFN-γ resulted in dramatically decreased PD-L1 expression, whereas treatment with PI3K inhibitor LY294002, P38 inhibitor SB203580, and NFκB inhibitor SN50 had no such effect[44]. The importance of MAPK in PD-L1 was also demonstrated in a study by Falchook et al (2012) in which blocking MEK inhibited PD-L1 transcription in AML[45]. The modulation of PD-L1 by MAPK has been observed in a number of different cancers including anaplastic large

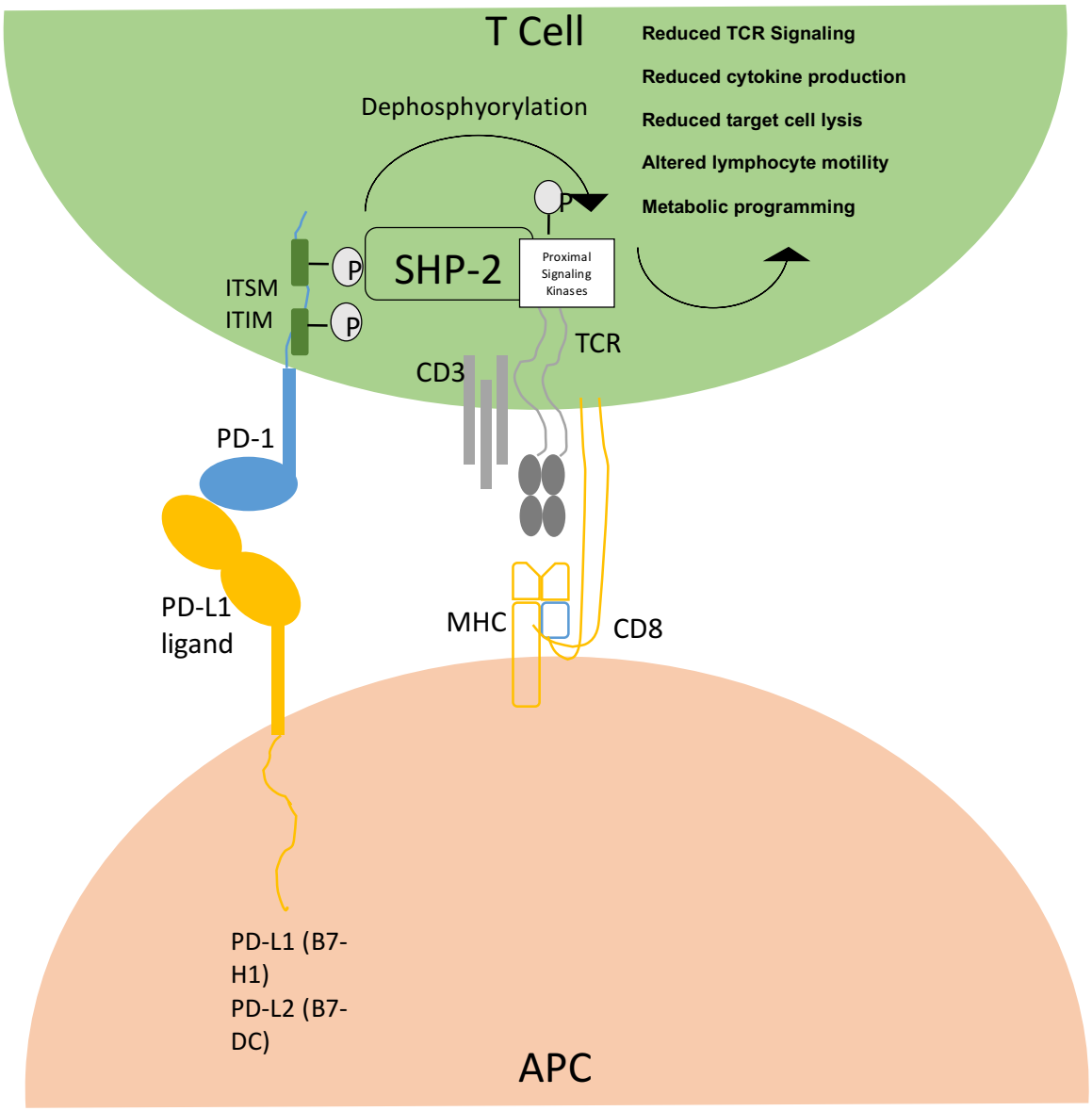

T Cell
Reduced TCR Signaling
Reduced cytokine production
Reduced target cell lysis
Altered lymphocyte motility
Metabolic programming
Dephosphyorylation
P
SHP-2
Proximal Signaling Kinases
ITSM
ITIM
P
P
TCR
CD3
PD-1
PD-L1 ligand
MHC
CD8
PD-L1 (B7-H1)
PD-L2 (B7-DC)
APC

Figure 1.7: Interaction of PD-1 on T-cell with Programmed Cell Death Ligand-1 expressed on tumour cell.

PD-L1 (B7-H1) expressed on tumour cell surface interacts with the corresponding PD-1 molecule on the CD8+ or CD4+ T-cell. Interaction leads to signal proprogation, which causes phosphorylation of ITIM and ITSM. ITSM interacts with SHP-2, which then becomes dephosphoylated, leading to phosphorylation of proximal signaling kinases. This interaction leads to inhibition of TCR mediated IL-2 production, and subsequent inhibition and deactivation, as well as diminishes cell growth and survival signals[51]. Adapted from (42).

cell lymphoma and Hodgkin lymphoma[43,46]. PD-L1 is transcriptionally modulated by c-jun and increased by STAT3 and through the inhibition of MEK, the expression of PD-L1 is subsequently decreased[47].

1.4.2 Programmed Cell Death Ligand-1 Expression in Breast Cancer

The expression of PD-L1 on tumour cells differs from cancer to cancer, however its presence has been documented in melanoma, ovarian, pancreatic, renal, lung, colon as well as breast cancer[48]. In one study, the presence of PD-L1 on breast tumours was observed in 30% of patients and was associated with TN status and high levels of TILs[49]. The mechanisms that regulate PD-L1 in tumour cells are not fully understood, however a few mechanisms have been described[48]: On myeloid-derived DCs, the PD-1 ligation acts by negatively regulating cytokines such as IL-6 and MIP-1α. On splenic DCs, PD-1 acts by inhibiting the production of IL-12 and TNF-α. When PD-1 on T-cells interacts with PD-L1 on DCs along with the TCR, inhibition of cytokine production, proliferation and cytolytic activity occurs. Another study has shown that interaction of PD-1 and PD-L1 cause anergy in naïve T-cells and apoptosis in effector T-cells[50].

In breast cancer, the role of PD-L1 is less understood: PD-L1 is expressed only on tumour tissue and completely absent in normal tissue of the same breast[52]. The expression of PD-L1 is associated with a poorer prognosis as well as hormone negative breast cancer and high grade status[52,53]. There have also been links between the mutation in PTEN and the induction of PD-L1. PTEN is a protein that regulates cell proliferation and acts as a tumour suppressor and is also important in the regulation of

the PI3K pathway. In a study by Seo et al (2006), increase in PD-L1 has been shown when PTEN-function is lost or mutated in prostate cancer cells[50,54]. There also exists a correlation between the proliferation marker Ki-67 and PD-L1 expression. Ki-67 is expressed on both normal as well as cancerous proliferating cells[2].

In a study by Ghebeh (2006), PD-L1 was shown on 41% of TILs in breast cancer patients[52]. PD-L1 expression is associated with larger tumours, and worse prognosis. Immune defects such as low numbers of peripheral blood lymphocytes, high numbers of T-regulatory cells, as well as reduced expression of both HLA-class II and co-stimulatory signal marker B-7 all contribute to a decrease in an effective immune response[48]. The expression of PD-L1 on TILs can inhibit T cell activation and proliferation by a reverse signalling mechanism or through binding to the PD-1 receptors in order to inhibit other T-cells[55,56]. The presence of TILs in breast cancer specimens has shown to be an important prognostic predictor of pCR or responsiveness to NAC, a commonly used TNBC therapy[8,10]. Testing involving patients with advanced cancers such as non-small-cell lung cancer, melanoma and renal-cell cancer, showed that treatment with anti-PD-L1 antibody contributed to durable tumour regression as well as prolonged stabilization of disease[57].

1.5 Rationale for Targeting the MAPK for Treatment of TNBC Overview

There are a number of factors that contribute to the poor prognosis of TNBC; these include activation of pathways that contribute to excessive growth and survival of tumour cells, one such pathway is the MAPK pathway. MAPK is one of the most

important pathways for regulating cell function due to its various roles in cell differentiation, cellular proliferation, cell migration and adhesion and cell death[58]. Mammalian MAPK signaling cascades are separated into four major groups: ERK or MAPK, JNK, p38 and ERK 5. The remaining focus will be on the "classical" pathway or the ERK/MAPK pathway (Figure 1.8).

The classical pathway is activated by the binding of various ligands such as growth factors, mitogens, or hormones to the cell surface receptor. The cell surface receptor then sends a signal from the cell surface to the nucleus through a series of phosphorylation events starting with the dimerization of the receptor that triggers the autophosphorylation of specific tyrosine kinases[6]. This allows adaptor proteins to bind and recruit the guanine nucleotide exchange factor Sos at the plasma membrane. Sos then activates Ras by catalyzing the exchange of GDP for GTP. Ras then activates the serine threonine kinase Raf family made up of A-Raf, B-Raf and Raf-1, which in turn phosphorylate MEK 1 and MEK 2 (MAPK kinase/ERK kinase). Like Ras, Raf can send signals through MEK-dependent or MEK-independent pathways; however, the only downstream targets of MEK 1 and MEK 2 are ERK 1 and ERK 2[59]. MEKs share 79% amino acid identity and are both equally able to phosphorylate ERK 1/2. Once phosphorylated, ERK 1/2 are able to enter the nucleus and activate transcription factors and regulatory proteins that are responsible for a diverse number of cellular responses such as cell proliferation, survival, differentiation, motility and angiogenesis[60,61]. ERK 1 and ERK 2 are 85% homologous with two phosphoacceptor sites, tyrosine and threonine, which are

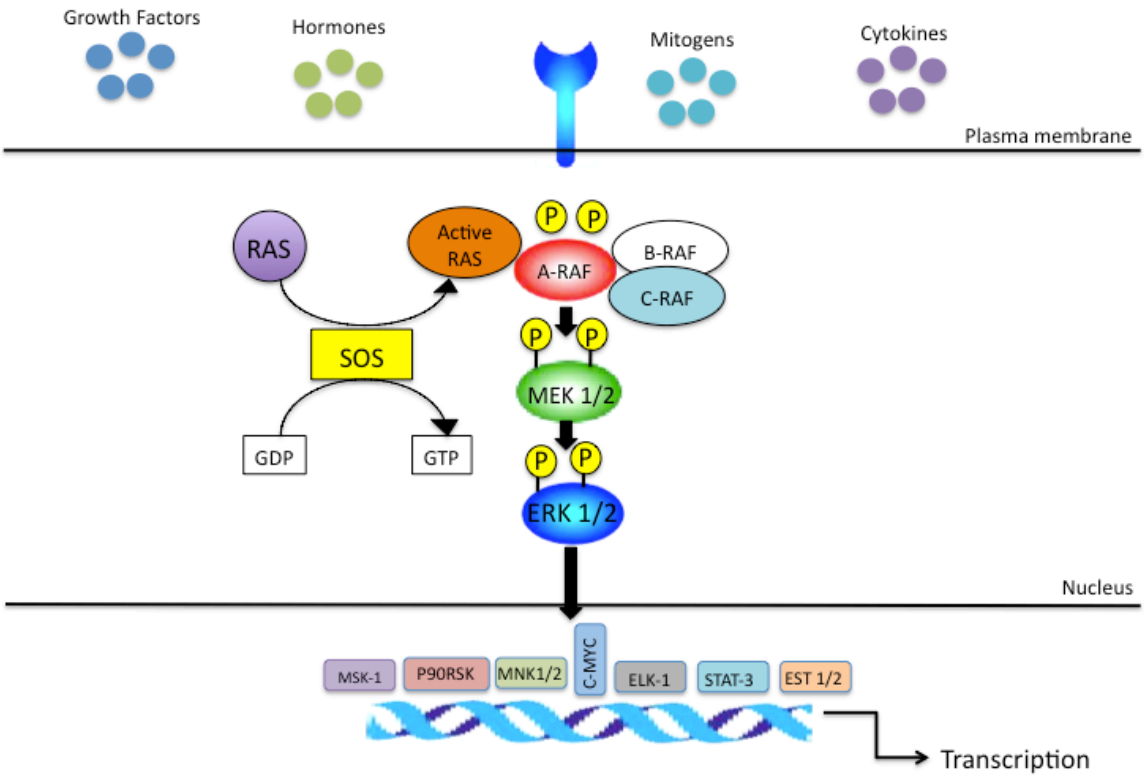

Growth Factors
Hormones
Mitogens
Cytokines
Plasma membrane
RAS
Active RAS
SOS
GDP
GTP
P
A-RAF
B-RAF
C-RAF
MEK 1/2
ERK 1/2
Nucleus
MSK-1
P90RSK
MNK1/2
C-MYC
ELK-1
STAT-3
EST 1/2
Transcription

Figure 1.8: The mitogen-activated protein kinase pathway.

MAPK pathway is activated by attachment of ligands (growth factors, mitogens, hormones) to the cell surface EGFR. The signal causes the dimerization of the EGFR that then triggers autophosphorylation of specific tyrosine kinases. Sos is bound and recruited by adaptor proteins and then activates Ras by exchanging GDP for GTP. Ras then activates the Raf family, which phosphorylates MEK 1 and MEK 2, and in turn phosphorylates ERK 1 and ERK 2, which can then enter the nucleus and activate transcription factors and regulatory proteins. Adapted from (43).

phosphorylated to activate the kinases. Both ERK 1 and ERK 2 are ubiquitously expressed, but their abundance in tissue is varied[62].

Ras belongs to a group of GTPases that help mediate the integration of signals generated by the pathway and therefore is a therapeutic target with therapies aimed at disrupting its expression and/or function. There are three Ras family genes (KRas, NRas, and HRas) and mutations in the Ras family are known to be crucial for the development of human tumours. Indeed the Ras oncoprotein is one of the main regulators of growth factor-induced cell survival in both normal and cancer cells[63,64]. In the cascade, Ras activates Raf1 in a GTP-dependent manner[59]. Given the complexity of this pathway and its importance in the regulation of cellular processes, scaffolding proteins are responsible for pathway organization. The scaffold proteins bind and sequester various MAPK components and help coordinate efficient activation of the pathway in response to stimuli[65].

1.5.1 Mitogen-Activated Protein Kinase Pathway and Breast Cancer

The MAPK pathway is found to directly contribute to the protection of tumours through two ways: 1) Growth and 2) Cell survival[59]. Of the three Ras family genes only small minorities of tumours from BC patients have mutations (KRas is mutated in just 1.5% of BCs)[66]. Raf mutations have also been linked to human cancers, and regardless of its mutation status, Raf is activated in tumours causing increased proliferation and survival. Of the three isoforms of Raf, BRaf is mutated in approximately 3% of the tumours in BC patients[67]. Despite the rarity of these mutations, many in the literature have discussed the MAPK pathway and its importance in breast cancer: For instance the

majority of basal-like breast cancers show high levels of EGFR and due to these high levels, results in hyperactivation of the MAPK pathway[6]. Interestingly, in mouse models, mammary tissue-specific expression of activated Ras is enough to generate breast carcinoma[68]. In one study by Loi and colleagues (2015), genomic or transcriptomic activation of the MAPK is also associated with decreased levels of TILs, which has been shown to lead to a poorer prognosis for TNBC patients[8].

1.5.2 Implications of Targeting the Mitogen-Activated Protein Kinase Pathway

There are drawbacks to inhibiting such a widespread pathway. Because a large number of normal cells rely on the continued activation of MAPK, inhibition could prove toxic. However, cross-talk between the pathways, such as the PI3K pathway, may become activated in response to MAPK inhibition helping to preserve homeostasis[69]. One of the benefits of MEK-specific inhibitors is that their only direct downstream targets are ERK 1/2 making it an attractive target for pathway inhibition.

1.6 The Mitogen- Activated Protein Kinase Pathway Inhibitors

There are a number of inhibitors on the market that can inhibit the MAPK pathway, and these vary depending on the part of the pathway targeted. There are 19 MEK specific inhibitors, as well as many others for RAF, p38, JNK, and ERK. In the course of this research, five MAPK inhibitors were used: PD98059, U0126, PD0325901, Selumetinib (AZD6244) and Trametinib (GSK1120212)[70]. These inhibitors are specific for

the MEK proteins, however their specific targets vary; with some specific for MEK 1 and others inhibiting both MEK 1 and 2. Of these five, Sel and Tram are the most clinically relevant in current cancer research.

1.6.1 U0126

U0126 is a pan MEK inhibitor, effectively blocking both MEK 1 and MEK 2 with an IC50 of 0.07 μM. U0126 is a non-competitive inhibitor with respect to MEK, ATP and ERK[71]. In vitro, U0126 functionally antagonizes AP-1 transcriptional activity and blocks a variety of cytokines and metalloproteinases involved in the inflammatory process, also, U0126 decreases T-cell activation and it down-regulates IL-2 mRNA levels[72].

U0126 has been tested extensively both *in vitro* and *in vivo* and has shown consistent results in its ability to inhibit the MAPK pathway. U0126 also has the ability to rescue estrogen receptor expression in BC cells[73]. However, in many TNBC lines such as MDA-MB-231, BT-20, MDA-MB-468 and HS578T cells, which exhibit site-specific methylation of CpG islands in the ERα promoter, treatment with a demethylating agent is required to obtain re-expression of the estrogen status[74].

U0126 has also been shown to increase CIITA levels especially when in the presence of IFN-γ[75,76]. These studies also suggest that CIITA expression is negatively regulated by ERK and p38 MAPK signals in macrophages and dendritic cells. Previous research in the Drover lab has shown that the use of U0126 results in the decrease of surface HLA-DR expression[77]. Explanations for this could be that MAPK activation up-regulates cell surface markers such as CD40, CD80, CD86 and HLA-DR therefore the

antigen presentation process is enhanced[78]. U0126 has also been shown to decrease PD-L1 levels in melanoma cells[47].

1.6.2 PD98059

PD98059 is a highly selective MEK 1 inhibitor with no direct inhibition of ERK 1 or ERK 2. It is non-ATP competitive and has an IC50 of 2 μM. PD98059 inhibits neither MAPK homologs JNK and p38, nor Raf kinase, cAMP-dependent kinase, protein kinase C, v-Src, epidermal growth factor (EGF) receptor kinase, insulin receptor kinase, PDGF receptor kinase, or phosphatidylinositol 3-kinase[70].

PD98059 is incapable of inhibiting wild type MEK 1 that has been activated through Raf-mediated phosphorylation, suggesting that PD98059 can only bind to the inactive or unphosphorylated form of MEK 1[71]. PD98059 has also been shown to increase HLA class I in gastric and ESCC cell lines[79].

1.6.3 PD0325901

PD0325901 is a pan inhibitor of MEK with an IC50 of 0.33 nM and is non-ATP competitive. PD0325901 is approximately 500-fold more potent than 1st generation MEK inhibitor CI-1040[70]. A single oral dose at 25 mg/kg shows greater than 50% inhibition of ERK at 24 hours post-dose. In mouse studies, a week of oral administration shows no tumour growth in those inoculated with BRAF-mutation-containing papillary thyroid cancer (PTC) cells[80]. The mechanism of action for PD0325901 allows for inhibition of

MEK without perturbing the ATP site. It has been proposed that this inhibitor induces a conformational change in the protein that traps the kinase activation loop, and when bound, the inhibitor partially occludes the MEK 1 catalytic site therefore not allowing ERK to access the catalytic pocket and become phosphorylated[63].

In human melanoma clinical trials, when given a dosage of ≥2 mg bi-daily (BID), PD0325901 decreased phosphorylated ERK by 60% as well as decreased the proliferation marker, Ki67[80]. Despite its advances, PD0325901 was discontinued from clinical trials due to its toxicities including retinal vein occlusions and neuropathy due to its ability to cross the blood brain barrier[81]. Because of the discontinuation of this drug in clinical trials, it was used only in the beginning of this research.

1.6.4 Selumetinib (AZD6244)

Selumetinib (AZD6244) (Astra Zeneca) is a highly selective second generation MEK 1 inhibitor with an IC50 of 14 nM. Selumetinib is not competitive with ATP and can inhibit ERK phosphorylation at concentrations of 40 nM or less. Selumetinib is highly selective and does not affect p38, c-jun, PI3K or MEK5/ERK5 pathways[70]. Selumetinib is used in clinical trials for treatment of cancers ranging from stage 1-3 including NSCLC, gallbladder carcinoma, rectal cancer, thyroid cancer, colon cancer, as well as metastatic breast cancer (among others)[82].

In a study by Favata and colleagues (1998), a correlation between sensitivity to Selumetinib and Ras and Raf mutations was shown[71]. Although less common in breast cancer, with only 4% harboring Ras mutations and 7% for Raf, they are associated with a

poor prognosis[83]. TNBC cell line MDA-MB-231 has mutations present in both BRAF and KRAS, which suggests it will be sensitive to Selumetinib treatment[84].

1.6.5 Trametinib (GSK1120212)

Trametinib (GSK1120212) is a highly specific and potent MEK 1/2 inhibitor with an IC50 0.92 nM-3.4 nM. Trametinib inhibits the kinase activity of MEK 1/2 and thereby prevents Raf-dependent MEK phosphorylation. Like Selumetinib, results using this inhibitor are most pronounced in cell lines with BRAF or Ras mutation[70]. It is more potent than both Selumetinib and PD0325901 and when administered in vivo orally at 0.3 -1 mg/kg for a two-week duration, is able to inhibit tumour growth[85].

This inhibitor has benefits compared to the others by having a long circulating half-life with continued inhibition after 24 hours. Trametinib differs from PD0325901 in that it does not significantly penetrate intact brain[81]. Trametinib has been shown to have a 33% response rate in Braf-mutant melanoma, with median progression-free survival in patients of 5-7 months, and in the subset without brain metastases it was 7-4 months[86].

When AT3ova and 4T1.9 mouse TNBC cells were treated with 100 nM Trametinib, it was shown that it could potentiate the effect of interferon-gamma on MHC class I, MHC class II and PD-L1 expression in vivo and in vitro, indicating its usefulness as a potential immunotherapeutic drug for TNBC[8].

1.7 Rationale and Hypothesis

Immunotherapy has been gaining more attention and importance in cancer research, due to its promising ability to target and destroy tumours that may otherwise lack target molecules or be resistant to current treatment options. For cancers with limited targets available for treatment, such as TNBCs, additional options in the form of immunotherapy such as checkpoint and pathway inhibitors that can modulate immune markers, is fast becoming a real possibility. By helping our immune systems better identify tumour cells while simultaneously decreasing immune escape, even cancers that lack targets for conventional therapies have the potential for pharmaceutical intervention.

With down-regulation of HLA class I as a mechanism of immune escape in so many tumour cells, the rescue or up-regulation of HLA status could have potential immunotherapeutic effects by increasing tumour-antigen presentation to surrounding TIL's. Conversely, the up-regulation of certain inhibitory molecules such as PD-L1 on the tumour surface could have adverse effects by decreasing T-cell activation and proliferation, thereby aiding the tumour in immune escape.

As previous studies have shown, MAPK becomes hyperactivated in TNBC and may play a role in the modulation of HLA and PD-L1 expression. Thus, we hypothesized that MEK inhibition and, subsequently, inactivation of phosphorylated ERK 1/2 may alter tumour expression of these markers, possibly resulting in desirable effects, that is, increased HLA expression and diminished PD-L1 expression. Also, due to the importance of the IFN-γ pathway in anti-tumour immunity and its role in the regulation of HLA and

PD-L1 expression, we queried the effects of inhibition of MAPK on IFN-γ inducible, as compared to constitutive HLA and PD-L1 expression.

Objectives:

1. As previous research in the Drover laboratory showed that the MEK*i*, U0126, diminished cell surface, but not intracellular HLA-DR expression on the TNBC line, MDA-MB-231c10A, it was important to determine the effects (if any) of U0126 on HLA class I and PD-L1 expression in the same cell line. The second part of this objective, is to determine if other MEK*i*(s) similarly modulate of these molecules in MDA-MB-231c10A.
2. To determine the effects of MEK*i*(s) on both constitutive and IFN-γ induced HLA and PD-L1 expression in other TNBC lines.
3. To determine the effects of MEK*i*(s) on constitutive and IFN-γ -induced HLA and PD-L1 expression in other cancer lines.

Chapter 2: Materials and methods

2.1 Cell Culture

Several established TNBC cell lines were used in this research: MDA-MB-231, MDA-MB-468, BT-20, HS578T, normal breast line: HS578Bst and three non-breast cancer lines: MDA-MB-435, a melanoma line[87], colon cancer line HT-29 and EBV transformed lymphoblastoid line SAVC. A clone of MDA-MB-231, henceforth referred to as MDA-231c10A, was also used. MDA-231c10A was grown in MEM (supplemented with 2 mM L-glutamine (Invitrogen, Cat# 25030-164), and 2 mM antibiotic antimycotic, (A/A) (Invitrogen, Cat# 15240-112) and estrogen-depleted FBS. the remaining lines were grown in CM consisting of IMDM, supplemented with 10% heat inactivated FCS (Invitrogen, Cat# 12600-44), 2 mM L-glutamine (Invitrogen, Cat# 25030-164), and 2 mM antibiotic antimycotic, (A/A) (Invitrogen, Cat# 15240-112). The cancer cell lines were originally purchased form the ATCC, while the B-cell line was obtained through the 10th International Histocompatibility Workshop. Cultures were grown in 10 cm^2 tissue culture plates (Falcon, Becton Dickinson (BD) Biosciences) at 37°C in 7% CO_2 atmosphere. Cultures were refreshed every 5 days with CM and cells were harvested when 80-90% confluent. B-cell line was grown as suspension cultures in IMDM.

2.2 Harvesting of Adherent Cells

Media was aspirated from culture plates using vacuum filtration. Cells were washed using 5-6mls of 1X sterile PBS. Cells grown in IMDM were detached from plate

using 0.25% Trypsin-EDTA (Invitrogen, Cat# 25200-114) and incubated at 37°C for 1-2 mins after which, reaction was ceased by addition of CM. Additionally, cells grown in MEM used phenol-red free 2.5% Trypsin. Medium was pipetted back and forth to ensure maximum cell detachment and collection, The cells were then transferred to 15 mL centrifuge tube and centrifuged at 500 x g at 8°C for 7 minutes. After centrifugation and removal of supernatant, the pellet was re-suspended in 6-7 mls of medium and centrifuged again at the same settings. After the second wash, cells were re-suspended in 6mls of CM and counted using a haemocytometer and phase contrast microscope. Cells were re-plated at a density of approximately $5x10^5$ in a 10 cm^2 culture dish containing 10 mls of CM.

2.3 MEK Inhibitor Treatment

Cell lines, plated in 6-well tissue culture plates (Falcon, Corning Life Sciences, Cat# 353046) at a density of $2.5X10^5$ cells/well were incubated for 24 hours, after which the medium was exchanged for fresh CM. Preparations of MEK inhibitors, as described below were added directly to wells. MEK inhibitors; Selumetinib (AZD6244) (Cat# S1008, 50 mg), Trametinib (GSK1120212) (Cat# S2673, 5 mg), and PD0325901 (Cat# S1036, 10 mg), PD98059 (Cat# S1177) were purchased from Selleckchem.com and U0126 was purchased from Calbiochem (Cat # CAS 109511-58-2).

Table 2.1:Human cell lines used in this study

Cell Line	Identification	Cell Type	Cancer classification	Receptor Expression
MDA-MB-231	ATCC HTB-26	TNBC	Adenocarcinoma Basal B	ER^-, PR^-, $HER2^-$
MDA-MB-468	ATCC HTB-132	TNBC	Adenocarcinoma Basal A	ER^-, PR^-, $HER2^-$
BT-20	ATCC HTB-19	TNBC	Carcinoma Basal A	ER^-, PR^-, $HER2^-$
HS578T	ATCC HTB-126	TNBC	Carcinoma Basal B	ER^-, PR^-, $HER2^-$
MDA-MB-435	ATCC HTB-129	Melanoma	Ductal carcinoma[1]	N/A[2]
HT-29	ATCC HTB-38	Colon Cancer	Colorectal adencarcinoma	N/A
HS578Bst	ATCC HTB-125	Normal Breast Tissue	Normal breast tissue	N/A
SAVC	10^{th} international histocompatibility workshop	EBV transformed lymphoblastoid line	Blood sample	N/A

[1] MDA-MB-435 was previously classified as a TNBC, but is now accepted as a melanoma line.

[2] "Not applicable" for this section

5 mM Stock solutions were achieved by dissolving each drug in DMSO and stored in aliquots at -80°C. Cells were treated for a time period ranging 4-72 hours, with most data collected from 72hr experiments. Drugs were used at clinically relevant and optimal concentrations suggested by literature and product website as well as determined by crystal violet viability assays done in lab to determine drug-induced cytotoxicity (Chapter 2.9). All drug treatments were matched with DMSO vehicle controls 1hr prior to IFN-γ addition.

2.4 Interferon Gamma Stimulation

Since IFN-γ is known to up-regulate HLA class I, HLA class II and PD-L1, its addition/absence was used to compare constitutive and induced expression of HLA and PD-L1 in cell lines treated with MEK*i*(s). One 1hr after drug treatment, 100 units of human recombinant IFN-γ (BD Biosciences, 55461) was added to each well and incubated for the desired time period. Optimal IFN-γ concentration for up-regulation of HLA was achieved through previous experiments (Ahmed Mostafa, PhD thesis, 2014).

2.5 Flow cytometry

2.5.1 Cell Surface Assay

The effects of MEK*i*(s) on HLA class I, HLA DR and PD-L1 surface expression was detected by flow cytometry. Adherent cells were washed in PBS, detached with trypsin

and harvested according to above procedure (Chapter 2.2). After the first centrifugation, cells were resuspended in Fluorescence Activated Cell Sorting Buffer (FACS buffer) comprised of 0.2% FBS, and 0.02% sodium azide (NaN_3) in PBS. After washing, 100 μl of cell suspension containing a minimum of $1X10^5$ was added to 5 ml polystyrene round bottom tubes (Falcon, BD). Primary antibodies were diluted in FACS buffer to their optimal concentrations and 25 μl was added to 100 μl cell suspension and incubated for 30 minutes on ice at 4°C.

After this primary incubation, cells were washed twice in 2 mls FACS buffer and centrifuged at 600 x g for 5 minutes at 8 degrees. Supernatant was decanted and 25 ul of secondary antibody goat anti-mouse immunoglobulin-G labeled with phycoerythrin fluorochrome, diluted 1/40 in FACs buffer was added to each tube. Cells were incubated on ice in the dark for a further 30 minutes. After this incubation, cells were washed and centrifuged twice and then fixed with 150 μl 1% paraformaldehyde (PFA) (Sigma) diluted in PBS. Cells were analyzed for 10,000 events using a FACStarPlus flow cytometer (Becton-Dickinson) and CellquestPro software (Becton-Dickinson).

2.5.2 Data Analysis and Interpretation

MFI and the percentage of positive cells were measured used Cellquest Pro software in order to quantify the amount of cell surface expression of HLA-DR, HLA class I and PD-L1. Test results were considered positive if MFI was at least twice the negative control MFI. Ratio of treated/untreated was obtained by dividing relative test values by

the relative DMSO control given by the formula: (treatment MFI – control MFI) / (DMSO MFI - control MFI). Test values were compared to that of the relative controls, which were given the value of 1. Those with values of 1 were designated “no effect”, and those with values greater than 1 were concluded to have a positive or, up-regulatory effect on surface expression. A Student T-Test was used to determine statistical significance between the controls and the drug-treated samples. A one-way Anova paired with a Tukey HSD test was used to compare the inhibitor-mediated effects to one another. Statistics were assessed on Microsoft Excel. A p-value of less than or equal to 0.05 was determined to be of significance.

2.6 SDS-PAGE Western Blotting

2.6.1 Lysate Preparation

Cells were plated in 6-well tissue culture dishes at a density of $2.5x10^5$ and treated with optimal concentrations of MEK*i*, controls and/or IFN-γ as previously described in section(s) 2.3 & 2.4. Once ready, medium was removed via vacuum filtration, cells were rinsed in 2 mls PBS, followed by the addition of 100-200 μl RIPA was added to each well. RIPA buffer prepared immediately before use, consisted of 0.5% sodium deoxycholate (Biochemical, 43035), 0.1% SDS (BioRAD, 161-0301),1% NP40 (Sigma, 100F-39211) in PBS at a pH of 7.3, and protease inhibitors aprotinin (5 mg/ml) (Sigma, A4529), leupeptin (10 mg/ml) (Sigma, L202), pepstatin A (2 mg/ml) (Sigma, P5318), 2 M PMSF (Sigma, P7626) and halt phosphate inhibitor cocktail

(Thermoscientific, 78420). Cells were then scraped from the plate using a sterile, plastic cell scraper, transferred to 1.5 ml microfuge tubes and centrifuged at 11,000 x g for 15 mins at 4°C. The pellet was discarded and the supernatant was collected, labeled and stored at -80°C for electrophoresis and Western blotting.

2.6.2 Quantification of Cell Lysate Protein Concentration(s)

Protein concentration of cell lysates was quantified using a BCA protein assay kit (ThermoScientific), according to the manufacturer's instructions. The assay was set up by adding 50 μl of each sample, diluted 1:10 to 5 ml polystyrene round-bottom tubes (Falcon, Becton Dickson Bioscience) and 50 μl of BSA standards, to ten separate 5 ml polystyrene round-bottom tubes. BSA concentrations, 0 μg, 31.25 μg, 62.5 μg, 125 μg, 250 μg, and 500 μg, were done in duplicate. 1ml of BCA protein assay working reagent was added to each tubes at a ratio of 50:1 Reagent A: Reagent B. All tubes were incubated at 37°C for 30mins and then read using Spectrophotometer (Beckman Coulter DU→ 530) at wavelength 562 nM. Protein concentrations were calculated from the standard curve.

2.6.3 Electrophoresis of Cell Lysates

An 8% running gel was prepared using 1.25 ml water, 1ml acrylamide, 1 ml running buffer (1.5 M Tris HCl Buffer (BioRad)) pH 8.8, 40 μl 10% SDS, 40 μl 10% APS, and 2 μl Temed. The stacking gel was prepared using 860 μl water, 250 μl acrylamide

(BioRad), 380 µl stacking buffer (0.5 M Tris HCl buffer (BioRad)) pH 6.8, 15 µl 10% SDS, 15 µl APS (BioRad), 1.5 µl Temed (BioRad))

Samples were loaded using equal amounts of protein in 10-40 µl depending on the amount of protein in the appropriate well of each lane alongside 4 µl of protein ladder (BLUeye Prestained Protein Ladder Cat# PM007-0500) in order to determine band-size. Electrophoreses was performed at 100V for 1hr 45mins using a Mini-PROTEIN® Cell electrophoresis chamber (BioRad) containing running buffer (consisting of 25 mM Tris HCl pH 8.8, 0.1% SDS, 190 mM gylcine (Sigma)).

2.6.4 Electrophoretic Protein Transfer

Proteins were transferred to nitrocellulose membrane as follows: nitrocellulose membranes, filter paper and foam pads were pre-soaked in transfer buffer (25 mM- Tris HCL pH 8.8, 190 mM glycine, 20% methanol) for 15mins prior to transfer. Gels were carefully removed from electrophoresis apparatus and submersed in transfer buffer. The transfer sandwich cassette was then assembled in this order: black plastic base, foam pad, filter paper, filter paper with gel, nitrocellulose membrane, filter paper, clear plastic base. The sandwich cassette was then placed into the Trans-blot cell (BioRad) with a cool pack, set upon a magnetic stirrer and run at 100V for 1hr.

Table 2.2: Primary antibodies used in this study

Antibody	Isotype	Specificity	Concentration			Sources
			Flow	W.B	I.F	
W6/32	Mouse IgG_{2a}	HLA class I	1/25	1/25	1/25	In house
MUB2037P	Mouse IgG_{2a}	HLA class I heavy chain	NA[3]	1/1000	NA	Nordic MUbio
L243	Mouse IgG_{2a}	HLA-DR	2.5 μg/ml	NA	2.5 μg/ml	In house
MIH2 ab109052	Mouse IgG_1	PD-L1	5 μg/ml	NA	NA	Abcam
E1L3N #13684	Rabbit IgG	PD-L1	NA	1/1000		Cell Signaling
ab58810*	Rabbit IgG	PD-L1	NA	1 μg/ml	NA	Abcam
Phospho-p44/42 MAPK (ERK 1/2) (Thr 202/Tyr 204) 9101	Rabbit IgG	pERK	NA	1/500	NA	Cell Signaling
(E-4) sc-7383	Mouse IgG_{2a}	pERK	NA	1/500	NA	SantaCruz Biotechnology
K-23 sc94	Rabbit IgG	ERK-1 total	NA	1/5000	NA	SantaCruz Biotechnology
IgG1	Mouse IgG_1	Control	5 μg/ml	NA	5μg/ml	eBioscience Clone P3.6.2.8 Cat# 16-4714-85
NSG_{2a}	Mouse IgG2a	Control	5 μg/ml	NA	5μg/ml	Local source
B-7	Mouse IgG_{2a}	Alpha-tubulin	NA	250 ng/ml	NA	Santa Cruz Biotechnology
Ab8245	Mouse IgG_1	GAPDH	NA	1 ng/ml	NA	Abcam

[3] Not applicable to this section

2.6.5 Detection of Proteins

After transfer was complete, nitrocellulose membranes were treated with blocking buffer (5% milk powder in Tris-buffered saline (TBS-T)-Tween 20 (0.15 M NaCl, 0.05 M Tris pH 7.2-7.6, 0.05% Tween-20)) and set on a shaker for 1hr. Primary antibody optimally diluted in 5ml blocking buffer was added to each blot, in a sealed container and placed on a shaker at 4°C and left overnight. Membranes were then washed three times for 5mins each with TBS-Tween-20. Optimally-diluted secondary antibody, conjugated with horseradish peroxidase was added to the membrane and placed on a shaker for 1hr. Membranes were washed 3 times for ten minutes/wash followed by signal detection using Immobilon Western Chemiluminescent HRP Substrate (Millipore). ImageQuant LAS 4000 Station (GE Health Care) was used to image the blots.

2.6.6 Reprobing Membranes

To strip blots for reprobing, membranes were washed three times for 10mins with a TBS stripping buffer (pH 2.0), followed by three washes with TBS-Tween-20 for 10mins each.

2.7 Immunocytochemisty

2.7.1 Chamber Slide Set-Up

Cells were grown in 8-well or 16-well chamber slides (Cat # 154534 ThermoScientific) at $2.5x10^4$ or $1.5x10^4$ cells/well respectively with experimental

Table 2.3: Secondary antibodies used for Western blotting

Antibody	Isotype	Specificity	Concentration	Source
HRP-conjugated affiniPure $f(ab)_2$ fragment goat anti-mouse (GAM) Fc specific	Goat IgG	Mouse IgG	1/10,000	Jackson Immnoresearch
HRP-conjugated affiniPure $f(ab)_2$ fragment goat anti-rabbit (GAR) Fc specific	Goat IgG	Rabbit IgG	1/10,000	Jackson Immnoresearch

treatment added for a specific time period. Then media was aspirated and cells were washed with PBS and left to air dry overnight, followed by fixation with acetone (15mins at -20°C), methanol (10mins at -20°C or paraformaldehyde (15mins 4°C).

2.7.2 Cytocentrifuge Preparation

Cells were counted using haemocytometer and an aliquot containing $6x10^5$ cells was added to a 15 ml centrifuge tube and centrifuged at 500 x g for 7min at 8°C. After spinning, media was removed and the pellet was re-suspended in 6mls of PBS. 500 μl of cell sample ($5x10^4$ cells) was added to the cytocentrifuge sample chamber and cells were adhered to the slide by centrifuging at 500 x g for five minutes. Cells were adhered to a particular spot on the slide surface due to a piece of filter paper (Shandon Inc., Pittsburg, PA) assembled between the chamber and the slide. Once centrifuged, slides were left to dry overnight.

2.7.3 Immunofluorescence Staining

Cells were rehydrated by adding PBS for 5mins, then were blocked for 1hr using 15% goat serum in PBS. Then 100 μl of primary antibody, optimally diluted in washing buffer (PBS containing 0.5% bovine serum albumin (BSA) (Sigma), 0.05% Tween-20), was added to each well and incubated for 1hr at room temperature in a humid chamber. Slides were then washed 3 times for 5mins each with washing buffer in a Coplin jar on a stir plate with a small magnetic stirrer. Slides were then incubated for 1hr, in the dark,

with secondary antibody (Alexafluor-conjugated), followed by three 5min washes with washing buffer followed by a 20min wash with PBS. Slides were mounted using VECTASHEILD mounting medium with DAPI (Vector Labs). Cells were then examined using an Axioscope AI microscope (Zeiss)

2.8 Chemical Knockdowns to Silence ERK 1 and ERK 2 Using Small Interfering RNA

2.8.1 Cell Plating

Cells were plated in a 24-well culture dish at a density of $2.5x10^4$ cells/well in 0.5 mls culture medium without antibiotics and incubated at 37°C and 5% CO_2 overnight. Each knockdown experiment consisted of untreated cells, scrambled siRNA, ERK 1 siRNA (Santa Cruz Biotechnology sc-29307), ERK 2 siRNA (Santa Cruz Biotechnology sc-35335) and a combination of ERK 1 and ERK 2.

2.8.2 Transfection

The medium was changed approximately 2 hours before transfection. Both solution A (2μl of siRNA, ERK 1, ERK 2, or ERK 1/2 + 50 μl IMEM (no serum, no antibiotics) and solution B (2 μl of transfection buffer (Thermo Scientific DharmaFECT) + 50 μl IMDM (no serum, no antibiotics)) were made and left for 15mins at room temperature. They were then mixed and left for 15mins at room temperature before adding 100 μl directly to each appropriate well.

Table 2.4: Secondary antibodies used in immunocytochemistry

Antibody	Isotype	Stock Concentration	Concentration	Source
Alexafluor 555 goat anti-mouse A21127	$IgG_1(\gamma1)$	2 mg/ml	1/1000	Life Technologies
Alexafluor 594 goat anti-mouse A21125	$IgG_1(\gamma1)$	2 mg/ml	1/1000	Life Technologies
Alexafluor 555 goat anti-mouse A21137	IgG_{2a} ($\gamma2a$)	2 mg/ml	1/1000	Life Technologies
Alexafluor 488 goat anti-mouse A21131	IgG_{2a} ($\gamma2a$)	2 mg/ml	1/1000	Life Technologies
Alexafluor 555 goat anti-rabbit A21429	IgG (H+L)	2 mg/ml	1/1000	Life Technologies

Transfections were repeated at 24 hours, without removing any media. Protein samples were obtained 48-72 hours after first transfection by using RIPA buffer at 100 μl per well. Samples were stored at -80°C until Western blot analysis.

2.9 Viability Testing Using Crystal Violet Assay

Cells were plated in a 96-well plate at $2x10^4$ cells/well and incubated overnight at 37°C and 5% CO_2, followed by replacement of media containing treatment at the appropriate doses using three replicate wells per treatment and then incubated at the desired time. Medium was then aspirated and wells were washed with PBS. Cell viability was tested by adding 100 μl of crystal violet (0.5% crystal violet in 10% neutral buffered formalin (VWR-EMD) and incubated for 30mins at room temperature. After which, the crystal violet was aspirated and the plate was washed under the tap and blotted on filter paper. Plate was left to dry for 5mins at room temperature. The viable cells, represented by remaining crystal violet, were detected by dissolving it in 100μl of 30% acetic acid in water. The plate was shaken for 1min and the absorbance was measured on an ELISA reader (Bio-Rad model 3550) at 595 nM.

Table 2.5: Triple negative breast cancer cell lines and mutations

MDA-MB-231	BT-20	MDA-MB-468	HS578T	HS578Bst
TNBC epithelial cell line WNT7B oncogene KRAS (38G>A) & BRAF (1391G>T) mutation Basal B	TNBC epithelial cell line PIK3CA (1616C>G) mutation Basal A	TNBC epithelial cell line PTEN (IVS4+1G>T) mutation Basal A	TNBC epithelial cell line HRAS (35G>A) mutation Basal B	Normal mammary epithelial tissue

Table 2.6: Non-breast cancer cell lines and mutations

MDA-MB-435	HT-29
Metastatic melanoma BRAF mutation V600E	Colon adenocarcinoma BRAF mutation PIK3CA mutation

Chapter 3: Results

Previous research in the Drover laboratory suggested that MAPK activation leads to up-regulation of HLA class II in ERα⁻ breast cancer cell lines. Patients with ERα⁻ breast cancer have worse prognoses because ERa- tumors are resistant to hormonal therapies and frequently over-express growth factor receptors, leading to activation of several downstream signalling pathways such as NF-κB and MAPK signaling.

A previous student, Ahmed Mostafa, found inhibition of the MEK pathway using U0126 (10 μM) in the TNBC line MDA-MB-231c10A resulted in significantly decreased constitutive and IFN-γ induced HLA-DR expression on the cell surface. Ahmed Mostafa also confirmed these findings in VC5 and MC2, which are derived from the parent cell line, MDA-231c10A. Based on these results, it was hypothesized that the MAPK signaling is important for HLA-DR expression.

To further explore this hypothesis, Western Blot analysis was performed on lysates from MDA-231c10A, VC5 and MC2 treated or not with U0126 followed by IFN-γ stimulation or not for 24, 48 and 72 hours. Contrary to the extracellular surface flow results, treatment with U0126 increased intracellular HLA-DR expression in all cell lines at all time points examined. These contradictory results suggested that U0126 might be having an effect on cellular trafficking or recycling of the HLA-DR molecule.

Since MAPK signaling modulates protein stability and cell surface expression of some proteins[75,78,88], Ahmed hypothesized that U0126 destabilizes HLA-DR molecules, which might help explain the increase of HLA-DRα at the expense of a decrease in HLA-DR dimers. Indeed, immuncytochemistry and confocal microscopy revealed that U0126-

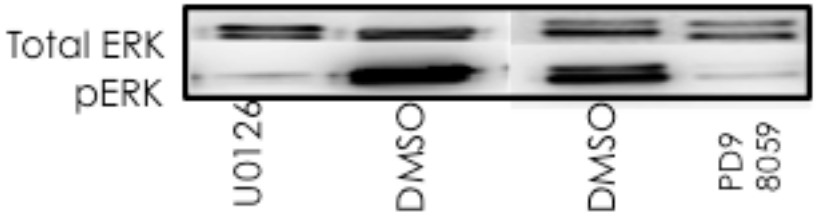
treated cells showed decreased surface HLA-DR compared to DMSO, with considerable deposits of HLA-DR localized to endocytic vesicles.

To further investigate whether the effect of MEK inhibition on HLA-DR expression was not unique to U0126, further experiments were performed using different MEK*i* and other breast cancer cell lines as described in the following sections.

3.1 U0126, But Not PD98059 Decreases Surface HLA-DR Expression in MDA-231c10A Cells

To confirm and expand the above findings, MDA-231c10A cells were treated with U0126 as well as an additional MEK*i*, PD98059, to see if the effects were similar. U0126 and PD98059 differ in their inhibition targets: U0126 is a pan MEK inhibitor whereas PD98059 is specific for MEK 1. Using the same conditions as described by AM (Ahmed PhD thesis, 2014), cells were plated in 6-well plates at $2.5x10^5$ cells/well and treated with the inhibitors or DMSO, followed by stimulation with 100units/ml of IFN-γ 1hr after MEK treatment. At 72 hours after IFN-γ stimulation, cells were analyzed for surface HLA-DR expression by flow cytometry. Western blotting for pERK was conducted on lysates prepared from cells treated in the same way to deduce the efficacy of the inhibitors (Figure 3.1. A). While flow cytometry results confirmed that U0126 decreased HLA-DR expression, PD98059 had only a marginal effect (Figure 3.1. B). This was not due to drug inefficiency since PD98059 inhibited pERK to the similar extent to U0126.

A.


Total ERK
pERK
U0126
DMSO
DMSO
PD98059

B.

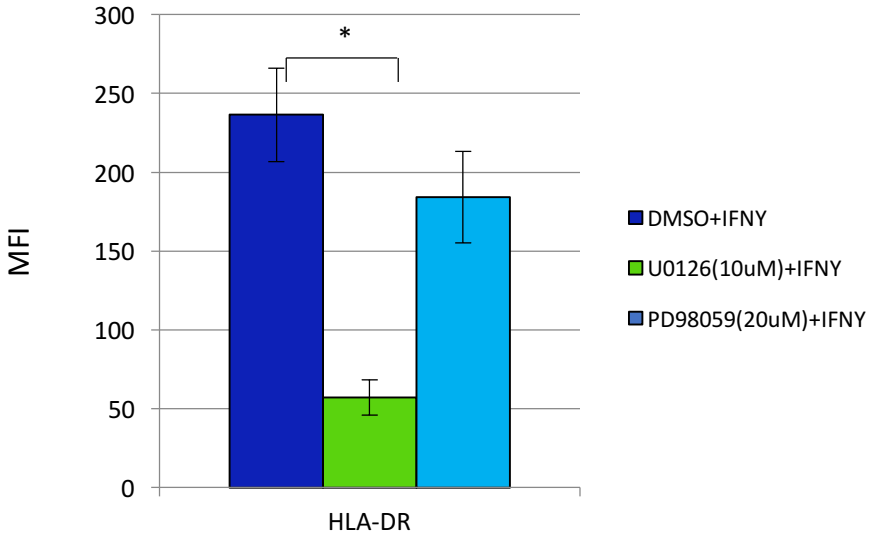

300
250
200
150
100
50
0
MFI
*
HLA-DR
DMSO+IFNY
U0126(10uM)+IFNY
PD98059(20uM)+IFNY

Figure 3.1: MEK inhibition decreases HLA-DR expression.

A) Western blot of whole cell lysates from MDA-231c10A cells (cultured in MEM) treated with U0126 (10 μM) or PD98059 (20 μM) or DMSO (vehicle control) followed by IFN-γ (100 u/ml) addition 1hr after MEKi treatment. Cells were then incubated for a total incubation time of 72 hours. Western blot analysis was conducted using antibodies against pERK (Thr2202/Tyr204) or total ERK (K-23). B) Flow cytometric analysis of MDA-231c10A treated with U0126 (10 μM) or PD98059 (20 μM) DMSO (vehicle control) to determine surface HLA-DR (L243) expression. Bar graphs represent mean fluorescence intensity (MFI) with error bars +/- SEM and are indicative of three independent experiments. Statistical analysis was done by student T-test and asterisks represent p value <0.05.

3.2 U0126 and PD98059 Differentially Modulate HLA class I and PD-L1 Expression

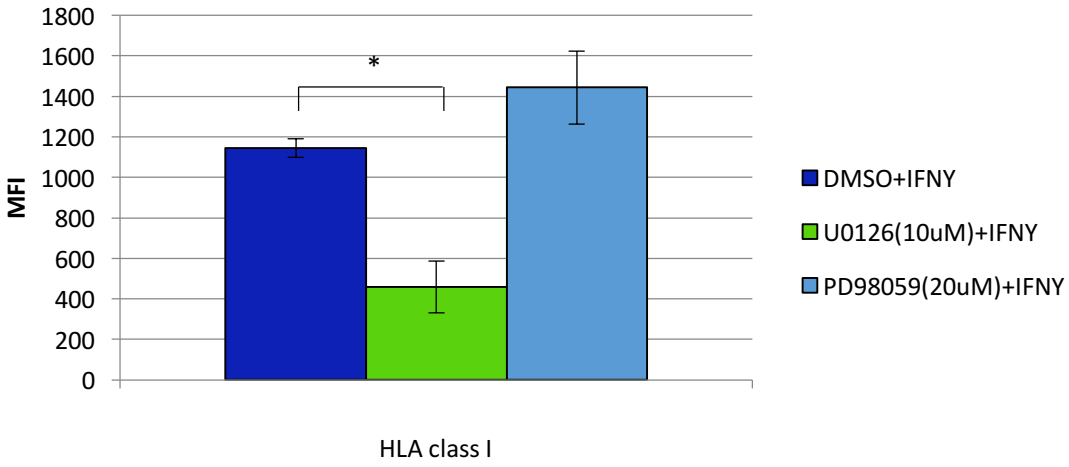
We expanded our findings to test the effects of MEK*i*(s) on another antigen presentation marker, HLA class I. HLA Class I is trafficked to the cell surface through a different pathway than HLA-DR, therefore we queried if U0126 would have the same effect on its expression. Results show that in the presence of INF-γ, U0126 significantly decreases surface HLA class I expression, whereas PD98059 does not (Figure 3.2. A).

After observing that MEK*i*(s) modulate the surface expression of HLA class I and HLA-DR, we questioned whether inhibition has effect on the inhibitory marker PD-L1. Results show modest decreases in PD-L1 expression with U0126 treatment, although not statistically significant, whereas PD98059 increases expression significantly (Figure 3.2. B)

3.3 Effects of MEKi(s) on HLA-DR, HLA class I and PD-L1 Expression of B-Cell Line SAVC

Up to this point, experiments were conducted on MDA-231c10A. Next, we tested to see if these effects were transferrable to an APC line, more specifically, the B cell line SAVC. Cells were treated as above with U0126 (10 μM) or PD98059 (20 μM), and incubated for 72 hours in the absence of IFN-γ. As an APC, SAVC expresses high amounts of HLA class I and HLA-DR without stimulation. Three independent experiments were done and results showed limited effects of MEKi(s) on the HLA and/or PD-L1 expression (Figure 3.3. A, B & C). However, U0126 does decrease in HLA-DR and class I. Constitutive expression of PD-L1 was too low to deduce any trends.

A.


1800
1600
1400
1200
1000
800
600
400
200
0
MFI
*
DMSO+IFNY
U0126(10uM)+IFNY
PD98059(20uM)+IFNY
HLA class I

B.

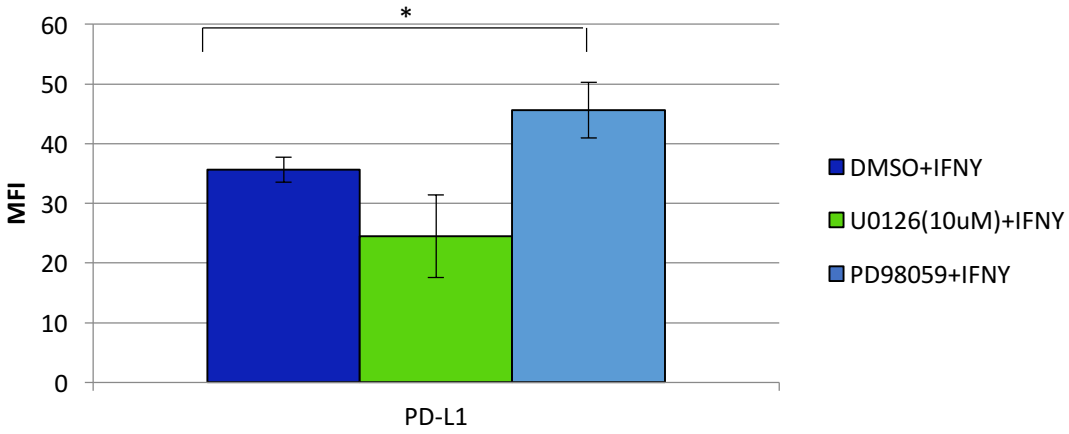
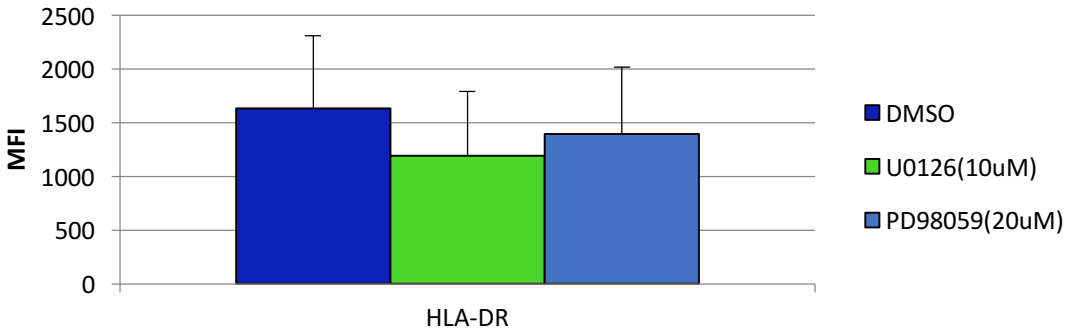

60
50
40
30
20
10
0
MFI
*
DMSO+IFNY
U0126(10uM)+IFNY
PD98059+IFNY
PD-L1

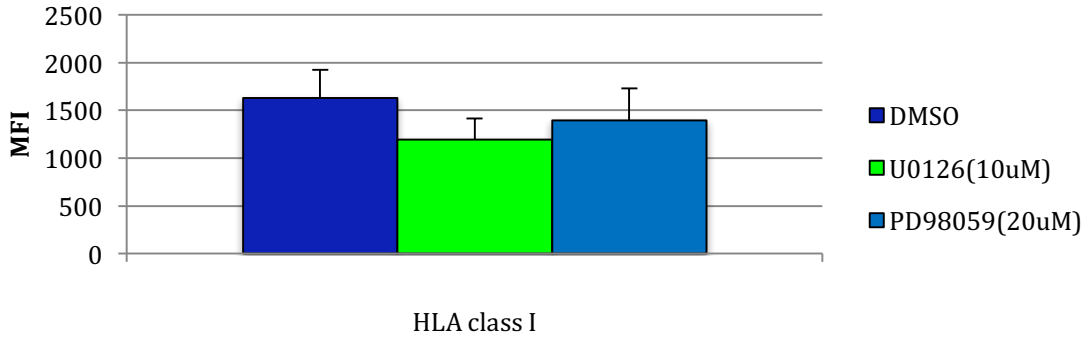
Figure 3.2*:* MEK*i*(s) differently modulate HLA class I and PD-L1 expression.

Flow cytometric analysis of MDA-231c10A treated with U0126 (10 μM) or PD98059 (20 μM) DMSO (vehicle control) in the presence of IFN-γ to determine A) surface HLA class I (W6/32) expression and B) PD-L1 (MIH2) expression. Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Statistical analysis was done by student T-test and asterisks represent p value <0.05.

A.



B.



HLA class I

C.

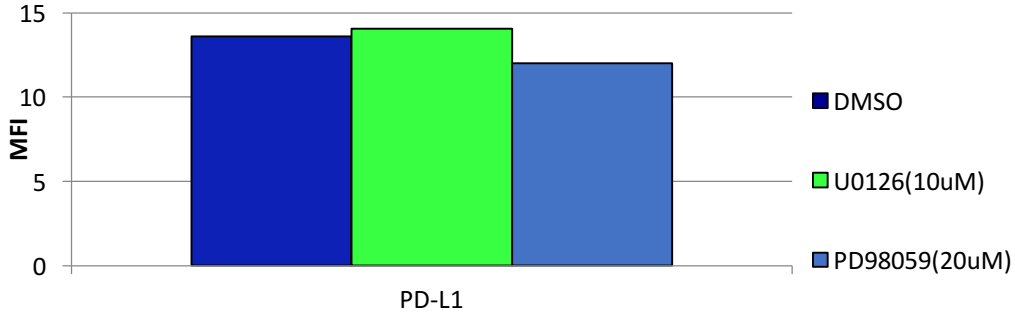


Figure 3.3: MEKi(s) have no significant effect on HLA and PD-L1 expression in the B-cell line, SAVC.

Flow cytometric analysis of SAVC treated with U0126 (10 μM) , PD98059 (20 μM) or DMSO (vehicle control) to determine surface HLA-DR (L243) expression (A), surface HLA class I (W6/32) expression (B) and Surface PD-L1 (MIH2) expression (C). Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Statistical analysis was done by student T-test.

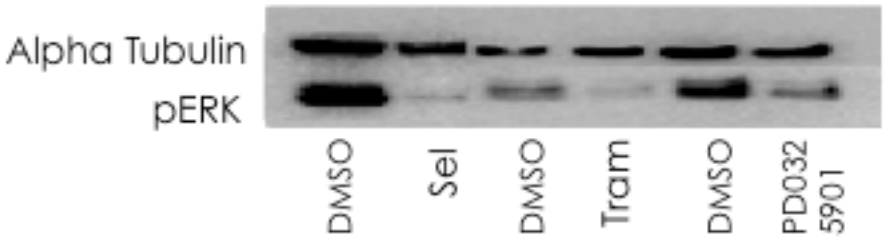
3.4 Viability Assay to Determine Dose Responses for New MEKi

MEK inhibitors Sel, Tram and PD0325901 were evaluated by viability assay to determine EC50 values. MDA-231c10A cells were plated in a 96-well plate and treated with three concentrations of inhibitor in triplicate. The assay was conducted according to the protocol described in Materials and Methods, Section 2.9, using concentrations similar to those recommended by the company and reported in the literature (Table 3.1).

3.4.1 Use of Clinically Relevant MEK Inhibitors Differentially Affect HLA-DR Expression in MDA-231c10A Cells

Since our initial experiments revealed conflicting results with U0126 and PD98059 on HLA-DR, HLA class I and PD-L1 expression, we added three additional MEK*i*(s): Selumetinib (Sel), Trametinib (Tram), and PD0325901. PD0325901 and Tram are pan MEK inhibitors like U0126 whereas Sel, like PD98059, is specific for MEK 1. The efficacy of the inhibitors was assessed by Western blotting (Figure 3.4. A). The effects of the MEK*i*(s) on surface HLA-DR in MDA-231c10A cells was assessed by flow cytometry (Figure 3.4. B). Cells were plated in 6-well plates at a density of $2.5x10^5$ and treated with MEK*i*(s) at the recommended concentrations of 15 nM, 2 nM and 0.5 nM for Sel, Tram and PD0325901 respectively, or DMSO and stimulated with 100 units of IFN-γ 1hr after drug treatment and then incubated for a total of 72 hours. All three inhibitors have limited effect(s) on surface expression of HLA-DR.

A.


Alpha Tubulin
pERK
DMSO
Sel
DMSO
Tram
DMSO
PD032
5901

B.

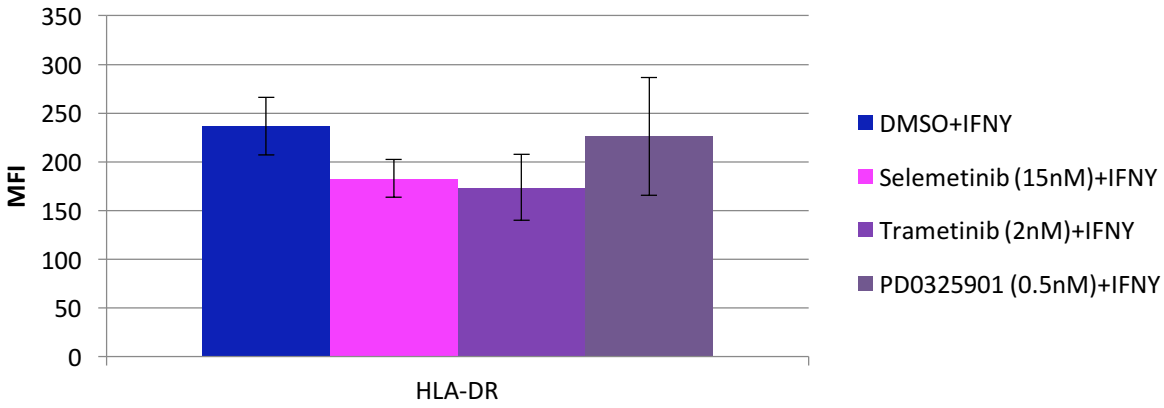

350
300
250
200
150
100
50
0
MFI
HLA-DR
DMSO+IFNY
Selemetinib (15nM)+IFNY
Trametinib (2nM)+IFNY
PD0325901 (0.5nM)+IFNY

Figure 3.4: Analysis of MEK-meditated effects via Western blotting and flow cytometry

A) Western blot of whole cell lysates from MDA-231c10A cells (cultured in MEM) treated with Selumetinib (15 nM), Trametinib (2 nM), PD0325901 (0.5 nM), or DMSO (vehicle control) for 72 hours and then with IFN-γ (100 u/ml) 1hr after MEK*i* treatment. Blots were probed with pERK (Thr202/Tyr204) or alpha tubulin (B-7). B) Flow cytometric analysis of MDA-231c10A treated with Sel (15 nM), Tram (2 nM), & PD0325901 (0.5 nM), or DMSO (vehicle control) for 72 hours and then with IFN-γ (100 u/ml) 1hr after MEK*i* treatment to determine surface HLA-DR (L243) expression. Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Statistical analysis was done by student T-test.

3.5 MEK Inhibitors Differentially Modulate Surface HLA class I and PD-L1 Expression in MDA-231c10A Cells

MDA-231c10A cells were plated and treated with Sel (15 nM), Tram (2 nM), PD0325901 (0.5 nM), or DMSO and stimulated with 100 units of IFN-γ for 72 hours. Surface expression of HLA class I was assessed by flow cytometry. Sel, and Tram show modest increases in HLA class I expression, whereas PD0325901 decreased expression (Figure 3.5). MDA-231c10A does not constitutively express surface PD-L1, however, IFN-γ can increase its expression . No MEK*i*(s) significantly modulated surface expression of PD-L1 in MDA-231c10A treated with IFN-γ (Figure 3.5. B).

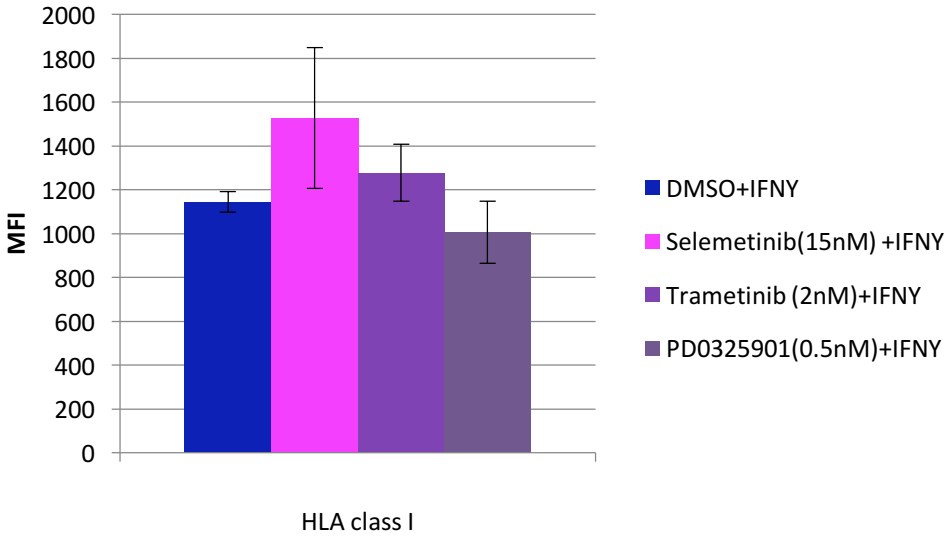
3.6 The Effects of ERK Knockdowns on HLA class I and PD-L1 Surface Expression

Due to the discrepancies in the effects the MEK inhibitors have on HLA and PD-L1 expression on the MDA-231c10A cell line, I decided to use siRNA knockdown of ERK 1 and ERK 2. ERK knockdowns were achieved by transfection of ERK 1 and ERK 2 siRNA three times. MDA-231c10A cells were seeded at $2.5x10^4$ cells/well in a 24 well plate. First transfection was performed at 24 hours after plating cells using serum-free and antibiotic-free IMDM media, transfection buffer and ERK 1 and ERK 2 siRNA. Second and third transfection was conducted at 48 and 72 hours after plating cells, the cells were harvested followed by analysis using flow cytometry and/or Western blotting.

Table 3.1: EC50 values of inhibitors as determined by viability assay

Inhibitor	Experimental Value
Selumetinib	48 hours: 11 nM 72 hours: 17.98 nM
Trametinib	48 hours: 0.64 nM 72 hours:2.98 nM
PD0325901	48 hours: 28.62 nM 72 hours: 0.8 nM

A.


2000
1800
1600
1400
1200
1000
800
600
400
200
0
MFI
HLA class I
DMSO+IFNY
Selemetinib(15nM) +IFNY
Trametinib (2nM)+IFNY
PD0325901(0.5nM)+IFNY

B.

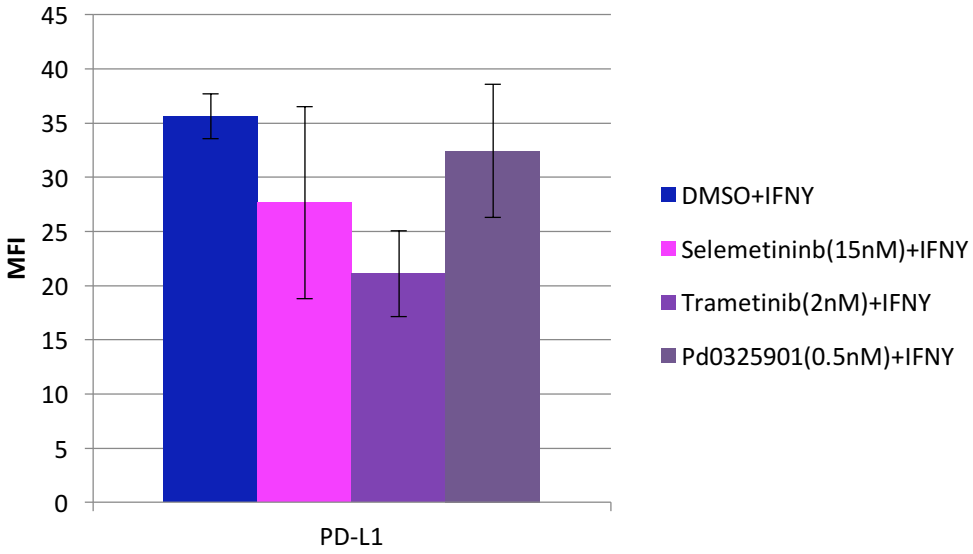

45
40
35
30
25
20
15
10
5
0
MFI
PD-L1
DMSO+IFNY
Selemetininb(15nM)+IFNY
Trametinib(2nM)+IFNY
Pd0325901(0.5nM)+IFNY

Figure 3.5: Flow cytometric analysis of HLA class I and PD-L1 expression in MDA-231 c10A.

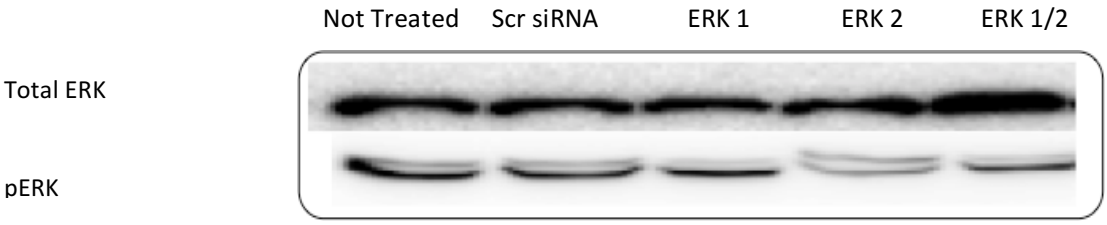
Cells were treated with Selumetinib (15 nM), Trametinib (2 nM), PD0325901 (0.5 nM), or DMSO (vehicle control) and induced with IFN-γ (100 u/ml) 1hour after MEK*i* treatment for a total incubation time of 72 hours. A) Surface HLA class I (W6/32) expression, and B) Surface PD-L1 (MIH2) expression was determined. Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Statistical analysis was done by student T-test.

siRNA knockdowns were conducted in the absence of IFN-γ, therefore HLA-DR was not examined due to low constitutive expression. The results show modest changes in class I (Figure 3.6. B) and PD-L1 expression (Figure 3.6. C), but the differences were not significant as shown by flow cytometry. Western blotting analysis confirmed knockdown of ERK 1 and 2 by siRNA (Figure 3.6. A).

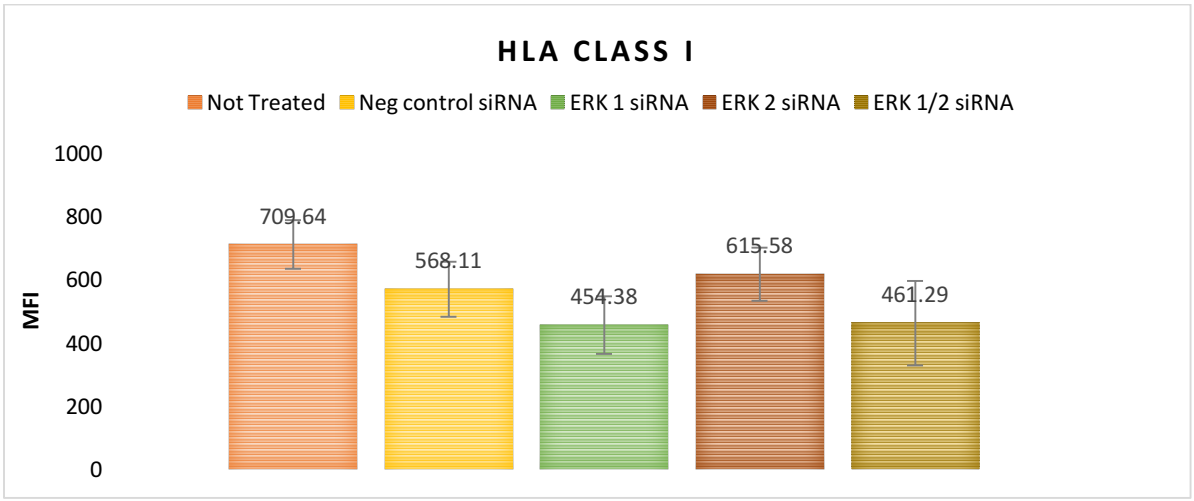
3.7 Determining the Effect(s) of MEKi(s) in Additional TNBC Lines

To test whether the effects of the MEKi (s) on HLA and PD-L1 expression were cell context dependent, we expanded the panel to include MDA-MB-231, BT-20, HS578T, and MDA-MB-468. Details of these cell lines can be seen in Table 2.5 in the materials and methods section. Both MDA-MB-231 and HS578T have a RAS mutation, with MDA-MB-231 having an additional BRAF mutation. BT-20 and MDA-MB-468 have PIK3CA and PTEN mutations respectively. Cells were plated in 6-well plates at a density of $2.5x10^5$ cells/well and treated with Sel, Tram or U0126 at 10 μM, 10 nM and 10 μM respectively for 72 hours. Drugs were used at higher concentrations due to updated literature[70]. We analyzed MEK*i*–mediated effects on constitutive expression levels (left panels of Figure 3.7) and inducible HLA-DR through stimulation with 100 units IFN-γ (Figure 3.7 right panel). The results are depicted as an overlay histogram representation with each coloured line matching the results summarized in a bar graph.

A.


Not Treated
Scr siRNA
ERK 1
ERK 2
ERK 1/2
Total ERK
pERK

B.


HLA CLASS I
Not Treated
Neg control siRNA
ERK 1 siRNA
ERK 2 siRNA
ERK 1/2 siRNA
MFI
1000
800
600
400
200
0
709.64
568.11
454.38
615.58
461.29

C.

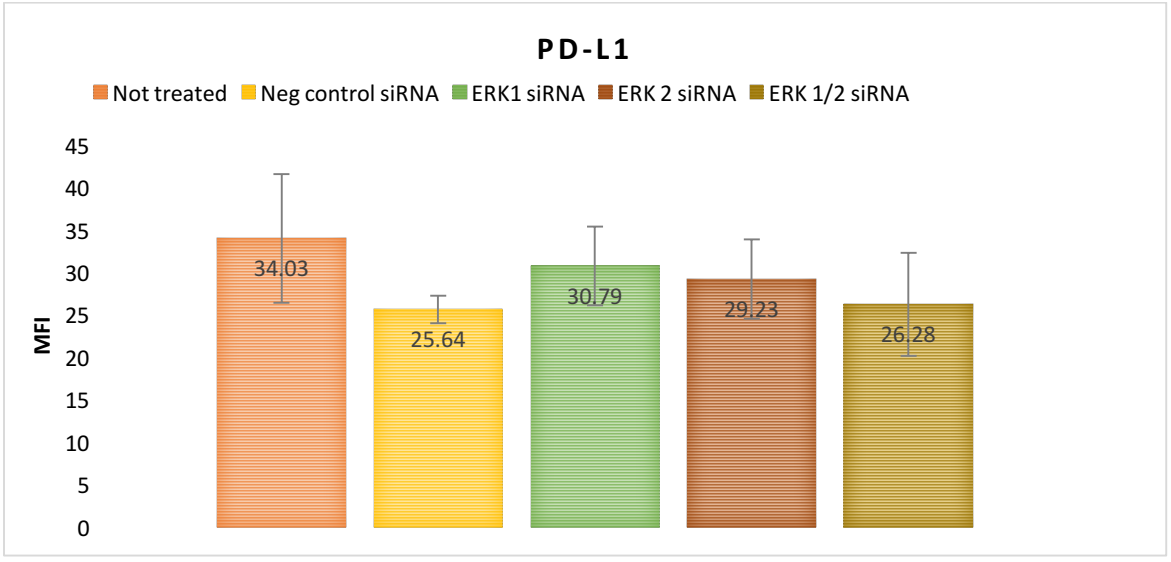

PD-L1
Not treated
Neg control siRNA
ERK1 siRNA
ERK 2 siRNA
ERK 1/2 siRNA
MFI
45
40
35
30
25
20
15
10
5
0
34.03
25.64
30.79
29.23
26.28

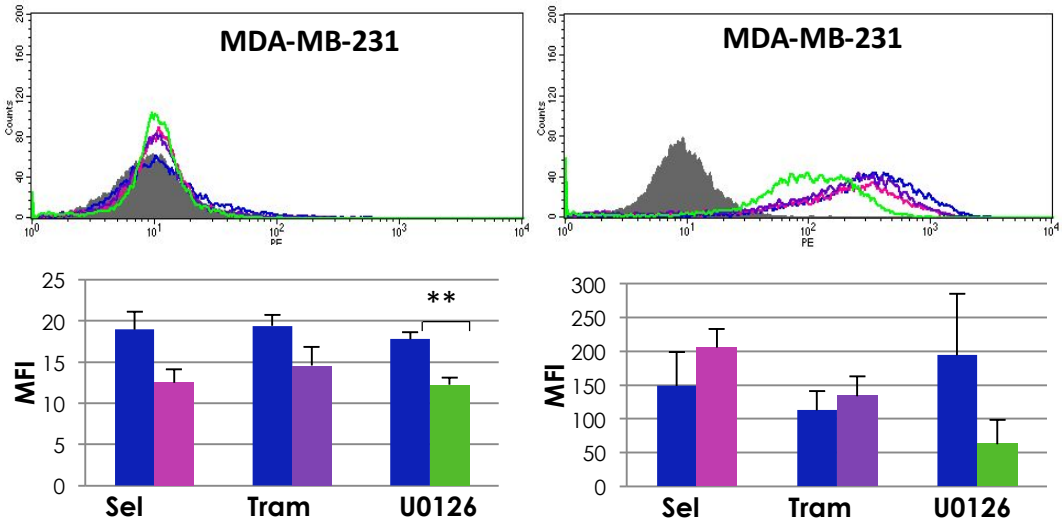
Figure 3.6: ERK silenced MDA-231c10A cells

A) Western blot analysis of MDA-231c10A cells. ERK 1 & 2 were silenced (ERK 1 & ERK 2 siRNA) or not (scrambled siRNA) in MDA-231c10A cells. Whole cell lysates were prepared and probed for total ERK (K-23) with alpha tubulin (B-7) as a loading control. B) Flow cytometry analysis of surface expression of HLA class I (W6/32) and (C) PD-L1 (MIH2) on MDA-231c10A cells treated with or without ERK siRNA oligos. Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Statistical analysis was done by student T-test.

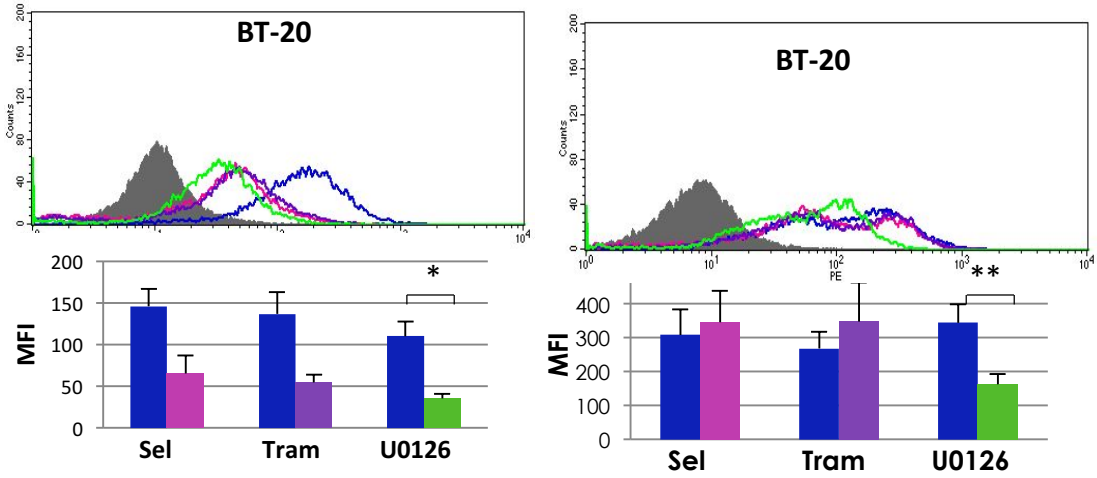
U0126 significantly decreases constitutive HLA-DR (Figure 3.7. A left panel), it also decreases induced HLA-DR expression, but not significantly. Neither Sel or Tram significantly affect constitutive or induced surface HLA-DR expression in MDA-MB-231. U0126 significantly decreases both constitutive and induced HLA-DR expression in BT-20 cells as shown in Figure 3.7. B, whereas neither Sel or Tram significantly impact constitutive or induced HLA-DR expression. Little effect is seen with any inhibitors in HS578T cells for constitutive or induced HLA-DR expression (Figure 3.7. C). Tram significantly decreases HLA-DR expression in MDA MB 468, however this cell line has very little inducible HLA-DR expression (Figure 3.10. A). Due to the low expression of HLA and PD-L1 in MDA-MB-468, constitutive expression was not analyzed.

The MEK*i* effects on constitutive HLA class I expression in MDA-MB-231, BT-20, and HS578T cells are shown on the left side panel(s) in Figure 3.8. A, B and C, whereas the inducible effects of MEK*i*(s) on HLA class I are shown on the right side panel(s) in Figure 3.8. A, B and C, respectively. Constitutive HLA class I was significantly increased in Sel- and Tram-treated MDA-MB-231 cells, whereas U0126 treatment resulted in decreased expression (Figure 3.8. A left panel). Similarly, Sel and Tram treatment increased inducible HLA class I in MDA-MB-231 cells, although this was only significant for Tram-treatment. By contrast, U0126 mediated significantly decreased inducible HLA class I expression. For constitutive expression in BT-20 cells, Tram treatment significantly increased HLA class I (Figure 3.8. B left panel), however when induced, (Figure 3.8. B right panel), HLA class I was significantly reduced by U0126 treatment, but insignificantly increased by Sel and Tram. MEK*i*(s) show limited effects on HS578T cells (Figure 3.8. C

A.


MDA-MB-231
MDA-MB-231
Counts
PE
MFI
**
Sel
Tram
U0126

B.


BT-20
BT-20
Counts
PE
MFI
*
**
Sel
Tram
U0126

C.

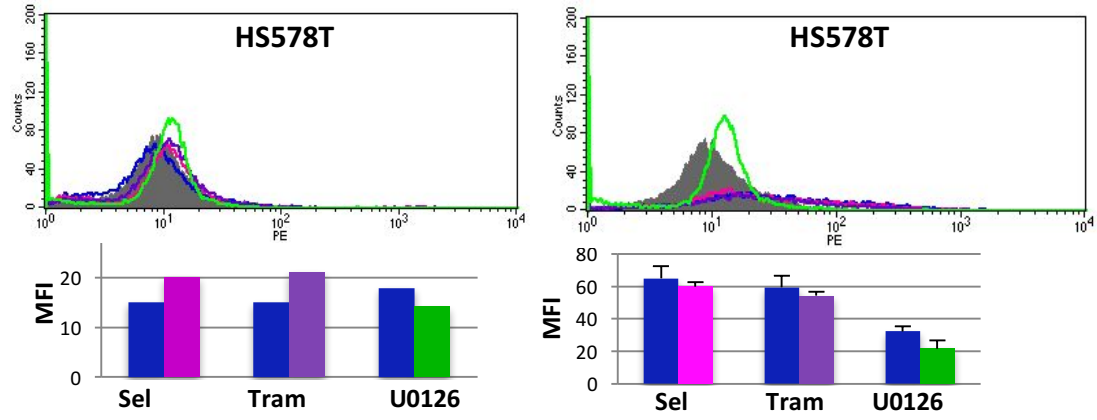

HS578T
HS578T
Counts
PE
MFI
Sel
Tram
U0126

Figure 3.7: Analysis of HLA-DR expression in MEK*i*-treated TNBC cells.

Cells were treated with Selumetinib (10 μM), Trametinib (10 nM), U0126 (10 μM), or DMSO (vehicle control) in absence or presence of IFN-γ (100 U/ml), added 1 hour after MEK*i*(s), for a total incubation of 72 hours. Constitutive (left panels) or Induced (right panels) expression of surface HLA-DR (L243) expression in A) MDA-MB-231 cells B) BT-20 cells and C) HS578T cells were determined by flow cytometry. Constitutive expression in HS578T is the result of one experiment. Histogram overlay is representative of one experiment. In overlays: isotype control, DMSO control, sel, tram and U0126. Bar graphs are colour-matched accordingly. Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Asterisks represent *p* value <0.05 (*) and <0.01 (**) by student T-test.

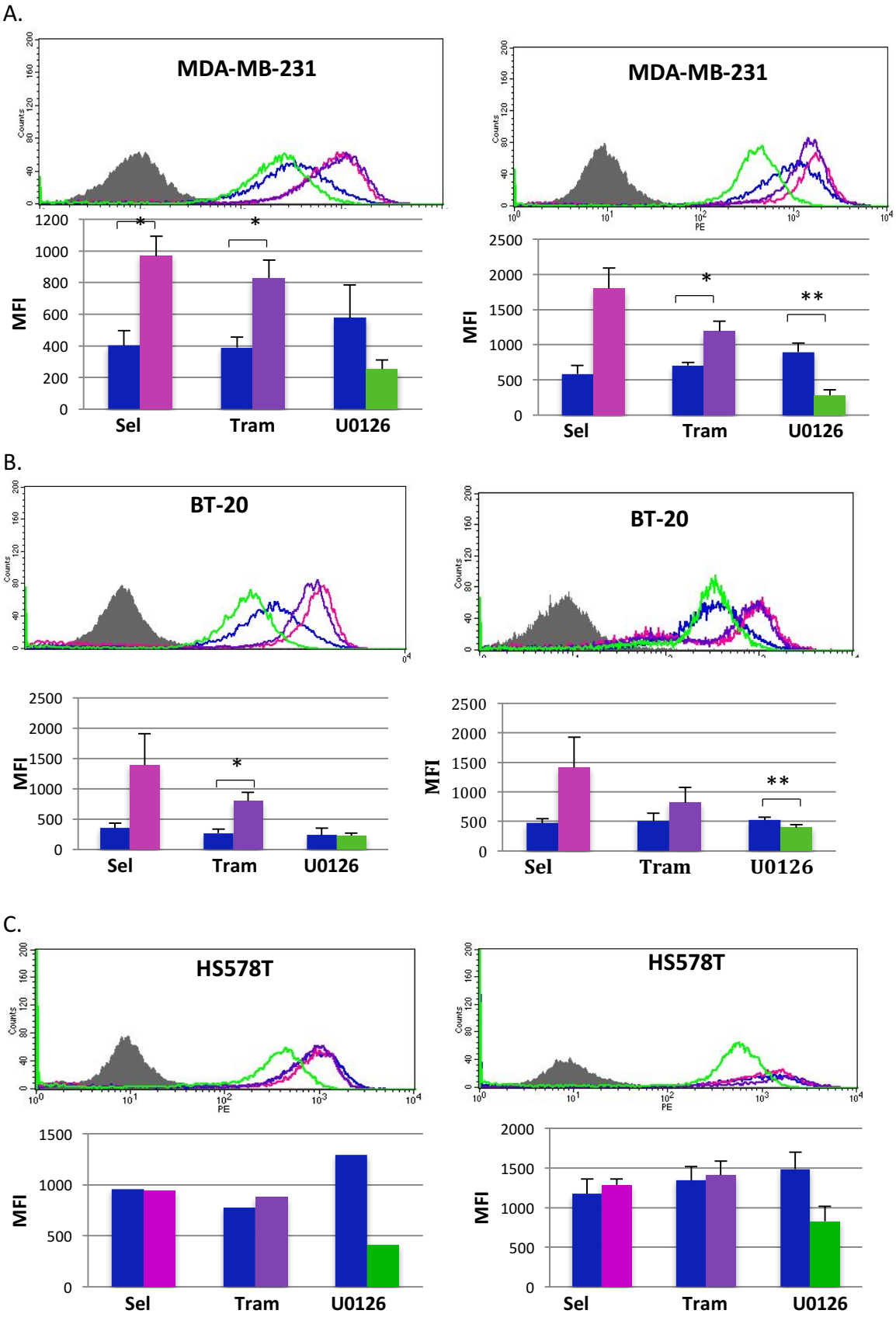

A.
MDA-MB-231
Counts
MFI
Sel
Tram
U0126
MDA-MB-231
PE
MFI
Sel
Tram
U0126
B.
BT-20
Counts
MFI
Sel
Tram
U0126
BT-20
Counts
MFI
Sel
Tram
U0126
C.
HS578T
Counts
PE
MFI
Sel
Tram
U0126
HS578T
Counts
PE
MFI
Sel
Tram
U0126

Figure 3.8: Analysis of HLA class I expression in MEK*i*-treated TNBC cells.

Cells were treated with Selumetinib (10 μM), Trametinib (10 nM), U0126 (10 μM), or DMSO (vehicle control), +/- IFN-γ (100 u/ml), added 1 hr after MEK*i*(s) for a total incubation of 72 hours, to determine constitutive (left panal) or induced (right panels) of surface HLA class I (W6/32) expression in A) MDA-MB-231 cells B) BT-20 C) HS578T cells. Constitutive expression in HS578T is the result of one experiment. Histogram overlay is representative of one experiment. In overlays: isotype control, DMSO control, sel, tram and U0126. Bar graphs are colour-matched accordingly. Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Asterisks represent *p* value <0.05 (*) and <0.01 (**) by student T-test.

left and right panels). In contrast to the other cell lines, MDA-MB-468 significantly decreased inducible HLA class I using all three inhibitors (Figure 3.10. B).

The effects of the MEK*i*(s) on constitutive PD-L1 expression in MDA-MB-231, BT-20 and HS578T cells are shown in the left side panel(s) in Figure 3.9. A, B & C, respectively. U0126 treatment significantly decreased constitutive PD-L1 expression in MDA-MB-231 cells, while both Sel and Tram somewhat decreased levels. Tram and U0126-treated BT-20 cells displayed decreased PD-L1, but the effect was only significant for Tram-treated cells (Figure 3.9. B left panel). All three inhibitors decreased PD-L1 in IFN-γ treated MDA-MB-231 and BT-20, these decreases were significant for U0126 while Tram and Sel mediated significant decreases in MDA-MB-231 and BT-20, respectively (Figures 3.9. A & B right panel(s)). Interestingly, MEK*i*(s) have limited effects on both constitutive and induced PD-L1 expression in HS578T cells (Figure 3.9. C), as well as induced expression in MDA-MB-468 (Figure 3.10. C). These results suggest that although IFN-γ stimulation increases levels of HLA and PD-L1, the effects of MEK inhibition are consistent for both constitutive and induced cells.

3.10 Effects of MEKi(s) on HLA and PD-L1 Expression in Other TNBC Lines as Determined by Western Blotting

Cells plated in 6-well plates at a density of $2.5x10^5$ cells/well were treated with Sel, Tram or U0126 for 72 hours. Whole cell lysates were prepared as described in section 2.6.1 and subjected to Western blot analysis using antibodies against PD-L1, HLA class I, pERK and total ERK.

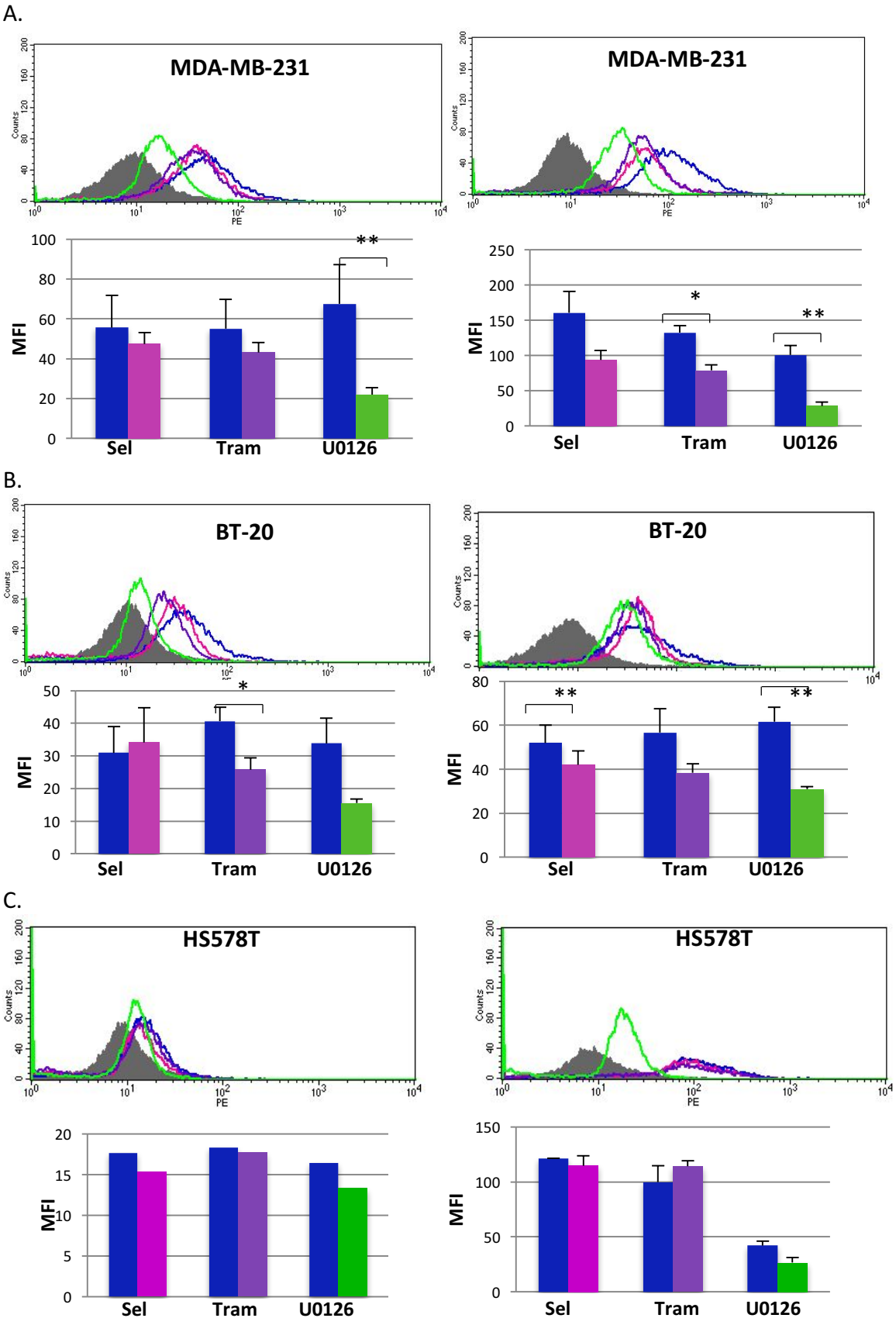
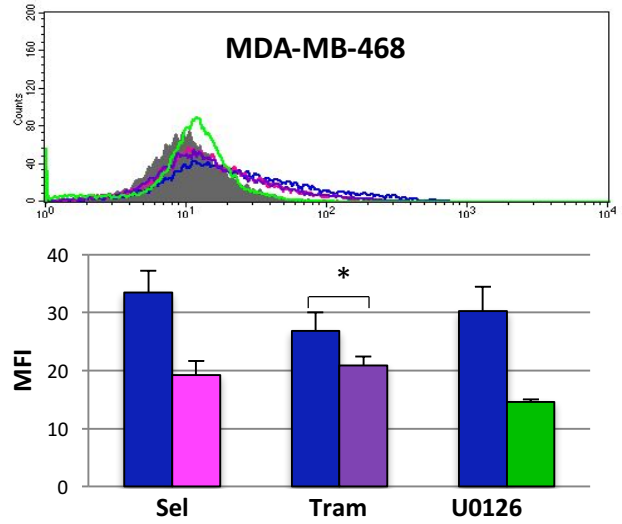

A.
MDA-MB-231
MDA-MB-231
Counts
PE
MFI
Sel
Tram
U0126
B.
BT-20
BT-20
C.
HS578T
HS578T

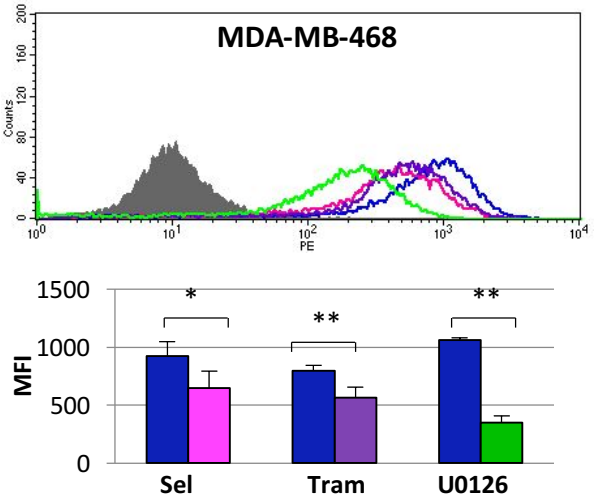
Figure 3.9: Analysis of PD-L1 expression in MEK*i*-treated TNBC.

Cells were treated with Selumetinib (10 μM), Trametinib (10 nM), U0126 (10 μM), or DMSO (vehicle control), +/- IFN-γ (100 u/ml), added 1 hr after MEK*i*(s), for a total incubation time of 72 hours to determine constitutive (left panels) or induced (right panels) to determine surface PD-L1 (MIH2) expression in A) MDA-MB-231 cells B) BT-20 cells and C) HS578T cells. Constitutive expression in HS578T is the result of one experiment. Histogram overlay is representative of one experiment. In overlays: isotype control, DMSO control, sel, tram and U0126. Bar graphs are colour-matched accordingly. Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Asterisks represent *p* value <0.05 (*) and <0.01 (**) by student T-test.

A.


MDA-MB-468
Counts
MFI
*
Sel
Tram
U0126

B.


MDA-MB-468
Counts
PE
MFI
*
**
**
Sel
Tram
U0126

C.

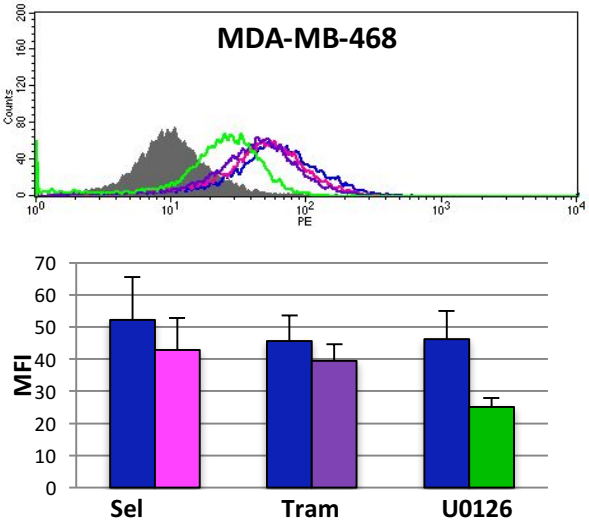

MDA-MB-468
Counts
PE
MFI
Sel
Tram
U0126

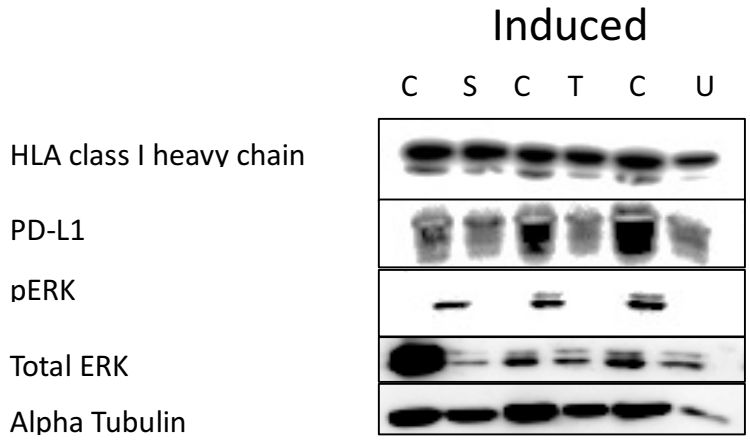
Figure 3.10: Analysis of HLA and PD-L1 expression in MDA-MB-468 cells.

Flow cytometry analysis of cells that were treated with Selumetinib (10 μM), Trametinib (10 nM), U0126 (10 μM), or DMSO (vehicle control) and induced with IFN-γ (100 u/ml) 1hr after MEK*i* treatment, and incubated for 72 hours showing mean fluorescence intensity (MFI) of A) Surface HLA-DR (L243) expression. B) Surface HLA class I (W6/32) expression. C) Surface PD-L1 (MIH2) expression. Histogram overlay is representative of one experiment. In overlays: isotype control, DMSO control, sel, tram and U0126. Bar graphs are colour-matched accordingly. Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Asterisks represent p value <0.05 (*) and <0.01 (**) by student T-test.

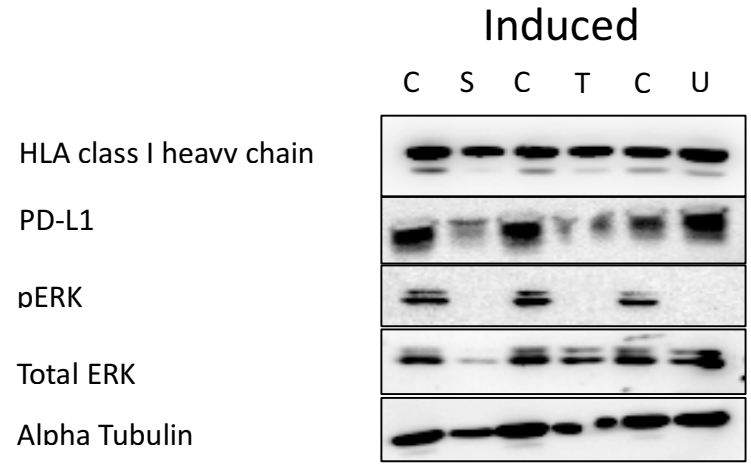
All MEK*i*(s) efficiently inhibited phosphorylation of ERK at 72 hours after treatment in MDA-MB-231 and BT-20, but only partially in HS578T (Figure 3.11. A, B & C). At 72 hours, both MDA-MB-231 and BT-20 show decreased expression in PD-L1 through treatment with all three MEK*i*(s). The effects of U0126 treatment on HLA class I expression in MDA-MB-231 and BT-20 cells as seen through Western blot analysis, do not show a decrease in expression, in contrast to what we have seen in flow cytometry analysis.

Time course experiments were also done at 4, 24 and 48 hours on MDA-MB-231 (Figure 3.12. A) and MDA-MB-468 (Figure 3.12. B) to determine effects of time on regulation of HLA and PD-L1 expression by MEK*i*(s). The results of the time course show that HLA class I increased from 4 to 48 hours in MDA-MB-231, regardless of the inhibitor used (Figure 3.12. A). However, both Sel and Tram treated MDA-MB-231 cells decrease PD-L1 levels at 24 and 48 hours. Although in Sel-treated cells, PD-L1 initially increased at 4 hours before expression decreased as incubation continued. In MDA-MB-468 cells, HLA class I increased from 4 to 48 hours, regardless of inhibitor used, however PD-L1 was not detected (Figure 3.12. B).

A.


Induced
C S C T C U
HLA class I heavy chain
PD-L1
pERK
Total ERK
Alpha Tubulin

B.


Induced
C S C T C U
HLA class I heavy chain
PD-L1
pERK
Total ERK
Alpha Tubulin

C.

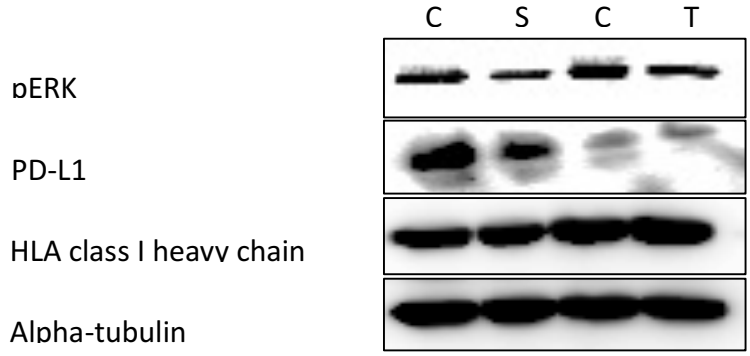
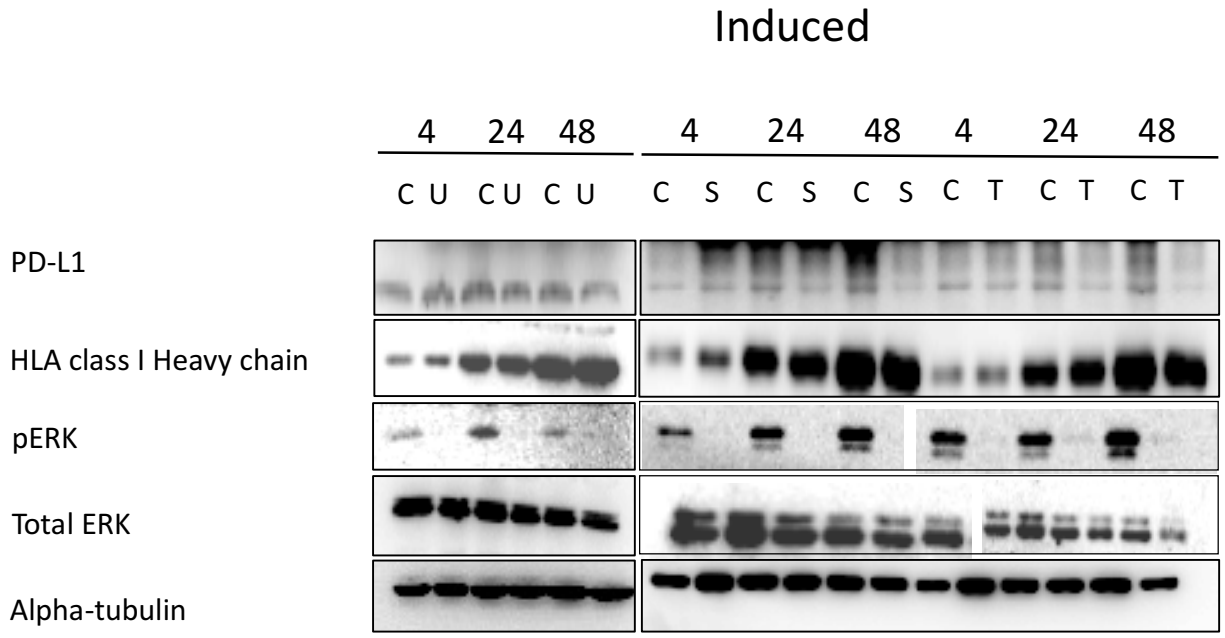

C S C T
pERK
PD-L1
HLA class I heavy chain
Alpha-tubulin

Figure 3.11: Western blot analysis of whole cell lysates from MEK*i*-treated MDA-MB-231, BT-20 and HS578T cells.

A) MDA-MB-231; B) BT-20, and C) HS578T cells treated with Selumetinib (S) (10 μM), Trametinib (T) (10 nM), U0126 (10 μM) or DMSO (vehicle control (C)), and induced with IFN-γ (100 u/ml) 1hr after MEK*i* treatment and incubated for a total of 72 hours. Blots were probed with PD-L1 (E1L3N), HLA class I heavy chain (MUB2037P), pERK (Thr202/Tyr204), total ERK (K-23) and alpha tubulin (B-7). Figure is a representative chosen out of 3 experiments.

A.


Induced
4 24 48 4 24 48 4 24 48
C U C U C U C S C S C S C T C T C T
PD-L1
HLA class I Heavy chain
pERK
Total ERK
Alpha-tubulin

B.

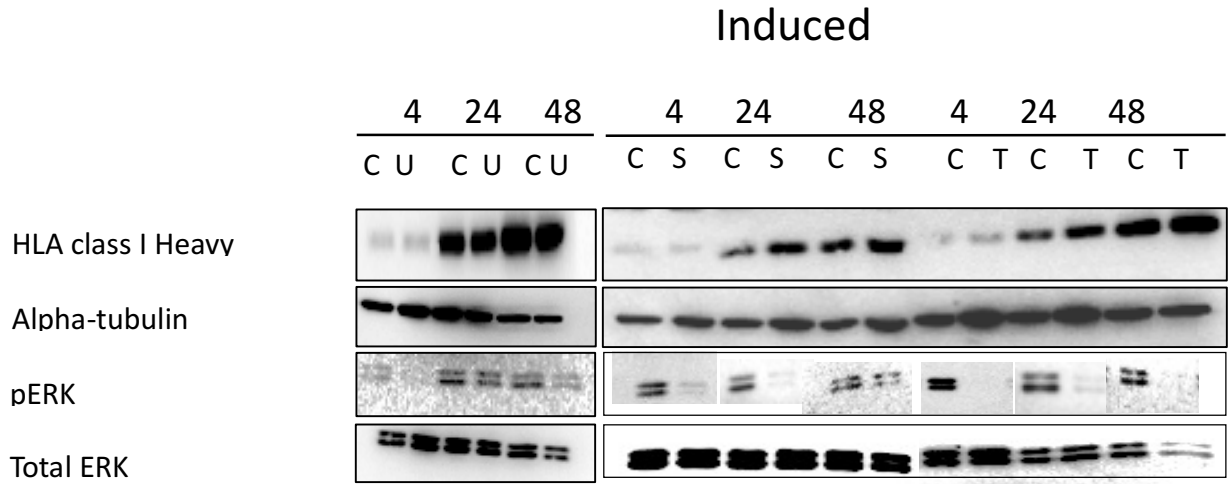

Induced
4 24 48 4 24 48 4 24 48
C U C U C U C S C S C S C T C T C T
HLA class I Heavy
Alpha-tubulin
pERK
Total ERK

Figure 3.12: Western blot analysis of whole cell lysates from MEK*i*-treated MDA-MB-231 and MDA-MB-468 cells.

A) MDA-MB-231 cells and B) MDA-MB-468 cells treated with Selumetinib (S)(10 μM), Trametinib (T) (10 nM), U0126 (U) (10 μM) or DMSO (vehicle control (C)) and treated with with 100 u/ml of IFN-γ 1hr after MEK*i*(s). Cells were incubated for 4, 24 or 48 hours. Blots were probed with PD-L1 (E1L3N), HLA class I heavy chain (MUB2037P), pERK (Thr202/Tyr204), total ERK (K-23) and alpha tubulin (B-7). Figure is a representative chosen out of 3 experiments.

3.11 Effects of MEKi(s) on HLA and PD-L1 Expression in Non-Breast Cancer Lines

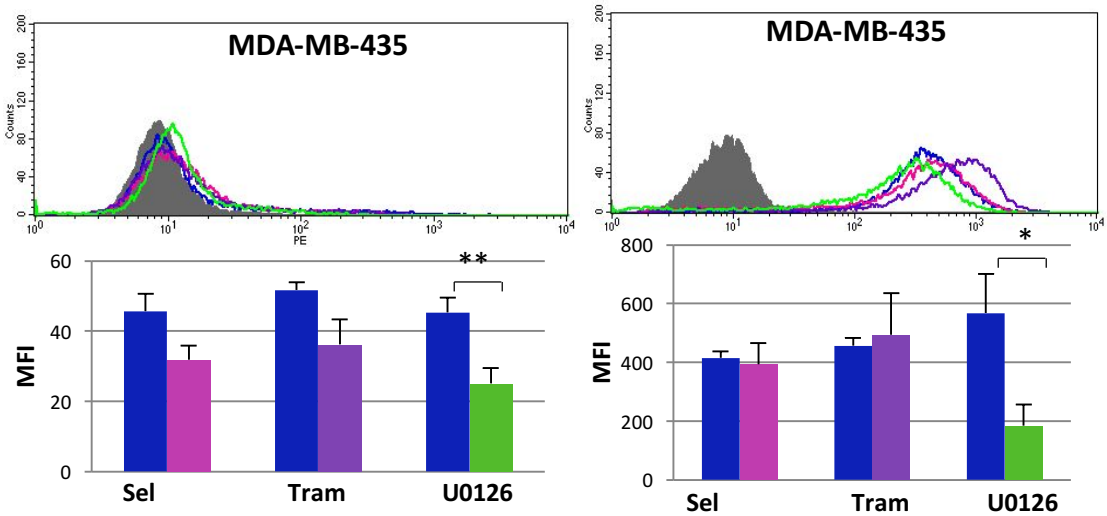
The next question we asked was if these MEK*i*-effects of altered HLA and PD-L1 expression are unique to TNBC lines. We chose the melanoma line MDA-MB-435 because, like MDA-MB-231, it contains a BRAF mutation. The BRAF mutation in MDA-MB-435 is due to the switching of a valine amino acid at position 600 to a glutamic acid[87]. We also chose the colon cancer line HT-29, as it also contains a BRAF mutation[87]. We set up both constitutive and IFN-γ induced experiments and assessed via flow cytometry to determine the effects of inhibition on surface HLA and PD-L1 expression.

U0126 significantly decreases constitutive (left panel) and induced (right panel) HLA-DR expression in MDA-MB-435 (Figure 3.13. A). There are no significant effects of the MEK*i*(s) on induced HLA-DR expression (right panel) in HT-29, whereas the expression of constitutive HLA-DR (left panel) was too low to see any effect (Figure 3.13. B).

U0126 treated MDA-MB-435 cells show significant decreases in constitutive HLA class I (Figure 3.14. A left panel), whereas there are no significant effects seen in the induced cells. However, both Sel and Tram show modest increases in both constitutive and induced expression (Figure 3.14. A). There are no significant MEK*i*-mediated effects seen in either constitutive (Figure 3.14. B left panel) or induced (right panel) expression.

Constitutive expression of PD-L1 was significantly reduced in MDA-MB-435 when treated with U0126 (Figure 3.15. A left panel) while induced expression of PL-D1 was significantly reduced by Tram (Figure 3.15. right panel). However, all three inhibitors in

A.


MDA-MB-435
Counts
PE
MFI
**
*
Sel
Tram
U0126

B.

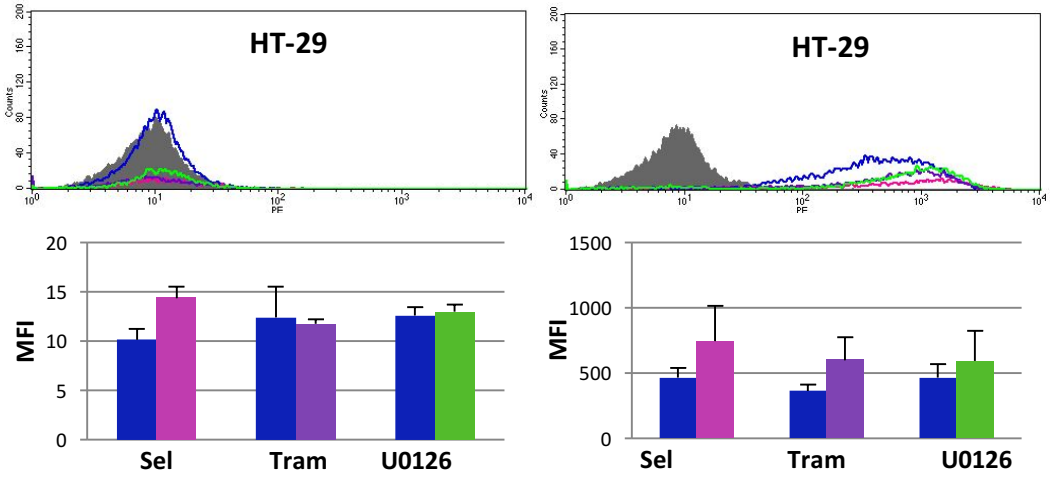

HT-29
Counts
PE
MFI
Sel
Tram
U0126

Figure 3.13: Analysis of HLA-DR expression in MDA-MB-435 and HT-29 cells.
Cells were treated with Selumetinib (10 μM), Trametinib (10 nM), U0126 (10 μM), or DMSO (vehicle control), followed by IFN-γ (100 u/ml) 1hr after MEK*i* treatment for induced expression (right panel), or none, for constitutive expression (left panel). Cells were incubated for a total of 72 hours. Flow cytometry analysis shows surface HLA-DR (L243) expression in A) MDA-MB-435 cells and B) HT-29 cells. Histogram overlay is representative of one experiment. In overlays: isotype control, DMSO control, sel, tram and U0126. Bar graphs are colour-matched accordingly. Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Asterisks represent p value <0.05 (*) and <0.01 (**) by student T-test.

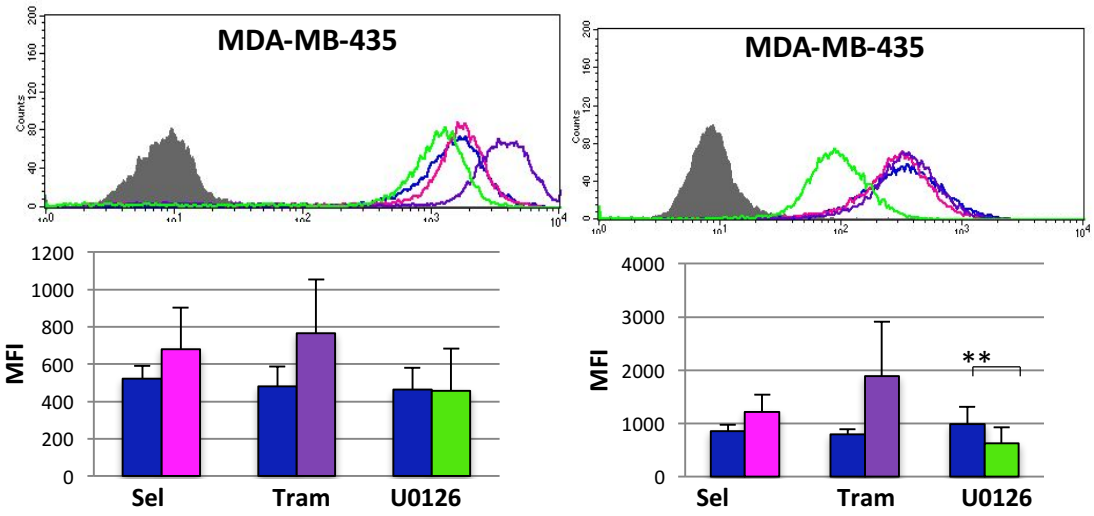
both constitutive and induced experiments show decreases in PD-L1 expression. As was the case for HLA class I expression, no obvious MEK*i*-mediated effects are seen in either constitutive (3.15 left panel) or induced (right panel) HT-29 cells.

3.12 Effects of MEKi(s) on HLA and PD-L1 Expression in MDA-MB-435 and HT-29 as Determined by Western Blotting

MDA-MB-435 and HT-29 cells were plated at $2.5x10^5$ cells/well and treated with Sel (10 μM), Tram (10 nM), U0126 (10 μM), or DMSO as control and induced with 100 u of IFN-γ for 4, 24 and 48 hours. All three MEK*i*(s) inhibited phosphorylated ERK and markedly decreased inducible PD-L1 expression in IFN-γ treated MDA-435 cells (Figure 3.16. A). HLA class I expression was not detectable at 4 hours, but increases at 24 and 48hr time point. Figure 3.16. B shows both induced and constitutive expression at 48hr time point. MDA-MB-435 cells have constitutive ERK expression, but little HLA class I heavy chain is seen. PD-L1 is not shown as there was too little constitutive expression.

To confirm the results in Figure 3.16, I conducted additional experiments with another time point at 72 hours after MEK*i* treatment. In MDA-MB-435, clear increases in HLA class I are seen in lanes treated with inhibitors. Decreases in PD-L1 expression are also seen in inhibitor-treated lanes. (Figure 3.17. A). The effects of MEK inhibition on HT-29 cells show clear decreases in pERK in treated lanes (Figure 3.17. B), but little difference is seen in HLA class I heavy chain expression. There was no PD-L1 detected in HT-29 cells.

A.


MDA-MB-435
Counts
MFI
Sel
Tram
U0126
MDA-MB-435
MFI
**
Sel
Tram
U0126

B.

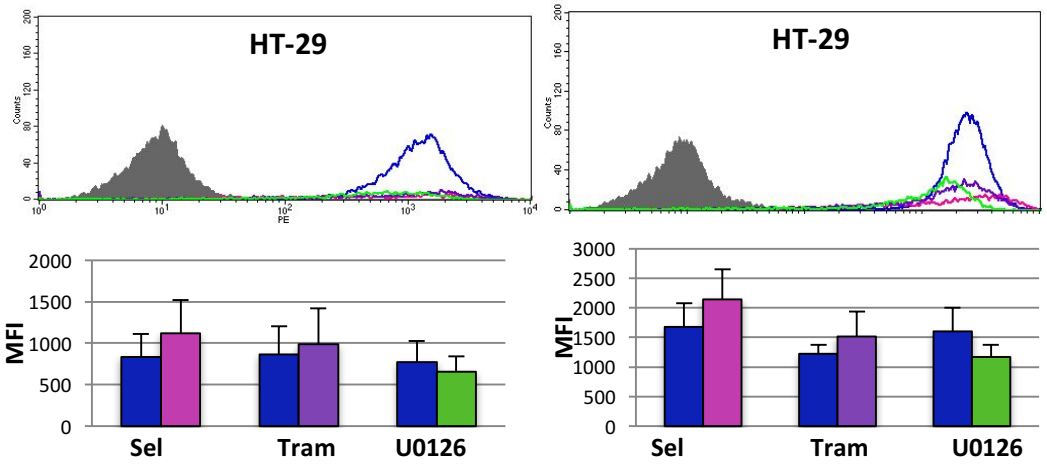
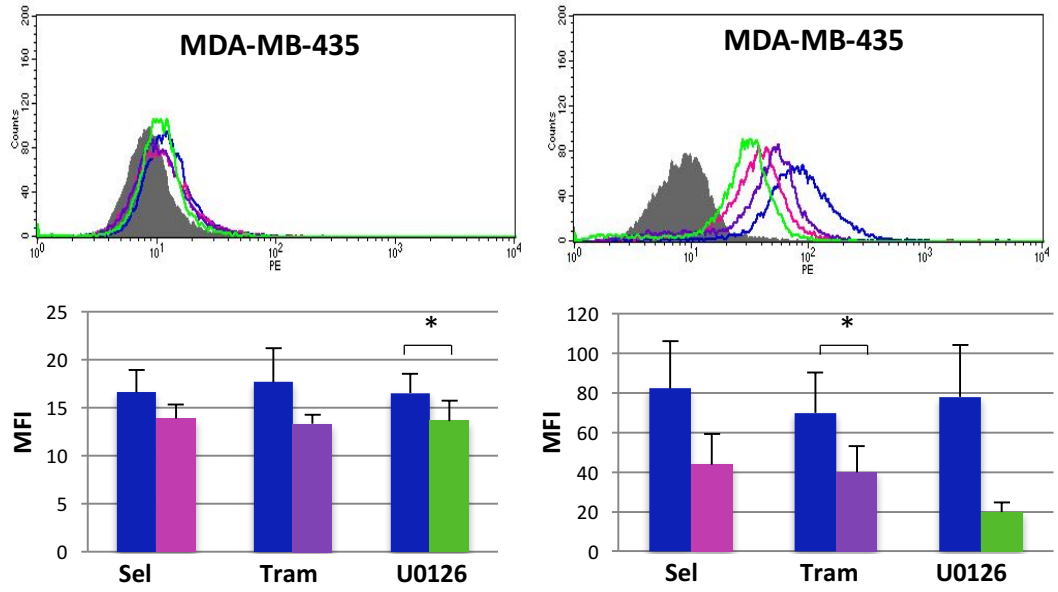

HT-29
Counts
MFI
Sel
Tram
U0126
HT-29
MFI
Sel
Tram
U0126

Figure 3.14: Analysis of HLA class I expression in MDA-MB-435 and HT-29 cells. Cells were treated with Selumetinib (10 μM), Trametinib (10 nM), U0126 (10 μM), or DMSO (vehicle control), with IFN-γ (100 u/ml) 1hr after MEK*i* treatment for induced expression (right panel), or none, for constitutive expression (left panel). Cells were incubated for a total of 72 hours. Flow cytometric analysis shows. Surface HLA class I (W6/32) expression for A) MDA-MB-435 cells and B) HT-20 cells. Histogram overlay is representative of one experiment. In overlays: isotype control, DMSO control, sel, tram and U0126. Bar graphs are colour-matched accordingly. Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Asterisks represent p value <0.05 (*) and <0.01 (**) by student T-test.

A.


MDA-MB-435
MDA-MB-435
Counts
PE
MFI
*
*
Sel
Tram
U0126
Sel
Tram
U0126

B.

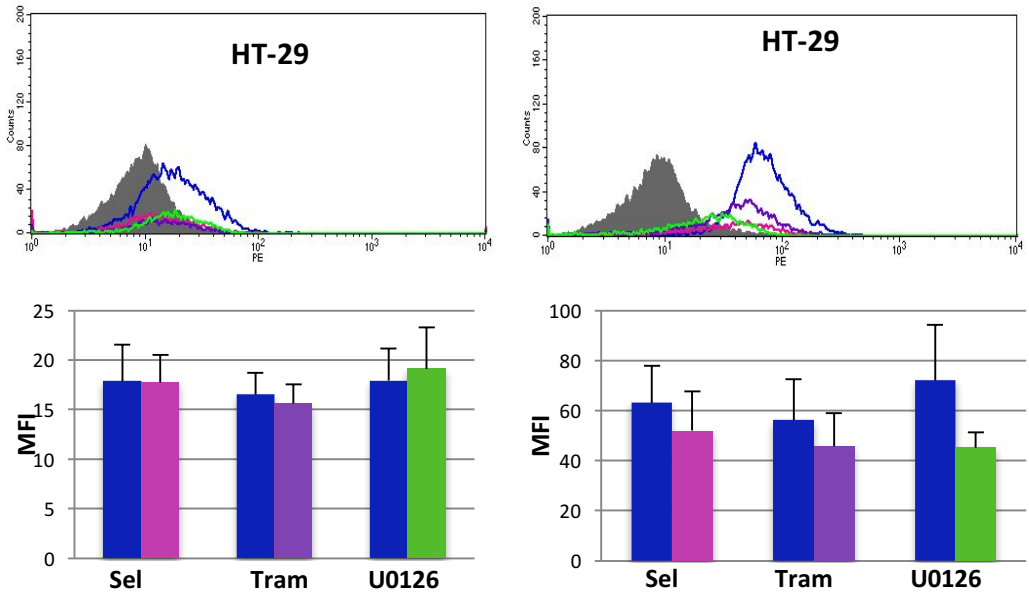
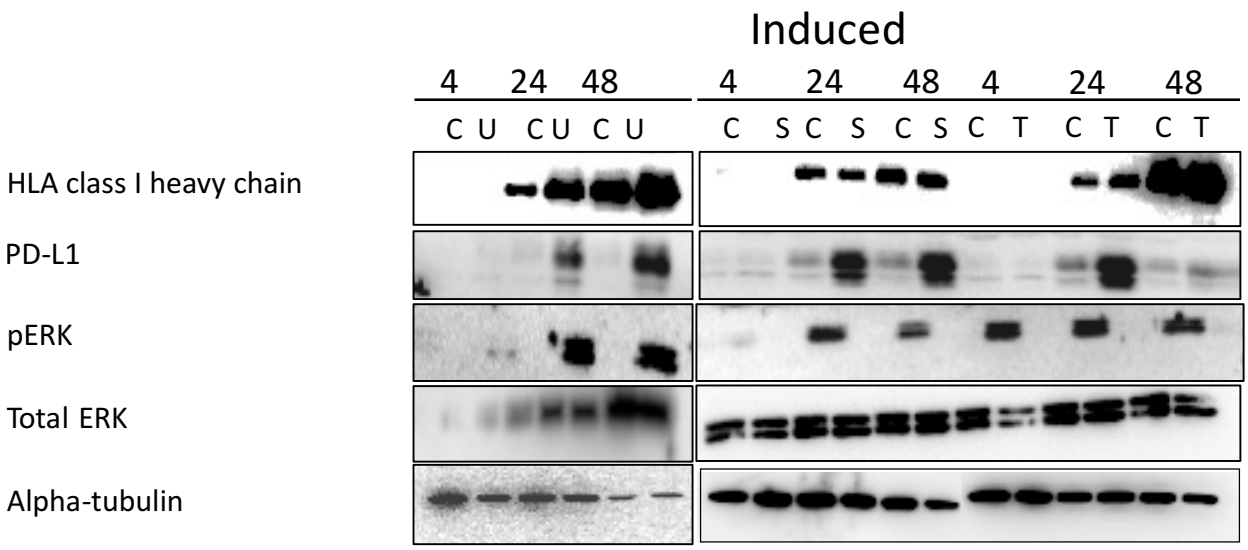

HT-29
HT-29
Counts
PE
MFI
Sel
Tram
U0126
Sel
Tram
U0126

Figure 3.15: Analysis of PD-L1 expression in MDA-MB-435 and HT-29 cells.
Cells were treated with Selumetinib (10 μM), Trametinib (10 nM), U0126 (10 μM), or DMSO (vehicle control), with IFN-γ (100 u/ml) 1hr after MEK*i* treatment for induced expression (right panel), or none, for constitutive expression (left panel). Cells were incubated for a total of 72 hours. Flow cytomtryic analysis shows surface PD-L1 (MIH2) expression for A) MDA-MB-435 cells and B) HT-20 cells. Histogram overlay is representative of one experiment. In overlays: isotype control, DMSO control, sel, tram and U0126. Bar graphs are colour-matched accordingly. Bar graphs represent mean MFI with error bars +/- SEM and are indicative of three independent experiments. Asterisks represent p value <0.05 (*) and <0.01 (**) by student T-test.

A.


Induced
4 24 48 4 24 48 4 24 48
C U C U C U C S C S C S C T C T C T
HLA class I heavy chain
PD-L1
pERK
Total ERK
Alpha-tubulin

B.

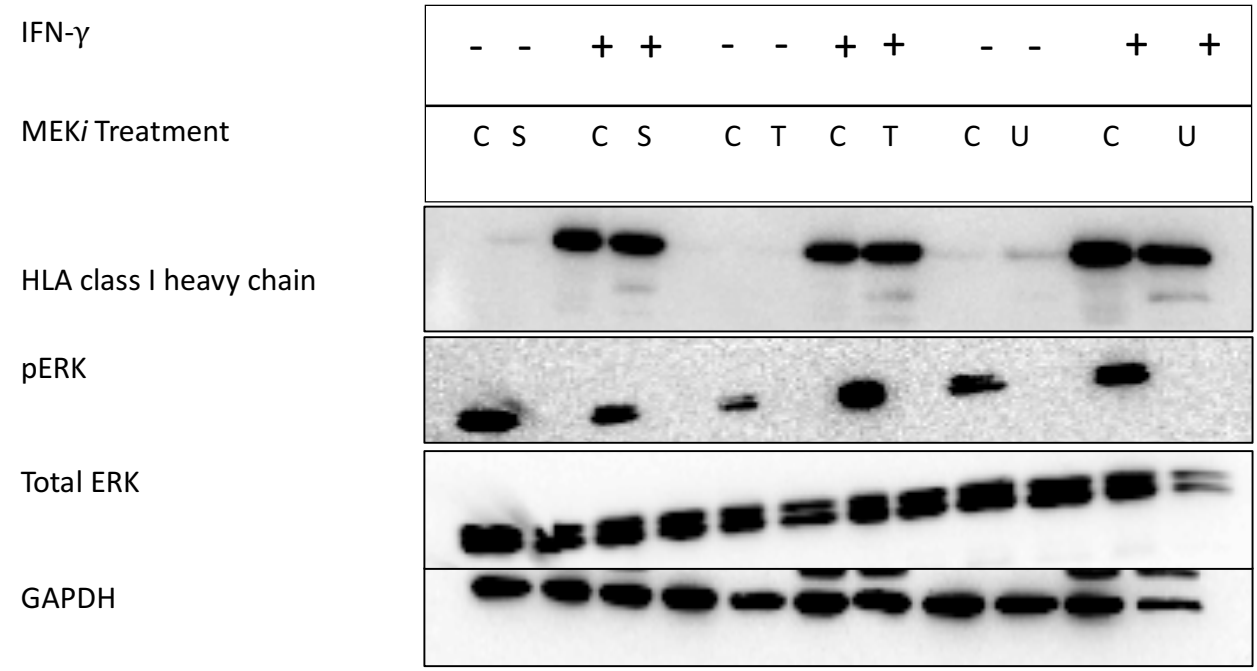
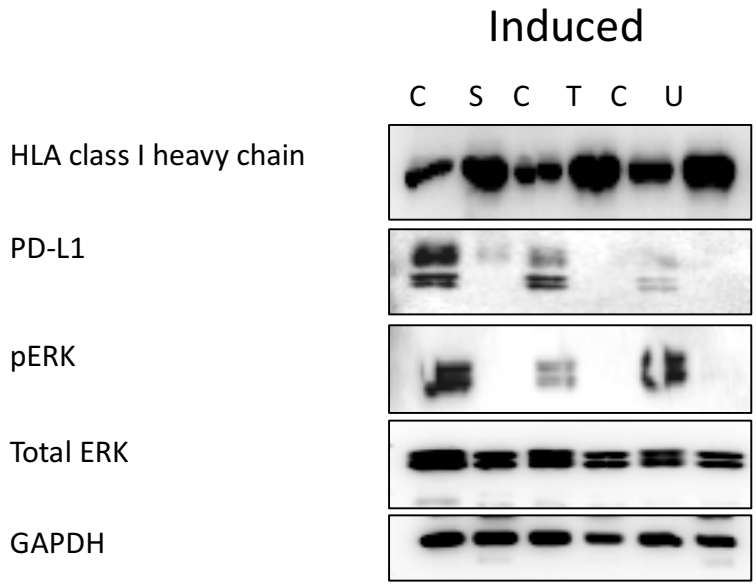

IFN-γ
- - + + - - + + - - + +
MEKi Treatment
C S C S C T C T C U C U
HLA class I heavy chain
pERK
Total ERK
GAPDH

Figure 3.16: Western blot of whole cell lysates from time response of MDA-MB-435 cells.

Cells were treated with Selumetinib (S) (10 μM), Trametinib (T) (10 nM), U0126 (U) (10 μM) or DMSO (vehicle control (C)) and induced or not with IFN-γ (100 u/ml) 1hr after MEK*i* treatment. A) MEK*i*-treated induced MDA-MB-435 cells for 4, 24 and 48hr time points and B) MEK*i*-treated induced and constitutive MDA-MB-435 cells for 48 hours. Blots were probed with PD-L1 (E1L3N), HLA class I heavy chain (MUB2037P), pERK (Thr202/Tyr204), total ERK (K-23) and alpha tubulin (B-7). Figure is a representative chosen out of 3 experiments.

A.


Induced
C S C T C U
HLA class I heavy chain
PD-L1
pERK
Total ERK
GAPDH

B.

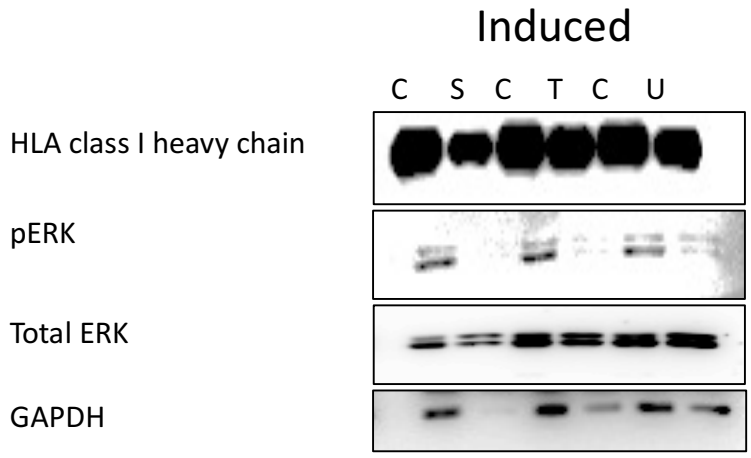

Induced
C S C T C U
HLA class I heavy chain
pERK
Total ERK
GAPDH

Figure 3.17: Western blot data of whole cell lysates from MEK*i*-treated MDA-MB-435 and HT-29 cells.

Cells were treated with Selumetinib (S) (10 μM), Trametinib (T) (10 nM), U0126 (U) (10 μM) or DMSO (vehicle control (C)) and induced with IFN-γ (100 u/ml) 1hr after MEK*i* treatment for a total incubation time of 72 hours. Western blot analysis of MEK*i*-treated A) MDA-MB-435 and B) HT-29 cells. Blots were probed with PD-L1 (E1L3N), HLA class I heavy chain (MUB2037P), pERK (Thr202/Tyr204), total ERK (K-23) and GAPDH (AB8245). Figure is a representative chosen out of 3 experiments.

3.13 Effects of MEKi(s) on HLA class I and PD-L1 Expression in MDA-MB-435 cells as Determined by Immunofluorescence

After obtaining results through flow cytometry and Western blotting, we then treated MDA-MB-435 cells with MEK*i*(s) and analyzed via immunofluorescence. Cells were plated in 8-well chamber slides and treated with U0126 (10 μM), Selumetinib (10 μM) or Trametinib (10 nM) and incubated for 72 hours. Cells were fixed and stained for HLA class I and PD-L1 and analyzed on a Zeiss Axiovision immunofluorescence microscope.

When treated with Tram, there appears to be a higher intensity of HLA class I expression while simultaneously decreasing intensity of PD-L1 (Figure 3.18), confirming the results observed in flow cytometry analysis (Figure 3.14 and 3.15) and Western blot analysis (Figure 3.17)

3.14: U0126 Down-regulates Surface HLA and PD-L1 Expression Consistently Across all Cell Lines Tested.

After the previous flow cytometry experiments have shown that treatment with U0126 consistently decreased surface HLA-DR, HLA class I and PD-L1 expression regardless of the cell line used, a separate statistical analysis was used to compare the effects of each inhibitor to one another (chapter 2.5.2). Results shown in Figure 3.19 show that U0126-treatment decreased surface HLA-DR expression, and its effect are significantly different compared to Sel (Figure 3.19. A).

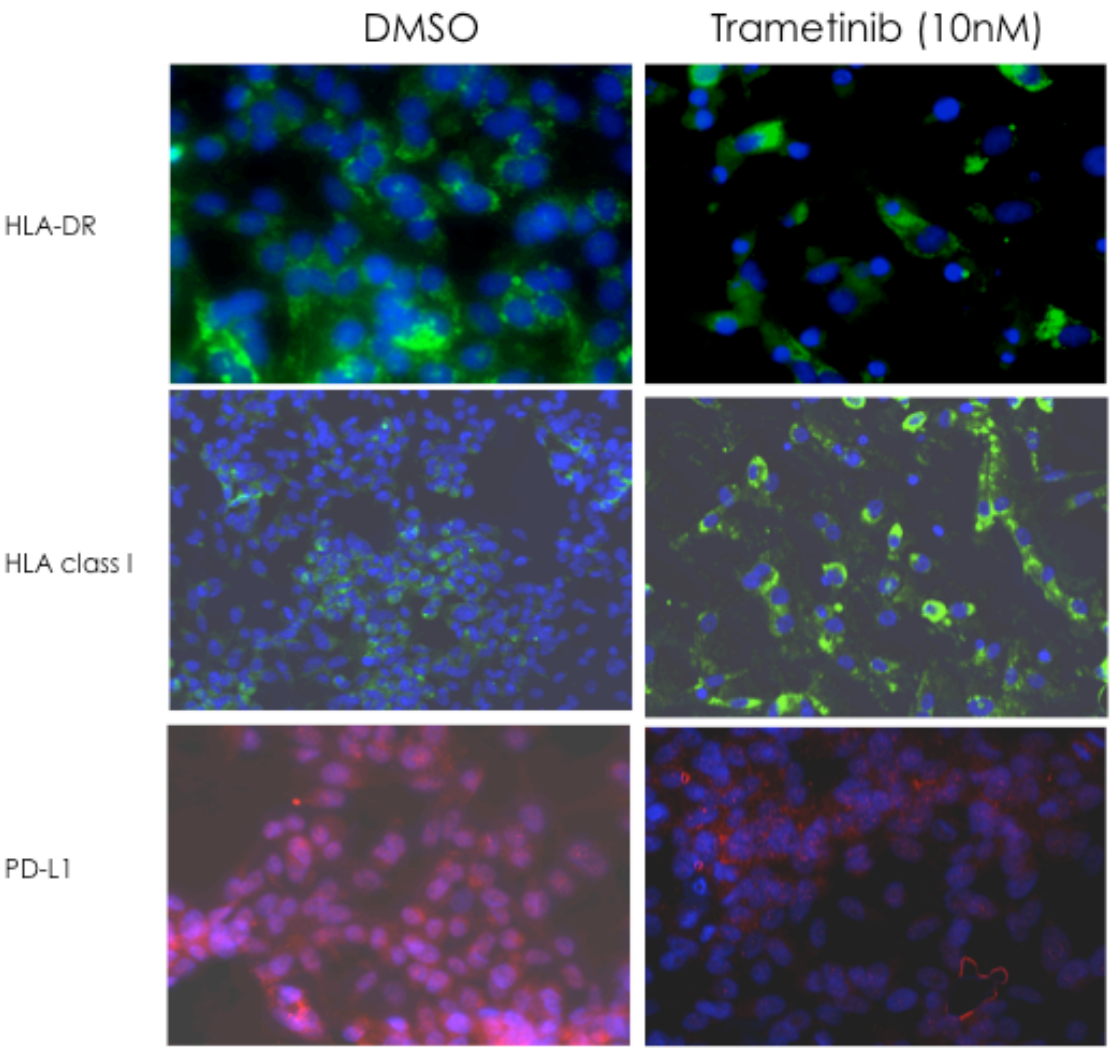
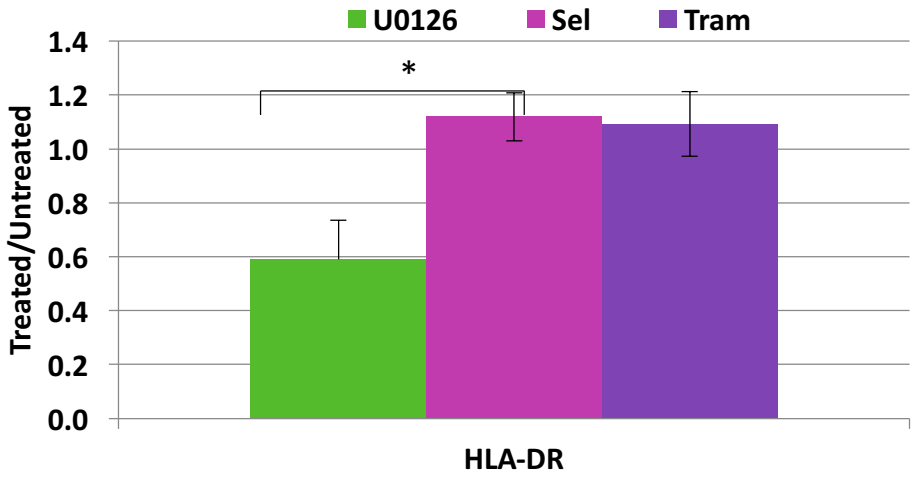

DMSO
Trametinib (10nM)
HLA-DR
HLA class I
PD-L1

Figure 3.18: Trametinib treated cells show increases in HLA class I expression and decreases in PD-L1 expression.

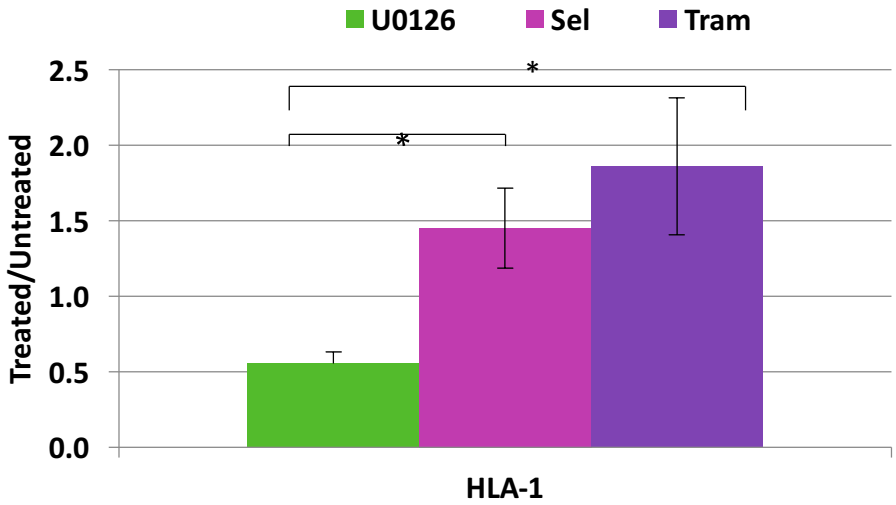
Immunofluoresence of MDA-MB-435 cells treated for 72 hours with DMSO or Trametinib (10 nM) in the presence of 100 units IFN-γ. Cells were methanol permeabilized and paraformaldehyde fixed, and stained with primary antibodies HLA-DR (L243), HLA class I (w6/32) and PD-L1 (E1L3N) and then with Alexa Fluor 488 G2A and Alexa Fluor 555 IgG1 as secondary antibodies with DAPI as blue nuclear stain.

The effects of U0126 treatment on HLA class I are also significantly different than both Sel and Tram (Figure 3.19. B), as well as significantly different compared to Tram in regards to PD-L1 expression (Figure 3.19. C). These results show that U0126 is unique in its effects on HLA and PD-L1 regulation as compared to Sel and Tram.

A.


U0126
Sel
Tram
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Treated/Untreated
HLA-DR

B.


U0126
Sel
Tram
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Treated/Untreated
HLA-1

C.

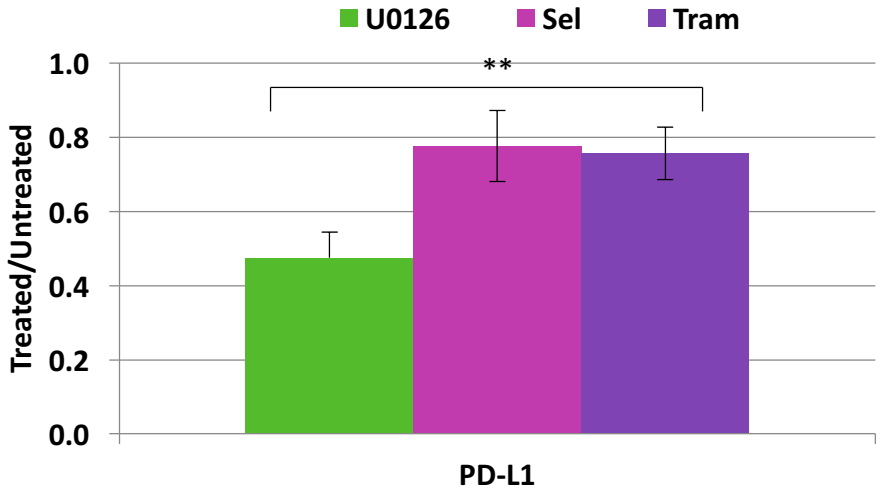

U0126
Sel
Tram
1.0
0.8
0.6
0.4
0.2
0.0
**
Treated/Untreated
PD-L1

Figure 3.19: U0126 modulates HLA and PD-L1 differently than other MEK*i*(S) in all cell lines.

Compilation of MEK-mediated effects of U0126 (10 μM), Selumetinb (10 μM) and Trametinib(10 nM) treatment in 100u/ml IFN-γ induced MDA-MB-231, BT-20, MDA-MB-468, MDA-MB-435, HS578T, and HT-29 cells as analyzed by flow cytometry to determine: A) Surface HLA-DR (L243) expression. B) Surface HLA class I (W6/32) expression. C) Surface PD-L1 (MIH2) expression. Statistical analysis was done by Anova and Tukey HSD test. Bar graphs represent mean MFI. Asterisks represent significance of p value <0.05 (*) and <0.01 (**).

Chapter 4: Discussion

4.1 Results Summary

In the course of this research, we demonstrated that the MAPK pathway plays a role in the regulation of the antigen presenting molecules HLA-DR and HLA class I, as well as the inhibitory molecule PD-L1. Through the use of several MEK inhibitors we have shown that these drugs differentially affect HLA and PD-L1 expression. We confirmed previous research in the Drover laboratory, that U0126-treated MDA-231c10A cells significantly decreased surface HLA-DR expression[77]. Furthermore, this down-regulation was observed in other TNBC lines: MDA-MB-231, BT-20, MDA-MB-468 as well as in the melanoma line, MDA-MB-435, indicating that the effect was not cell-context dependent.

Due to the differential effects of U0126 and PD98059 treatment on HLA-DR expression in MDA-231c10A cells, we questioned what effects they would have on HLA class I, which traffics through the endogenous pathway (Figure 1.3). Furthermore this was of clinical relevance since HLA class I is frequently down-regulated on tumor cells thereby contributing to immune evasion of the tumour[26]. We found that U0126 also down-regulated HLA class I and PD-L1 expression in MDA-231c10A as well as other TNBC and non-TNBC lines showing a stark contrast in its effects on HLA and PD-L1 modulation compared to the other inhibitors as confirmed in Figure 3.19.

Clinically relevant MEK inhibitors Selumetinib and Trametinib generally increased HLA class I and decreased PD-L1 expression in the majority of cell lines tested as assessed by flow cytometry and immunofluorescence, however their effects on HLA-DR

varied. Western blotting showed increased HLA class I intracellularly even in U0126-treated samples, whereas PD-L1 protein levels were decreased in all cell lines tested that showed sensitivity to MEK-inhibition.

The effects of MEK*i*(s) on HLA and PD-L1 expression were consistent in the cell lines used in this research, with Sel and Tram generally increasing surface HLA class I and decreasing PD-L1. In contrast, U0126-mediated effects are significantly different from the others. The mechanism(s) in which these inhibitors modulate expression needs to be addressed.

4.2 MEKi-mediated Effects on MDA-231c10A

The effects of MEK inhibition in MDA-231c10A differ slightly from the parent line MDA-MB-231, For example, although U0126 decreased HLA-DR, HLA class I and PD-L1 in both lines, the effect on HLA-DR was significant for c10A, while the effect on HLA class I and PD-L1 was more pronounced in MDA-MB-231. Similarly, Sel and Tram treatment increased HLA class I and decreased PD-L1 in both lines, but the effects were only significant for MDA-MB-231. These differential effects of the inhibitors are not due to technical issues, as we demonstrated using Western blotting that all inhibitors successfully inhibited phosphorylated ERK (Figure 3.1. A and Figure 3.4. A). Due to these variations, we took a closer look at the differences between both lines.

MDA-231c10A is grown in estrogen-depleted medium and differs from the parent line by lacking ERβ. Although previous research in the Drover laboratory showed that estradiol had little effect on HLA-DR expression in U0126-treated MDA-231c10A

cells[77], I performed a preliminary experiment to explore whether estradiol altered the effects of Sel and Tram on HLA and PD-L1 expression in c10A cells (Appendices 1 and 2). We observed limited effects of estradiol on HLA and PD-L1 expression in MDA-231c10A cells, however, when used in combination with either Sel or Tram the effects on HLA class I and HLA-DR were slightly diminished. While no differences were seen in PD-L1 expression. As this experiment was done once, it is hard to draw any conclusions, however these results support the theory that both Sel and Tram alter HLA and PD-L1 expression through different mechanisms, which could help explain why estradiol diminishes inhibitor-mediated effects on HLA but not PD-L1.

Although Sel and PD98059 are MEK 1-specific, Sel had the opposite effect as PD-L1 was decreased in Sel-treated MDA-231c10A cells, but increased with PD98059 treatment. Similarly, the effects on HLA and PD-L1 by the pan MEK inhibitors, Tram and PD0325901, were not analogous to those mediated by U0126. For example, Tram, unlike U0126, increased HLA class I, but like U0126 it decreased PD-L1-expression. PD0325901 has almost no effect on HLA and PD-L1. Because of these variations, we queried if the mechanisms employed by these inhibitors were similar for some molecules, such as PD-L1, yet varied for others (HLA). This also suggested to us that the effects of the MEK*i*(s) may not be specifically MEK 1- or MEK 2-dependent.

It is currently unclear why U0126 significantly differs from the other MEK*i*(s) in its effects on HLA and PD-L1 modulation. Although a pan MEK inhibitor, the reason for differential effects might be seen further downstream than MEK 1/2. As ERK 1/2 are the only downstream targets of MEK activation and are last in the MAPK pathway (Chapter

1.5), it was anticipated that by knocking down ERK 1 and ERK 2 using small interfering RNA, we might better understand the differential effects of the inhibitors. Due to MDA-231c10A showing moderate levels of both constitutive HLA class I and PD-L1, the experiments were done in the absence of IFN-γ. As the results of the knockdowns compared to the scrambled control siRNA, were not significant, we determined that the inhibitor mediated effects were not due to ERK inactivation. Furthermore, it would have been more relevant to have used MEK-specific siRNA as the inhibitors are MEK-, not ERK-specific and their effects might only be induced at that particular point in the MAPK pathway.

4.3 MEKi-mediated Effects on Other TNBC Lines

To determine whether the effects of MEK*i*(s) on HLA and PD-L1 in MDA-231c10a, were specific for TNBC, we performed the same treatments and experiments on four additional TNBC lines. If the effects of MEK*i*(s) were similar in other lines it would help determine mechanistically the reason for altered expression of HLA and PD-L1. The mutations in each cell line varies, as shown in Table 2.5: MDA-MB-231 has both KRAS and BRAF mutations, BT-20 has a PIK3CA mutation, MDA-MB-468 has a PTEN mutation, and HS578T has a HRAS mutation. Tumours that carry BRAF mutations have been shown to be more sensitive to Sel and Tram[83,89,90,91]. Due to the BRAF mutation expressed in MDA-MB-231, we hypothesized that Sel and Tram would have similar effects on HLA-DR expression in all cell lines that contained this mutation. However, we found that the

effects varied among the cell lines with BRAF mutations, whereas U0126 consistently decreased surface expression.

Both Sel and Tram increased constitutive and induced surface HLA class I expression in MDA-MB-231 and BT-20 despite differences in mutations, whereas both had no effects on HLA class I expression in HS578T. The limited efficacy in HS578T may be due to a high threshold in this cell line for these drugs, as some cell lines require higher concentrations. This was shown in a paper by Loi and colleagues (2015) that large concentrations of Trametinib were needed to inhibit phosphorylated ERK in the mouse tumor lines AT3ova and 4T1[8]. This potentially high threshold for Sel or Tram-mediated effects on HLA class I in HS578T appeared to be consistent with Western blot analysis, showing that lanes with Sel and Tram incompletely inactivated phosphorylated ERK at the 72hr time point.

The effects of MEK*i*(s) on MDA-MB-468 show stark contrasts, as it was the only cell line to show decreased HLA class I expression with inhibitor treatment other than U0126. Explanations for this could be its PTEN mutation, which could impact the inhibitor-mediated effects as links between the loss or mutation in PTEN and the induction of PD-L1 has been reported in prostate cancer cells[50,54]. A future direction would be to explore the effects of PI3K pathway inhibition on HLA and PD-L1 expression in MDA-MB-468 cells.

In MDA-MB-231 and BT-20, Sel, Tram and U0126 decreased both constitutive and induced surface PD-L1 and protein levels seen in Western blots. Despite different mutations, both cell lines have similar responses to the inhibitors. Western blots are

consistent with flow cytometric analysis, which indicates the inhibitors are decreasing both surface and intracellular protein levels. This suggests the inhibitors are not trapping PD-L1 intracellularly as may be the case with HLA.

4.4 MEKi-mediated Effects on Non-BC Lines

To determine if the MEK*i*-mediated effects on HLA and PD-L1 expression were common to cancers other than breast, we included two lines, MDA-MB-435 and HT-29, which also have dysregulated MAPK pathways. MDA-MB-435 has a BRAF mutation, and despite some confusion in the literature as to the source from which this line was originally derived, it is now commonly accepted as a melanoma line[87]. HT-29 is an adenocarcinoma colon cancer line also containing a BRAF mutation[87].

In MDA-MB-435, U0126 consistently decreased surface HLA-DR, HLA class I and PD-L1 expression, whereas Sel and Tram both increased HLA class I expression and decreased PD-L1 as was seen in MDA-MB-231 and BT-20. The effects on MDA-MB-435 are seen in both constitutively and IFN-γ induced cells. In HT-29, MEK inhibition with Sel and Tram similarly increased HLA class I and decreased PD-L1 expression. Western blotting analysis on these lines confirmed results seen from flow cytometry with a decrease in PD-L1 expression at the protein level. Interestingly, as was the case with the BC lines, HLA class I protein was not diminished with U0126 treatment, despite being decreased on the surface. The increase in protein HLA class I in cell lines treated with both Sel and Tram are consistent with surface expression via flow cytometry. This effect

is not novel, and it has been documented in the literature, however mechanisms regarding altered expression are still unknown[8].

4.5 Mechanisms

The mechanism through which U0126 down-regulates HLA-DR has not been fully elucidated, however, in previous Western blot and immunofluorescence analysis of MDA-231c10A cells, intracellular levels of HLA-DR were not decreased, nor were they reduced at transcriptional levels as shown by RT-PCR[77]. Decreased surface HLA-DR expression despite abundant levels of intracellular HLA-DR-proteins could be due to disruption in trafficking or recycling where U0126, either through a MEK-dependent or off-target mechanism, mediates trapping of HLA-DR in endosomal vesicles[92]. To further explore this potential mechanism, additional immunofluorescence analyses using markers for HLA-DR and specific endosomes were performed, but did not allow any clear conclusions (data not shown). Complicating this further was our finding that additional MEK inhibitors had variable effect(s) on HLA-DR in all cells, regardless of MEK-specific targets. These data indicate that mechanistically, U0126 modulates HLA-DR expression differently than the others.

HLA class I down-regulation in U0126-treated cells is not novel; Robertson and colleagues (2006) showed U0126-mediated decreased surface HLA class I expression in HeLa cells, however increased intracellular pools of “trapped” HLA was seen in the treated cells[92]. As U0126 shows similar effects of down-regulation of both HLA-DR and HLA class I, it hints that the mechanism employed for down-regulation is similar for both

molecules with our results reinforcing the potential trafficking mechanism employed by U0126[77,92]. Since the start of this thesis project, the increase in surface levels of HLA class I through treatment with Sel and Tram has been documented in the literature, with Loi and colleagues (2015) showing that treatment with Sel and Tram greatly increased constitutive and induced surface HLA class I expression in mouse model lines of breast cancer[8], whereas Liu et al (2015) showed increases through treatment with both Tram alone or in combination with the BRAF inhibitor Dabrafinib in human melanoma lines[93]. Liu and colleagues found this increase was associated with increases in apoptotic markers as well as down-regulation of immunosuppression factors. Additionally, Hu-Lieskovan and colleagues (2015) also saw increases in HLA class I through MEK inhibition with Tram treatment in a mouse BRAF V600E melanoma model[94]. Due to the general agreement that down-regulation of HLA class I leads to poorer prognosis and tumor escape[26,95], these results indicate increased chance of tumor destruction and decreased growth via treatment with MEK inhibitors. Incidentally, when observing micro dissected tumour regions, Garrido and colleagues (2012) showed that high levels of HLA class I gene expression could be observed in regression but not in progressing metastasis[95]. This finding supports that modulations in HLA class I on tumour cells could have therapeutic effects[96]. Since hyperactivation of MAPK allows cells to proliferate uncontrollably, it may be that negative regulation of HLA class I could be a mechanism employed by MAPK pathway-mutated tumor cells to escape immune surveillance. Therefore, by inhibiting the pathway, HLA class I expression is restored.

The mechanism by which MAPK regulates PD-L1 is not fully understood. However, PD-L1 is transcriptionally regulated by the c-jun protein[47], consequently U0126 works by functionally antagonizing AP-1 transcriptional activity, which is formed by c-jun and c-fos[70]. Therefore U0126 may interfere with PD-L1 at the transcriptional level, which is supported by the decreases in PD-L1 protein levels seen in Western blots of whole cell lysates (WCL) prepared from all cell lines in which the MEK*i*(s) successfully inhibited phosphorylated ERK. Decreases in transcriptional PD-L1 activity via MEK inhibition has been documented, with a study confirming both decreased PD-L1 and increased HLA class I and II in the melanoma lines A375 and sk-MEL-24 via RT-PCR and flow[93]. As these decreases in PD-L1 are also observed with both Sel and Tram treatment, the mechanism for decreased expression may be common to all three inhibitors. However, the effects of MEK inhibition on PD-L1 expression are controversial in the literature with some articles citing decreases in expression, while others cite large increases. Liu et al (2015) found treatment with Tram decreased surface PD-L1 levels in human melanoma lines both in the presence and absence of IFN-γ, however mRNA levels of PD-L1 increased steadily over the course of treatment. This group also found that once cells developed resistance to BRAF inhibition, PD-L1 levels increased[93]. A study by Atef and colleagues (2014) showed that Tram treatment decreased PD-L1 levels, however, induction with IFN-γ had the potential to reverse this effect[97]. Jiang et al (2013) also confirmed down-regulated PD-L1 expression in human melanoma cells through Tram treatment. Jiang's findings also complimented that of Liu with the decreases in PD-L1 expression combined with increases in apoptotic markers[47]. In a conflicting study, Loi and colleagues (2015) found

increasing surface PD-L1 levels with Sel and Tram treated mouse breast tumor lines[8] and Hu-Lieskovan et al (2015) also found increases in PD-L1 expression in a mouse melanoma model after Tram treatment[94].

4.6 How Our Findings Compare to Literature

Through the course of this research, our findings are synonymous with some literature regarding the MEK*i*-mediated effects of HLA and PD-L1 expression, but there are some conflicting reports. The effects of U0126-treatment on HLA class I expression in all cell lines is in agreement with Robertson and colleagues (2006) report of decreased expression in HeLa cells, as well with previous research from the Drover laboratory[77,92]. The increase of HLA class I through treatment with Sel and Tram is in agreement with the studies mentioned above[8,94,98]. Our results were consistent both in the presence and absence of IFN-γ, which is in agreement with several studies[8,98]. However, this is not in agreement with Atefi and colleagues (2014)[43].

Our research has shown repeatedly that MEK inhibition decreases both surface expression and protein levels of PD-L1. These data are in agreement with some in the literature[93,97,47], but are in disagreement with others[8,94]. It is worth noting that the concentrations of Tram used in the conflicting paper by Loi and colleagues is much higher than what we use[8], however experiments testing minor as well as log fold changes in concentrations did not perceptibly alter our findings (appendices 3,4, 8 & 9). Even at concentrations well beyond that of clinical relevance, we demonstrated that neither Sel or Tram increased PD-L1 expression in our TNBC or non-TNBC lines.

The conflicting studies regarding MEK inhibition on PD-L1 expression could be cell context dependent, including inhibition-resistant lines, species variations (human lines or mouse lines), as well as the cross-talk present between the MAPK pathway and others such as PI3K/AKT, and although marketed as specific for MEK, these inhibitors could have off target effects. Human breast tumours could also be modified the tumour stage, genetic background as well as the cell milieu in the tumour microenvironment [64,97,99,100]

4.7 Research Limitations

Despite careful attention to planning and executing this study, we recognize that there are some limitations that make it difficult to draw firm conclusions concerning the effects of MEKi on expression of HLA and PD-L1 in TNBC. Our study used eight human cell lines, a relatively small number, thus, additional lines would have increased the accuracy of our findings, especially as they pertain to the TNBC lines. In that regard, it would also have been informative to increase the number of other BCCL, such as ER+ and HER2+ cell lines to more clearly establish whether the modulating effects of MEKi on HLA and PD-L1 were specific for TNBC. We also found that siRNA did not completely knock down ERK 1 and ERK 2, as there were small amounts of ERK proteins still visible in the lanes containing samples treated with the siRNA (Figure 3.6 A). Indeed, the knockdowns with the chemical MEKi were more complete as pERK was barely present in most lines treated with the various inhibitors. Therefore, it would have been more informative to knock down MEK 1 and MEK 2 and to include antibodies that

were also specific for MEK 1 and MEK 2. There were also some issues with the antibodies used for Western blotting, with some showing non-specific bands and high amounts of background, as well as disruptions due to antibodies that were discontinued during the course of our research. As the clinically relevant inhibitors used were relatively new during the course of this research, the concentrations suggested by the manufacturers changed multiple times depending on new literature published. Another apparent limitation is the use of only cell lines, but analyzing tissues from tumour biopsies taken before and after treatment with MEKi, is beyond the scope of this Master's thesis. Access to a tumour bank and human tissue samples could help show the real-world relevance of our work to human breast cancer patients.

4.8 Future directions

Future directions for this research should be centered on understanding the underlying mechanisms responsible for the altered expression of HLA-DR, HLA class I and PD-L1 via MEK*i*(s). Once the mechanisms are elucidated, a greater understanding of tumour cell/T cell interactions within the tumour environment can be gained as well as information regarding potential new opportunities for combination treatments between pathway inhibitors and other treatments.

The effect of MEK inhibition on tumour-infiltrating T cells must also be addressed; Previous studies have shown MEK inhibitors to be immunosuppressive in vitro[101], however Liu and colleagues (2015) found that Tram-treated melanoma cells resulted in a partial/transient inhibition in T cell proliferation, but when treated with a combination of Tram and anti PD-1, TIL levels increased[98]. Also, Loi et al (2015)

suggested that MEK inhibitors can help recruit TILs[8]. This shows that the effects of MEK inhibition on immune cells is complex and context-dependent[93].

Future directions should also include RT-PCR to test whether transcription of PD-L1 is affected by MEK inhibition in TNBC cells, as this will help to understand the mechanism(s) underlying inhibitor-mediated decreases. As there exists discrepancies in MEK-inhibition on PD-L1 expression between studies using in vivo versus in vitro models[8,98], the use of in vivo TNBC models should be assessed to determine is the effects seen in human lines can be translated to animal models.

4.9 Significance

The potential to modulate the expression of immune markers on tumour cells could prove to be therapeutically beneficial for those with treatment-limited cancers, such as TNBC. Through hyperactivation of the MAPK pathway, cell growth and proliferation go unchecked, leading to tumour growth and metastasis[6,8,74,97]. Included in this rampant cellular proliferation, immune-escape mechanisms are also employed to down-regulate antigen presenting markers such as HLA class I[26]. Through inhibition of the MAPK pathway, the ability to increase surface HLA class I could aid in growth inhibition and tumour destruction. In addition to the increase in antigen presenting markers, the decreased expression of inhibitory molecules such as PD-L1 could exaggerate the chances of tumour destruction through decreased T cell inhibition and activation.

Selumetinib is currently being tested in 20 ongoing clinical trials[70]; and Trametinib is already FDA-approved in combination with an AKT inhibitor for the treatment of melanoma[70,86,90,102]. With our results with Sel and Tram consistently increasing HLA class I and decreasing PD-L1 levels across multiple cell lines, there exists the possibility that these drugs may also have therapeutic benefits in TNBC.

Chapter 5: References

1. *Breast Cancer Statistics*. at <http://www.cancer.ca/en/cancer-information/cancer-type/breast/statistics/?region=on>
2. Ghebeh, H. *et al.* Expression of B7-H1 in breast cancer patients is strongly associated with high proliferative Ki-67-expressing tumor cells. *Int. J. cancer.Journal Int. du cancer* **121,** 751–758 (2007).
3. Balko, J. M. *et al.* Molecular Profiling of the Residual Disease of Triple-Negative Breast Cancers after Neoadjuvant Chemotherapy Identifies Actionable Therapeutic Targets Molecular Profi ling of the Residual Disease of Triple-Negative Breast Cancers after Neoadjuvant Chemoth. *Cancer Discov.* **4,** 232–45 (2014).
4. Dent, R. *et al.* Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin. Cancer Res.* **13,** 4429–34 (2007).
5. Reis-Filho, J. S. & Tutt, A. N. Triple negative tumours: a critical review. *Histopathology* **52,** 108–118 (2008).
6. Giltnane, J. M. & Balko, J. M. Rationale for targeting the Ras/MAPK pathway in triple-negative breast cancer. *Discov. Med.* **17,** 275–283 (2014).
7. Balko, J. M. *et al.* Molecular profiling of the residual disease of triple-negative breast cancers after neoadjuvant chemotherapy identifies actionable therapeutic targets. *Cancer Discov.* **4,** 232–245 (2014).
8. Loi, S. *et al.* RAS/MAPK Activation Is Associated with Reduced Tumor-Infiltrating Lymphocytes in Triple-Negative Breast Cancer: Therapeutic Cooperation Between MEK and PD-1/PD-L1 Immune Checkpoint Inhibitors. *Clin. Cancer Res.* (2015).

9. Wilmes, L. J. *et al.* High-resolution diffusion-weighted imaging for monitoring breast cancer treatment response. *Acad. Radiol.* **20,** 581–589 (2013).

10. Adams, S. *et al.* Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. *J. Clin. Oncol.* **32,** 2959–2966 (2014).

11. Pieters, J. MHC class II-restricted antigen processing and presentation. *Adv. Immunol.* **75,** 159–208 (2000).

12. Cresswell, P. ss. *Annu. Rev. Immunol.* **12,** 259–293 (1994).

13. Kyi, C. & Postow, M. A. Checkpoint blocking antibodies in cancer immunotherapy. *FEBS Letters* **588,** 368–376 (2014).

14. Bodmer, W. F. The HLA system: structure and function. *J. Clin. Pathol.* **40,** 948–958 (1987).

15. Pratheek, B. M. *et al.* Mammalian non-classical major histocompatibility complex I and its receptors: Important contexts of gene, evolution, and immunity. *Indian J. Hum. Genet.* **20,** 129–141 (2014).

16. Guermonprez, P., Valladeau, J., Zitvogel, L., Théry, C. & Amigorena, S. Antigen presentation and T cell stimulation by dendritic cells. *Annu. Rev. Immunol.* **20,** 621–67 (2002).

17. Pamer, E. & Cresswell, P. Mechanisms of MHC class I--restricted antigen processing. *Annu. Rev. Immunol.* **16,** 323–358 (1998).

18. Hedley, M. L., Urban, R. G. & Strominger, J. L. Assembly and peptide binding of major histocompatibility complex class II heterodimers in an in vitro translation

system. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 10479–10483 (1994).

19. Oldford, S. A. *et al.* Tumor cell expression of HLA-DM associates with a Th1 profile and predicts improved survival in breast carcinoma patients. *Int. Immunol.* **18,** 1591–1602 (2006).

20. Ghosh, P., Amaya, M., Mellins, E. & Wiley, D. C. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature* **378,** 457–462 (1995).

21. Kropshofer, H. *et al.* Editing of the HLA-DR-peptide repertoire by HLA-DM. *EMBO J.* **15,** 6144–6154 (1996).

22. Seliger, B., Maeurer, M. J. & Ferrone, S. Antigen-processing machinery breakdown and tumor growth. *Immunol. Today* **21,** 455–464 (2000).

23. Peters, P. J., Neefjes, J. J., Oorschot, V., Ploegh, H. L. & Geuze, H. J. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* **349,** 669–676 (1991).

24. Reith, W., LeibundGut-Landmann, S. & Waldburger, J. M. Regulation of MHC class II gene expression by the class II transactivator. *Nat. Rev.* **5,** 793–806 (2005).

25. Jabrane-Ferrat, N. *et al.* Effect of gamma interferon on HLA class-I and -II transcription and protein expression in human breast adenocarcinoma cell lines. *Int. J. cancer.Journal Int. du cancer* **45,** 1169–1176 (1990).

26. Garcia-Lora, A., Algarra, I. & Garrido, F. MHC class I antigens, immune surveillance, and tumor immune escape. *Journal of Cellular Physiology* **195,** 346–355 (2003).

27. Koichi Kaneko, 1 Sumiya Ishigami, corresponding author1 Yuko Kijima, 1 Yawara

Funasako, 1 Munetsugu Hirata, 1 Hiroshi Okumura, 1 Hiroyuki Shinchi, 1 Chihaya Koriyama, 2 Shinichi Ueno, 1 Heiji Yoshinaka, 1 and Shoji Natsugoe1. Clinical implication of HLA class I expression in breast cancer. *BMC Cancer* **11,** (2011).

28. Turcotte, S. *et al.* Tumor MHC class I expression improves the prognostic value of T-cell density in resected colorectal liver metastases. *Cancer Immunol Res* **2,** 530–537 (2014).

29. Newman, R. A., Ormerod, M. G. & Greaves, M. F. The presence of HLA-DR antigens on lactating human breast epithelium and milk fat globule membranes. *Clin. Exp. Immunol.* **41,** 478–486 (1980).

30. Moller, P. *et al.* Expression of HLA-A, -B, -C, -DR, -DP, -DQ, and of HLA-D-associated invariant chain (Ii) in non-neoplastic mammary epithelium, fibroadenoma, adenoma, and carcinoma of the breast. *Am. J. Pathol.* **135,** 73–83 (1989).

31. Dadmarz, R., Sgagias, M. K., Rosenberg, S. A. & Schwartzentruber, D. J. CD4+ T lymphocytes infiltrating human breast cancer recognise autologous tumor in an MHC-class-II restricted fashion. *Cancer Immunol. Immunother.* **40,** 1–9 (1995).

32. Armstrong, T. D., Clements, V. K. & Ostrand-Rosenberg, S. Class II-transfected tumor cells directly present endogenous antigen to CD4+ T cells in vitro and are APCs for tumor-encoded antigens in vivo. *J. Immunother. (Hagerstown, Md. 1997)* **21,** 218–224 (1998).

33. Meazza, R., Comes, A., Orengo, A. M., Ferrini, S. & Accolla, R. S. Tumor rejection by gene transfer of the MHC class II transactivator in murine mammary

adenocarcinoma cells. *Eur J Immunol* **33,** 1183–1192 (2003).

34. Concha, A. *et al.* Different patterns of HLA-DR antigen expression in normal epithelium, hyperplastic and neoplastic malignant lesions of the breast. *Eur. J. Immunogenet.* **22,** 299–310 (1995).

35. Whitwell, H. L., Hughes, H. P., Moore, M. & Ahmed, A. Expression of major histocompatibility antigens and leucocyte infiltration in benign and malignant human breast disease. *Br. J. Cancer* **49,** 161–172 (1984).

36. Sheen-Chen, S. M., Chou, F. F., Eng, H. L. & Chen, W. J. An evaluation of the prognostic significance of HLA-DR expression in axillary-node-negative breast cancer. *Surgery* **116,** 510–515 (1994).

37. Mostafa, A. A. *et al.* Activation of ER?? signaling differentially modulates IFN-?? induced HLA-class II expression in breast cancer cells. *PLoS One* **9,** (2014).

38. Schroder, K., Hertzog, P. J., Ravasi, T. & Hume, D. A. Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* **75,** 163–189 (2004).

39. Ramana, C. V, Gil, M. P., Schreiber, R. D. & Stark, G. R. Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. *Trends Immunol.* **23,** 96–101 (2002).

40. Cheng, X. *et al.* The PD-1/PD-L pathway is up-regulated during IL-12-induced suppression of EAE mediated by IFN-gamma. *J. Neuroimmunol.* **185,** 75–86 (2007).

41. Zhang, P., Su, D. M., Liang, M. & Fu, J. Chemopreventive agents induce programmed death-1-ligand 1 (PD-L1) surface expression in breast cancer cells

and promote PD-L1-mediated T cell apoptosis. *Mol. Immunol.* **45,** 1470–1476 (2008).

42. Dong, H. *et al.* Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat. Med.* **8,** 793–800 (2002).

43. Atefi, M. *et al.* Effects of MAPK and PI3K pathways on PD-L1 expression in melanoma. *Clin. Cancer Res.* **20,** 3446–3457 (2014).

44. Liu, J. *et al.* Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-{gamma} and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. *Blood* **110,** 296–304 (2007).

45. Berthon, C. *et al.* In acute myeloid leukemia, B7-H1 (PD-L1) protection of blasts from cytotoxic T cells is induced by TLR ligands and interferon-gamma and can be reversed using MEK inhibitors. *Cancer Immunol. Immunother.* **59,** 1839–1849 (2010).

46. Ritprajak, P. & Azuma, M. Intrinsic and extrinsic control of expression of the immunoregulatory molecule PD-L1 in epithelial cells and squamous cell carcinoma. *Oral Oncol.* **51,** 221–228 (2015).

47. Jiang, X., Zhou, J., Giobbie-Hurder, A., Wargo, J. & Hodi, F. S. The activation of MAPK in melanoma cells resistant to BRAF inhibition promotes PD-L1 expression that is reversible by MEK and PI3K inhibition. *Clin. Cancer Res.* **19,** 598–609 (2013).

48. Hasan, A., Ghebeh, H., Lehe, C., Ahmad, R. & Dermime, S. Therapeutic targeting of B7-H1 in breast cancer. *Expert Opin. Ther. Targets* **15,** 1211–1225 (2011).

49. Wimberly, H. *et al.* PD-L1 expression correlates with tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy in breast cancer. *Cancer Immunol. Res.* (2014).

50. Dong, H., Zhu, G., Tamada, K. & Chen, L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat. Med.* **5,** 1365–1369 (1999).

51. Mahoney, K. M., Freeman, G. J. & McDermott, D. F. The Next Immune-Checkpoint Inhibitors: PD-1/PD-L1 Blockade in Melanoma. *Clin. Ther.* **37,** 764–782 (2015).

52. Ghebeh, H. *et al.* The B7-H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. *Neoplasia* **8,** 190–198 (2006).

53. Soliman, H., Khalil, F. & Antonia, S. PD-L1 expression is increased in a subset of basal type breast cancer cells. *PLoS One* **9,** e88557 (2014).

54. Crane, C. a *et al.* PI(3) kinase is associated with a mechanism of immunoresistance in breast and prostate cancer. *Oncogene* **28,** 306–312 (2009).

55. Dong, H. *et al.* Costimulating aberrant T cell responses by B7-H1 autoantibodies in rheumatoid arthritis. *J. Clin. Invest.* **111,** 363–370 (2003).

56. Seo, S. K. *et al.* Co-inhibitory role of T-cell-associated B7-H1 and B7-DC in the T-cell immune response. *Immunol. Lett.* **102,** 222–228 (2006).

57. Brahmer, J. R. *et al.* Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N. Engl. J. Med.* **366,** 2455–2465 (2012).

58. Pearson, G. *et al.* Mitogen-activated protein (MAP) kinase pathways: regulation

and physiological functions. *Endocr. Rev.* **22,** 153–183 (2001).

59. Herrera, R. & Sebolt-Leopold, J. S. Unraveling the complexities of the Raf/MAP kinase pathway for pharmacological intervention. *Trends Mol. Med.* **8,** S27–31 (2002).

60. Chung, C. & Reilly, S. Trametinib: A novel signal transduction inhibitor for the treatment of metastatic cutaneous melanoma. *Am. J. Health. Syst. Pharm.* **72,** 101–110 (2015).

61. Planz, O. Development of cellular signaling pathway inhibitors as new antivirals against influenza. *Antiviral Res.* **98,** 457–468 (2013).

62. Lefloch, R., Pouyssegur, J. & Lenormand, P. Single and combined silencing of ERK 1 and ERK 2 reveals their positive contribution to growth signaling depending on their expression levels. *Mol. Cell. Biol.* **28,** 511–527 (2008).

63. Sebolt-Leopold, J. S. & Herrera, R. Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat. Rev.* **4,** 937–947 (2004).

64. Saini, K. S. *et al.* Targeting the PI3K/AKT/mTOR and Raf/MEK/ERK pathways in the treatment of breast cancer. *Cancer Treat. Rev.* **39,** 935–946 (2013).

65. Kyriakis, J. M. & Avruch, J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* **81,** 807–869 (2001).

66. Haagenson, K. K. & Wu, G. S. The role of MAP kinases and MAP kinase phosphatase-1 in resistance to breast cancer treatment. *Cancer Metastasis Rev.* **29,** 143–149 (2010).

67. Davies, H. *et al.* Mutations of the BRAF gene in human cancer. *Nature* **417,** 949–954 (2002).

68. Joseph, E. W. *et al.* The RAF inhibitor PLX4032 inhibits ERK signaling and tumor cell proliferation in a V600E BRAF-selective manner. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 14903–14908 (2010).

69. Hoeflich, K. P. *et al.* In vivo antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models. *Clin. Cancer Res.* **15,** 4649–4664 (2009).

70. Selleckchem.

71. Favata, M. F. *et al.* Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* **273,** 18623–18632 (1998).

72. Duncia, J. V. *et al.* MEK inhibitors: The chemistry and biological activity of U0126, its analogs, and cyclization products. *Bioorganic Med. Chem. Lett.* **8,** 2839–2844 (1998).

73. Bayliss, J., Hilger, A., Vishnu, P., Diehl, K. & El-Ashry, D. Reversal of the estrogen receptor negative phenotype in breast cancer and restoration of antiestrogen response. *Clin. Cancer Res.* **13,** 7029–7036 (2007).

74. Oh, A. S. *et al.* Hyperactivation of MAPK induces loss of ERalpha expression in breast cancer cells. *Mol. Endocrinol.* **15,** 1344–1359 (2001).

75. Yao, Y. *et al.* ERK and p38 MAPK signaling pathways negatively regulate CIITA gene expression in dendritic cells and macrophages. *J. Immunol. (Baltimore, Md. 1950)* **177,** 70–76 (2006).

76. Voong, L. N., Slater, A. R., Kratovac, S. & Cressman, D. E. Mitogen-activated protein kinase ERK 1/2 regulates the class II transactivator. *J. Biol. Chem.* **283,** 9031–9039 (2008).

77. Mostafa, A. MECHANISMS INVOLVED IN THE REGULATION OF HUMAN LEUKOCYTE ANTIGEN CLASS II EXPRESSION IN ESTROGEN RECEPTOR ALPHA POSITIVE AND ESTROGEN RECEPTOR ALPHA NEGATIVE BREAST CANCER CELLS. (Memorial University of Newfoundland, 2014).

78. Martins, I. *et al.* Pathologic expression of MHC class II is driven by mitogen-activated protein kinases. *Eur. J. Immunol.* **37,** 788–797 (2007).

79. Mimura, K. *et al.* The MAPK Pathway Is a Predominant Regulator of HLA-A Expression in Esophageal and Gastric Cancer. *J. Immunol. Author Choice* **191,** 6261–6272 (2013).

80. LoRusso, P. M. *et al.* Phase I pharmacokinetic and pharmacodynamic study of the oral MAPK/ERK kinase inhibitor PD-0325901 in patients with advanced cancers. *Clin. Cancer Res.* **16,** 1924–1937 (2010).

81. Gilmartin, A. G. *et al.* GSK1120212 (JTP-74057) is an inhibitor of MEK activity and activation with favorable pharmacokinetic properties for sustained in vivo pathway inhibition. *Clin. Cancer Res.* **17,** 989–1000 (2011).

82. NIH National Cancer Institute. (2016). at <http://www.cancer.gov/about-cancer/treatment/clinical-trials/search/results?protocolsearchid=7686052>

83. Ciombor, K. K. & Bekaii-Saab, T. Selumetinib for the treatment of cancer. *Expert Opin. Investig. Drugs* **24,** 111–123 (2015).

84. Garon, E. B. *et al.* Identification of common predictive markers of in vitro response to the Mek inhibitor selumetinib (AZD6244; ARRY-142886) in human breast cancer and non-small cell lung cancer cell lines. *Mol. Cancer Ther.* **9,** 1985–1994 (2010).

85. Infante, J. R. *et al.* A phase 1b study of trametinib, an oral Mitogen-activated protein kinase kinase (MEK) inhibitor, in combination with gemcitabine in advanced solid tumours. *Eur. J. Cancer* **49,** 2077–2085 (2013).

86. Falchook, G. S. *et al.* Activity of the oral MEK inhibitor trametinib in patients with advanced melanoma: a phase 1 dose-escalation trial. *The Lancet.Oncology* **13,** 782–789 (2012).

87. ATCC.

88. Valledor, A. F. *et al.* Selective roles of MAPKs during the macrophage response to IFN-gamma. *J. Immunol. (Baltimore, Md. 1950)* **180,** 4523–4529 (2008).

89. Dry, J. R. *et al.* Transcriptional pathway signatures predict MEK addiction and response to selumetinib (AZD6244). *Cancer Res.* **70,** 2264–2273 (2010).

90. King, J. W. & Nathan, P. D. Role of the MEK inhibitor trametinib in the treatment of metastatic melanoma. *Future Oncol.* **10,** 1559–1570 (2014).

91. Solit, D. B. *et al.* BRAF mutation predicts sensitivity to MEK inhibition. *Nature* **439,** 358–362 (2006).

92. Robertson, S. E. *et al.* Extracellular signal-regulated kinase regulates clathrin-independent endosomal trafficking. *Mol. Biol. Cell* **17,** 645–657 (2006).

93. Liu, L. *et al.* The BRAF and MEK Inhibitors Dabrafenib and Trametinib: Effects on

Immune Function and in Combination with Immunomodulatory Antibodies Targeting PD1, PD-L1 and CTLA-4. *Clin. Cancer Res.* (2015).

94. Hu-Lieskovan, S. *et al.* Improved antitumor activity of immunotherapy with BRAF and MEK inhibitors in BRAF(V600E) melanoma. *Sci. Transl. Med.* **7,** 279ra41 (2015).

95. Garrido, C. *et al.* MHC class I molecules act as tumor suppressor genes regulating the cell cycle gene expression, invasion and intrinsic tumorigenicity of melanoma cells. *Carcinogenesis* **33,** 687–693 (2012).

96. Wilmott, J. S. *et al.* Dynamics of chemokine, cytokine, and growth factor serum levels in BRAF-mutant melanoma patients during BRAF inhibitor treatment. *J. Immunol.* **192,** 2505–13 (2014).

97. Atefi, M. *et al.* Effects of MAPK and PI3K pathways on PD-L1 expression in melanoma. *Clin. Cancer Res.* **20,** 3446–3457 (2014).

98. Liu, L. *et al.* The BRAF and MEK inhibitors dabrafenib and trametinib: Effects on immune function and in combination with immunomodulatory antibodies targeting PD-1, PD-L1, and CTLA-4. *Clin. Cancer Res.* **21,** 1639–1651 (2015).

99. Atefi, M. *et al.* Reversing melanoma cross-resistance to BRAF and MEK inhibitors by co-targeting the AKT/mTOR pathway. *PLoS One* **6,** e28973 (2011).

100. Dumble, M. *et al.* Discovery of novel AKT inhibitors with enhanced anti-tumor effects in combination with the MEK inhibitor. *PLoS One* **9,** e100880 (2014).

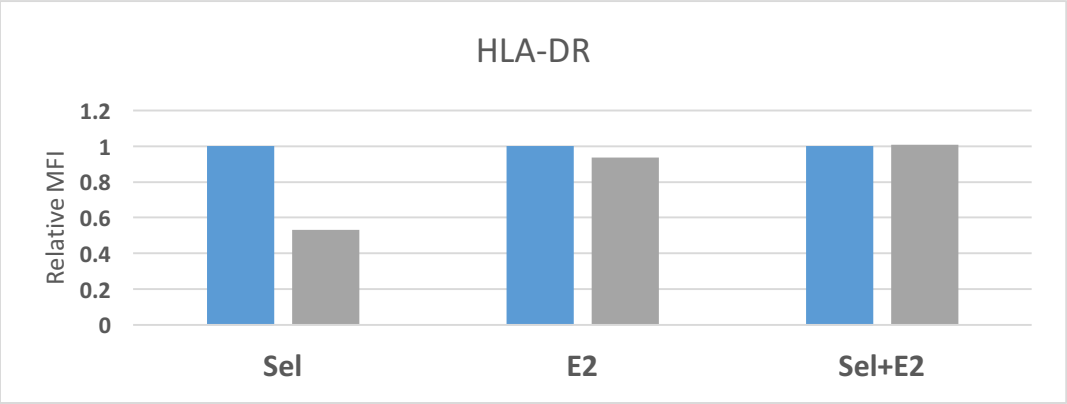
101. Vella, L. J. *et al.* MEK inhibition, alone or in combination with BRAF inhibition, affects multiple functions of isolated normal human lymphocytes and dendritic

cells. *Cancer Immunol. Res.* **2,** 351–60 (2014).

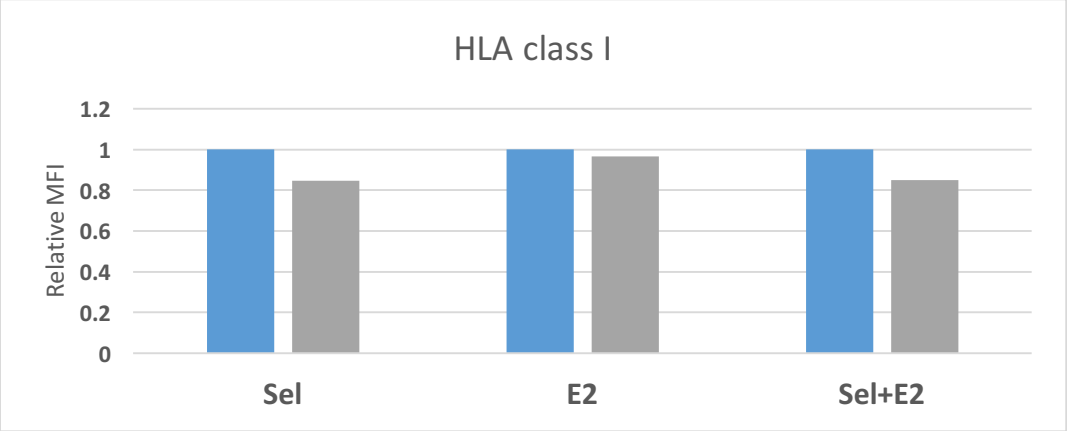
102. Wright, C. J. & McCormack, P. L. Trametinib: first global approval. *Drugs* **73,** 1245–1254 (2013).

Chapter 6: Appendices

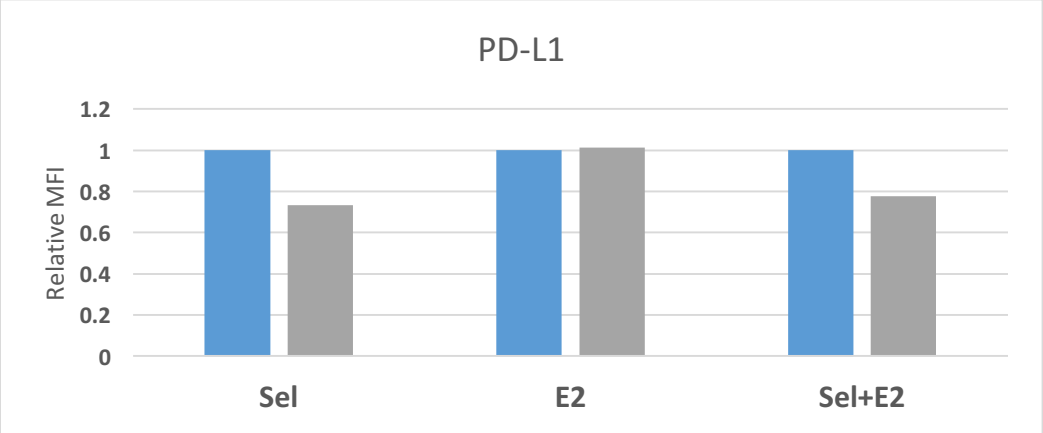
A.


HLA-DR
Relative MFI
1.2
1
0.8
0.6
0.4
0.2
0
Sel
E2
Sel+E2

B.


HLA class I
Relative MFI
1.2
1
0.8
0.6
0.4
0.2
0
Sel
E2
Sel+E2

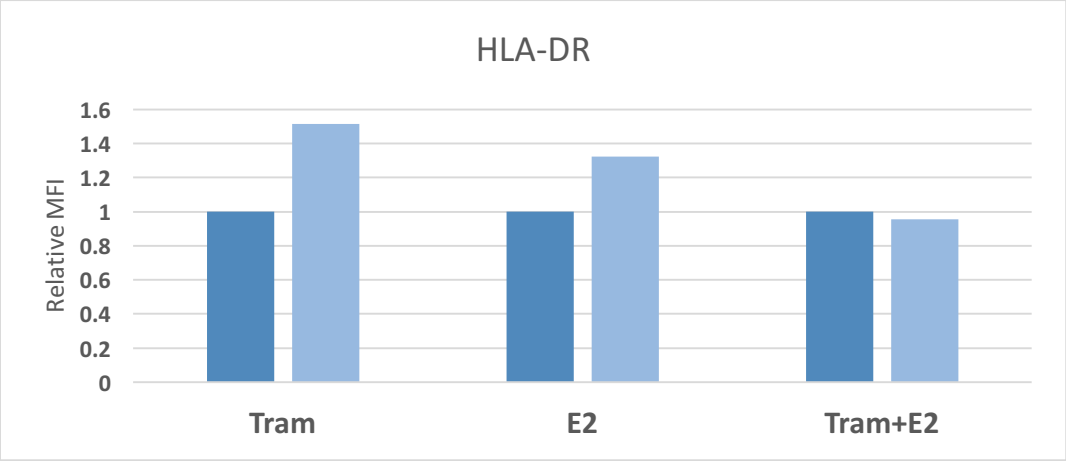
C.


PD-L1
Relative MFI
1.2
1
0.8
0.6
0.4
0.2
0
Sel
E2
Sel+E2

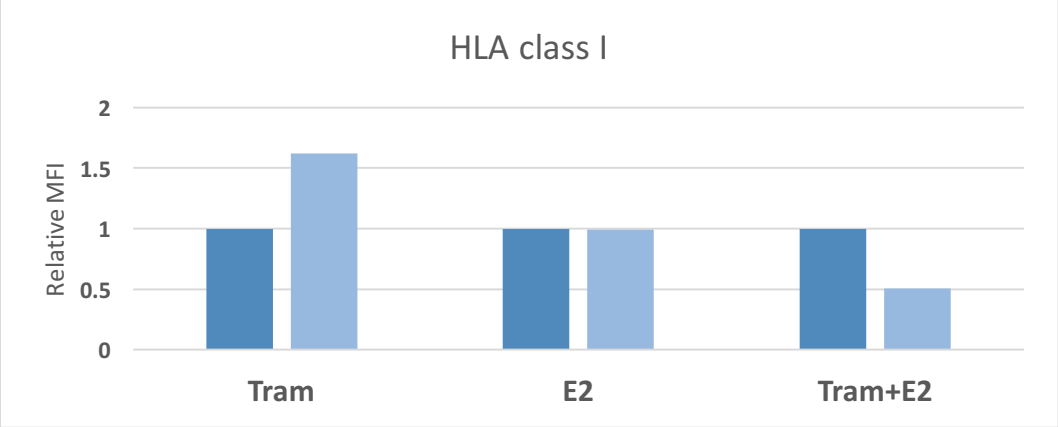
Appendix 1: Flow cytometric analysis showing treated/untreated values of MDA-231c10A cells treated with E_2 in combination or absence of Selumetinib.

Cells were treated with Selumetinib (15 nM) with DMSO as a vehicle control, E_2 (10^{9}M) in combination with or alone with ethanol as vehicle control, for 72 hours and then with IFN-γ (100 u/ml) 1hr after MEK*i* treatment to determine A) Surface HLA-DR (L243) expression. B) Surface HLA class I (W6/32) expression. C) Surface PD-L1 (MIH2) expression.

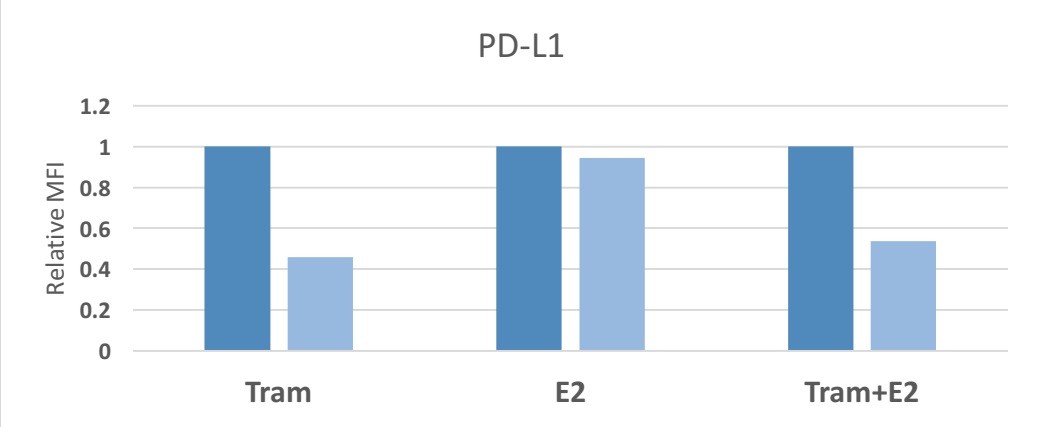
A.


HLA-DR
Relative MFI
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Tram
E2
Tram+E2

B.


HLA class I
Relative MFI
2
1.5
1
0.5
0
Tram
E2
Tram+E2

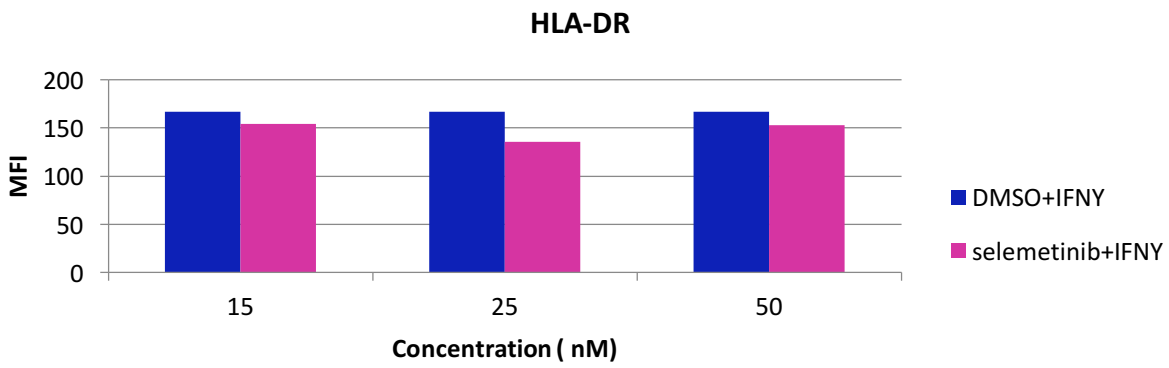
C.


PD-L1
Relative MFI
1.2
1
0.8
0.6
0.4
0.2
0
Tram
E2
Tram+E2

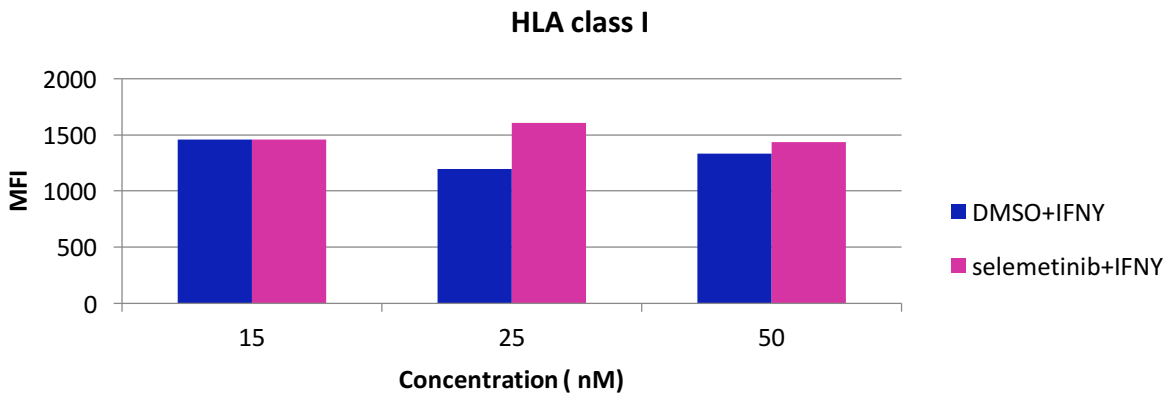
Appendix 2: Flow cytometric analysis showing treated/over untreated values of MDA-231c10A cells treated with E_2 in combination or absence of Trametinib.

Cells were treated with Trametinib (2 nM) with DMSO as a vehicle control, E_2 (10^9M) in combination with or alone with ethanol as vehicle control, for 72 hours and then with IFN-γ (100 u/ml) 1hr after MEK*i* treatment to determine A) Surface HLA-DR (L243) expression. B) Surface HLA class I (W6/32) expression. C) Surface PD-L1 (MIH2) expression.

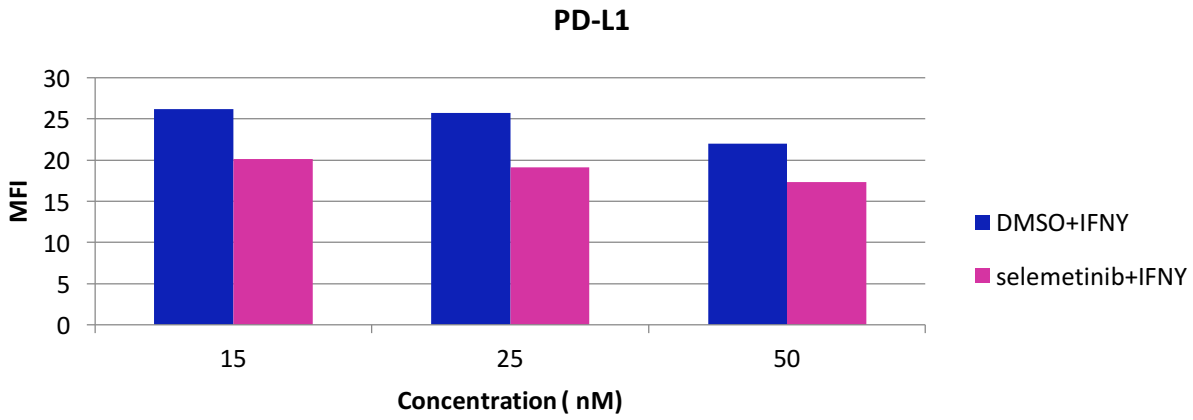
A.


HLA-DR
200
150
100
50
0
MFI
15
25
50
Concentration (nM)
DMSO+IFNY
selemetinib+IFNY

B.

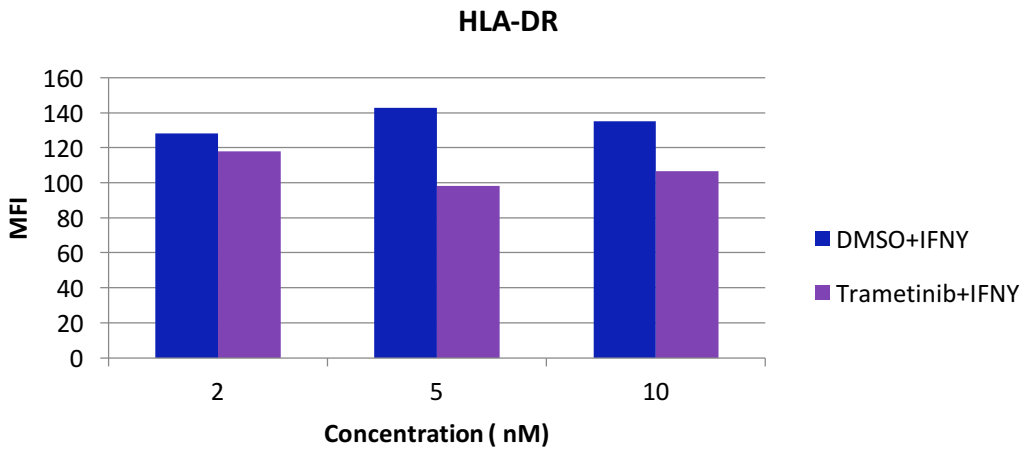

HLA class I
2000
1500
1000
500
0
MFI
15
25
50
Concentration (nM)
DMSO+IFNY
selemetinib+IFNY

C.

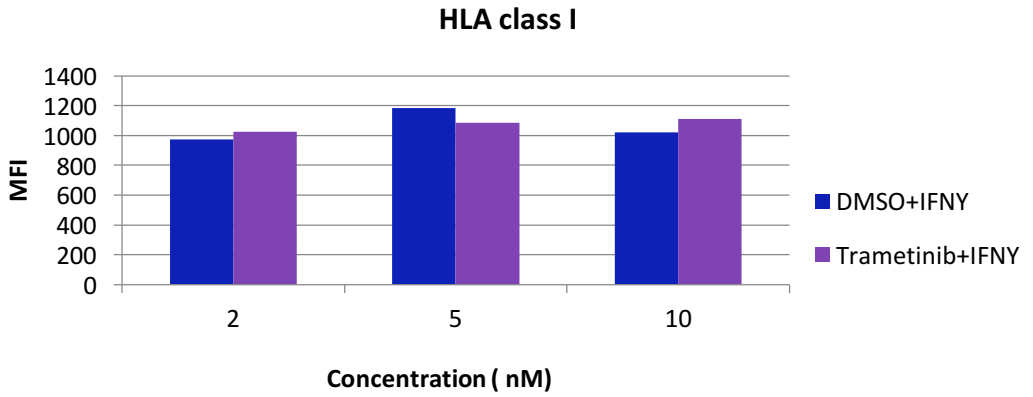

PD-L1
30
25
20
15
10
5
0
MFI
15
25
50
Concentration (nM)
DMSO+IFNY
selemetinib+IFNY

Appendix 3: Flow cytometric analysis of concentration experiment with MDA-231 c10A treated with Selumetinib (15 nM, 25 nM or 50 nM), or DMSO (vehicle control), induced with IFN-γ (100 u/ml) 1hr after MEKi treatment for a total incubation time of 72 hours to determine A) Surface HLA-DR (L243) expression. B) Surface HLA class I (W6/32) expression. C) Surface PD-L1 (MIH2) expression. Experiment shown was done once.

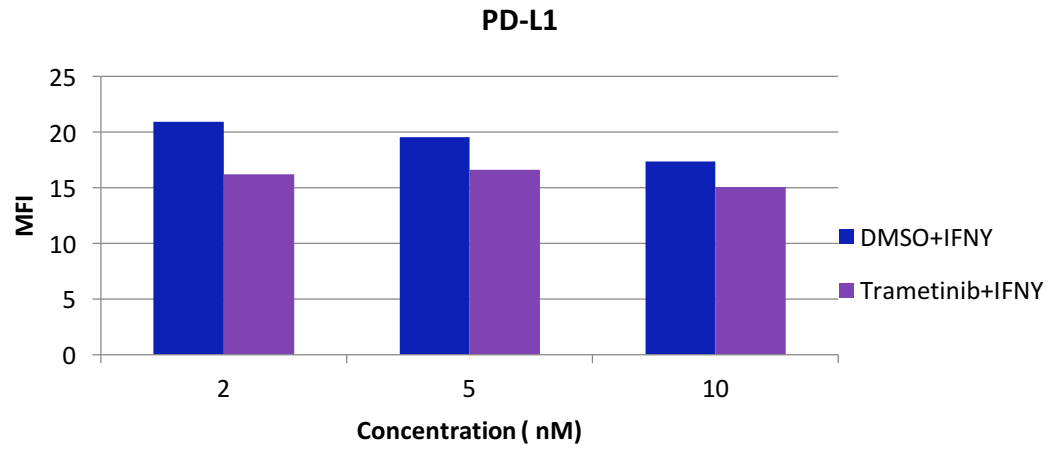
A.


HLA-DR
160
140
120
100
80
60
40
20
0
MFI
2
5
10
Concentration (nM)
DMSO+IFNY
Trametinib+IFNY

B.

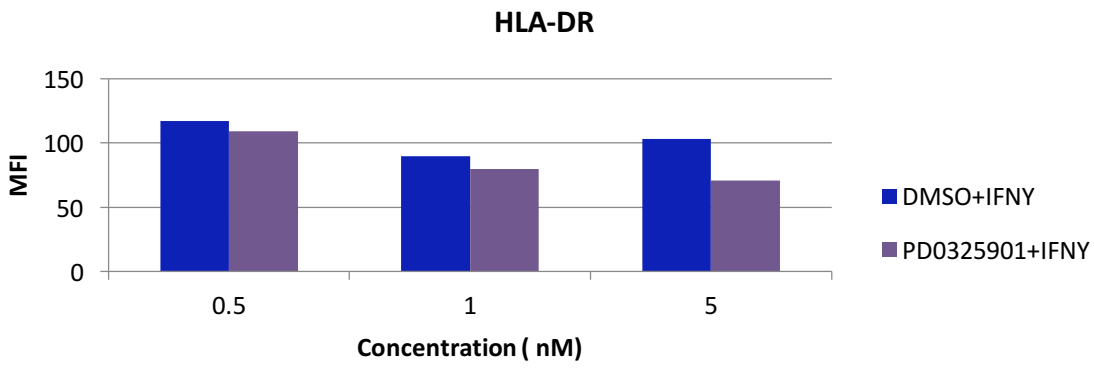

HLA class I
1400
1200
1000
800
600
400
200
0
MFI
2
5
10
Concentration (nM)
DMSO+IFNY
Trametinib+IFNY

C.

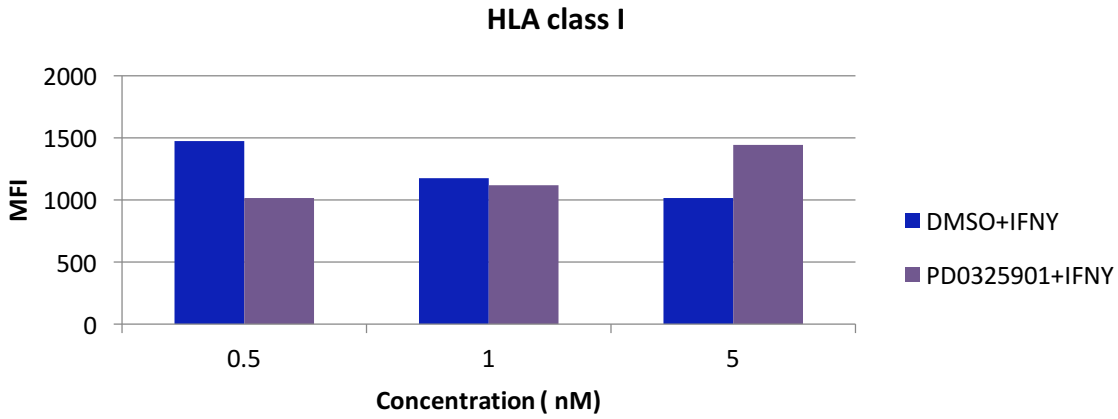

PD-L1
25
20
15
10
5
0
MFI
2
5
10
Concentration (nM)
DMSO+IFNY
Trametinib+IFNY

Appendix 4: Flow cytometric analysis of concentration experiment with MDA-231 c10A treated with Trametinib (2 nM, 5 nM or 10 nM), or DMSO (vehicle control), induced with IFN-γ (100 u/ml) 1hr after MEKi treatment for a total incubation time of 72 hours to determine A) Surface HLA-DR (L243) expression. B) Surface HLA class I (W6/32) expression. C) Surface PD-L1 (MIH2) expression. Experiment shown was done once.

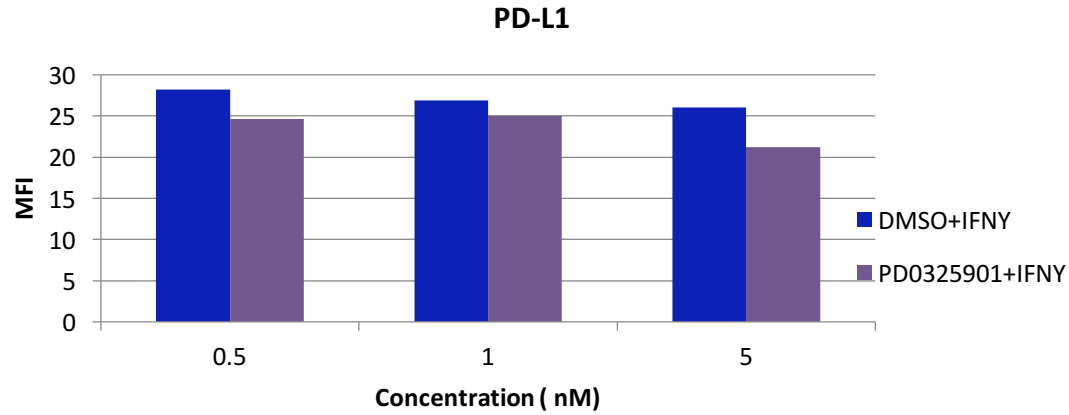
A.


HLA-DR
150
100
50
0
MFI
0.5
1
5
Concentration (nM)
DMSO+IFNY
PD0325901+IFNY

B.

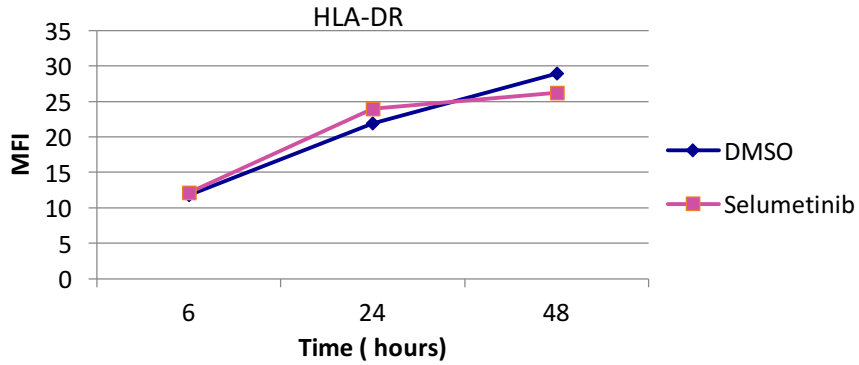

HLA class I
2000
1500
1000
500
0
MFI
0.5
1
5
Concentration (nM)
DMSO+IFNY
PD0325901+IFNY

C.

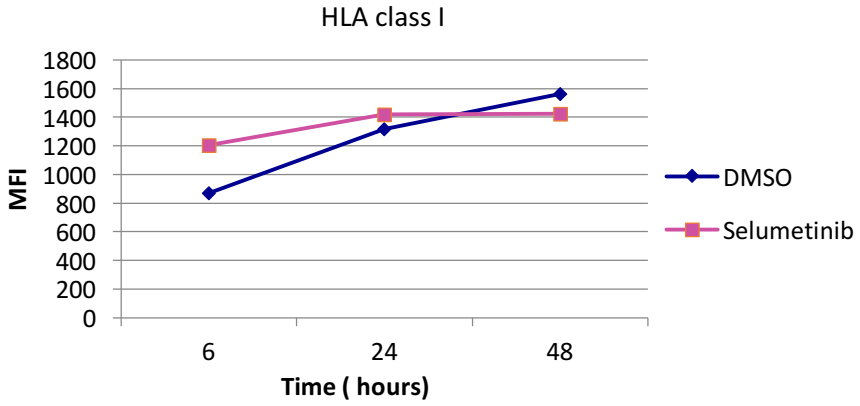

PD-L1
30
25
20
15
10
5
0
MFI
0.5
1
5
Concentration (nM)
DMSO+IFNY
PD0325901+IFNY

Appendix 5: Flow cytometric analysis of concentration experiment with MDA-231 c10A treated with PD0325901 (0.5 nM, 1 nM, or 5 nM), or DMSO (vehicle control), induced with IFN-γ (100 u/ml) 1hr after MEKi treatment for a total incubation time of 72 hours to determine A) Surface HLA-DR (L243) expression. B) Surface HLA class I (W6/32) expression. C) Surface PD-L1 (MIH2) expression. Experiment shown was done once.

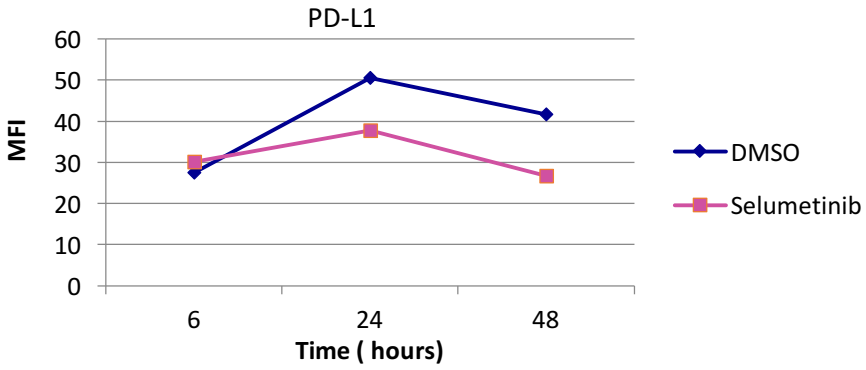
A.


HLA-DR
35
30
25
20
15
10
5
0
MFI
6
24
48
Time (hours)
DMSO
Selumetinib

B.

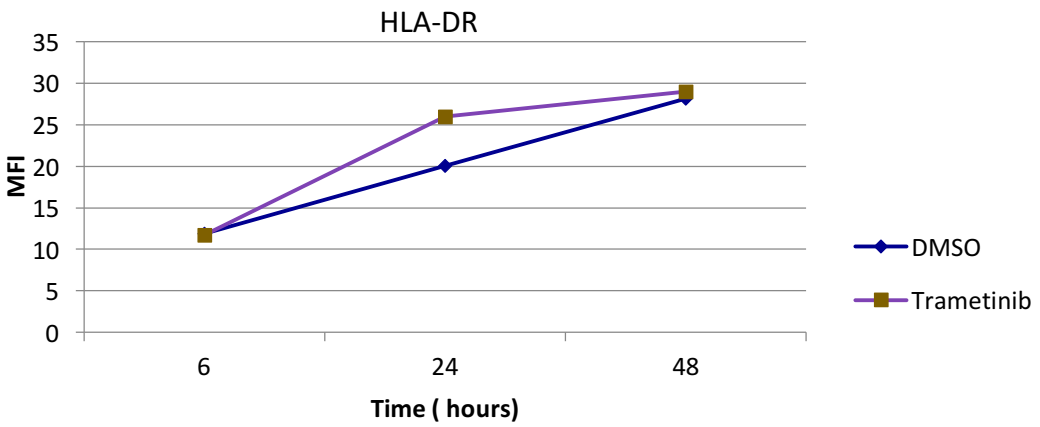

HLA class I
1800
1600
1400
1200
1000
800
600
400
200
0
MFI
6
24
48
Time (hours)
DMSO
Selumetinib

C.

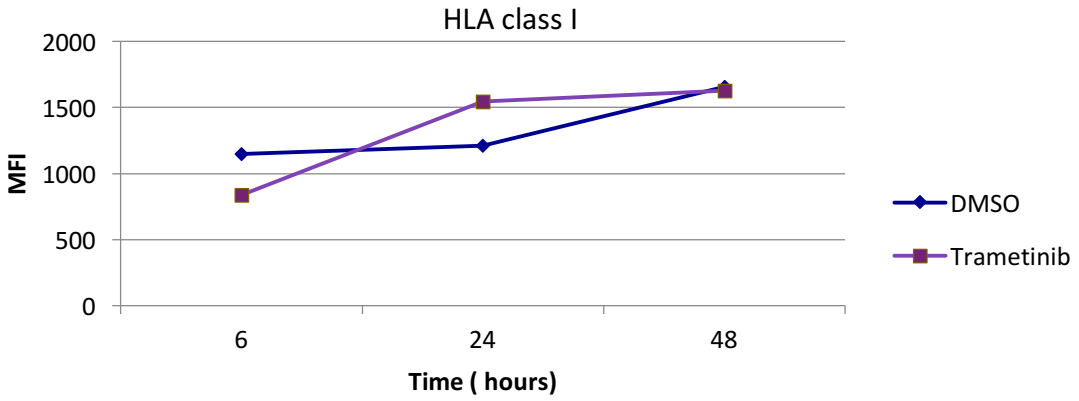

PD-L1
60
50
40
30
20
10
0
MFI
6
24
48
Time (hours)
DMSO
Selumetinib

Appendix 6: Flow cytometric analysis of time course experiment with MDA-231 c10A treated with Selumetinib (15 nM), or DMSO (vehicle control), induced with IFN-γ (100 u/ml) 1hr after MEKi treatment for total incubation time(s) of 6, 24, & 48 hours to determine A) Surface HLA-DR (L243) expression. B) Surface HLA class I (W6/32) expression. C) Surface PD-L1 (MIH2) expression. Experiment shown was done once.

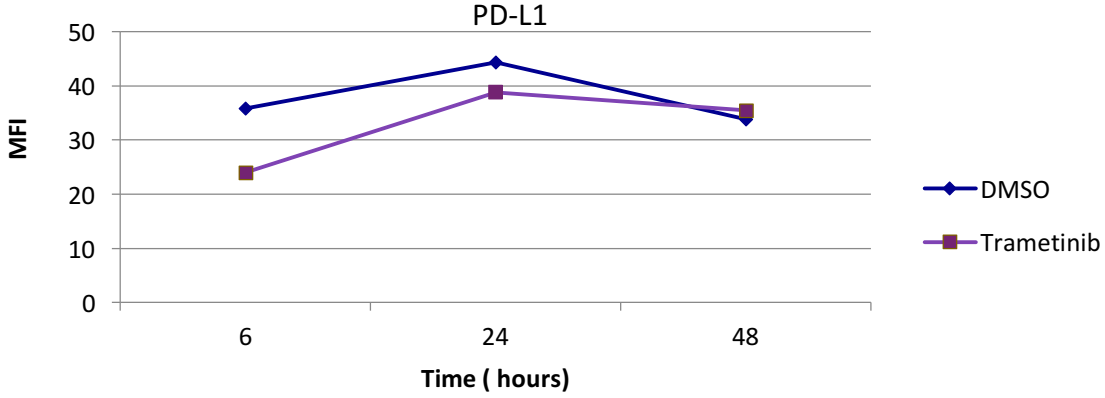
A.


HLA-DR
35
30
25
20
15
10
5
0
MFI
6
24
48
Time (hours)
DMSO
Trametinib

B.

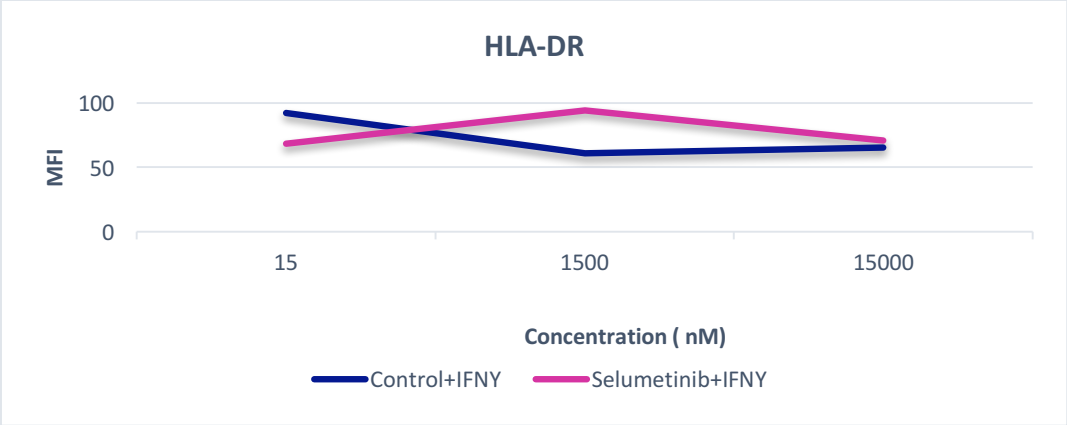

HLA class I
2000
1500
1000
500
0
MFI
6
24
48
Time (hours)
DMSO
Trametinib

C.

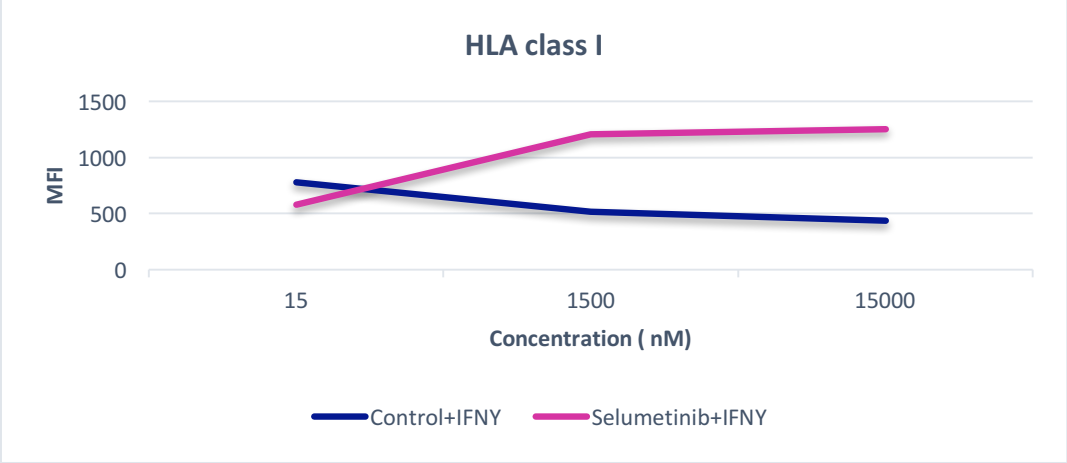

PD-L1
50
40
30
20
10
0
MFI
6
24
48
Time (hours)
DMSO
Trametinib

Appendix 7: Flow cytometric analysis of time course experiment with MDA-231 c10A treated with Trametinib (2 nM), or DMSO (vehicle control), induced with IFN-γ (100 u/ml) 1hr after MEKi treatment for total incubation time(s) of 6, 24, & 48 hours to determine A) Surface HLA-DR (L243) expression. B) Surface HLA class I (W6/32) expression. C) Surface PD-L1 (MIH2) expression. Experiment shown was done once.

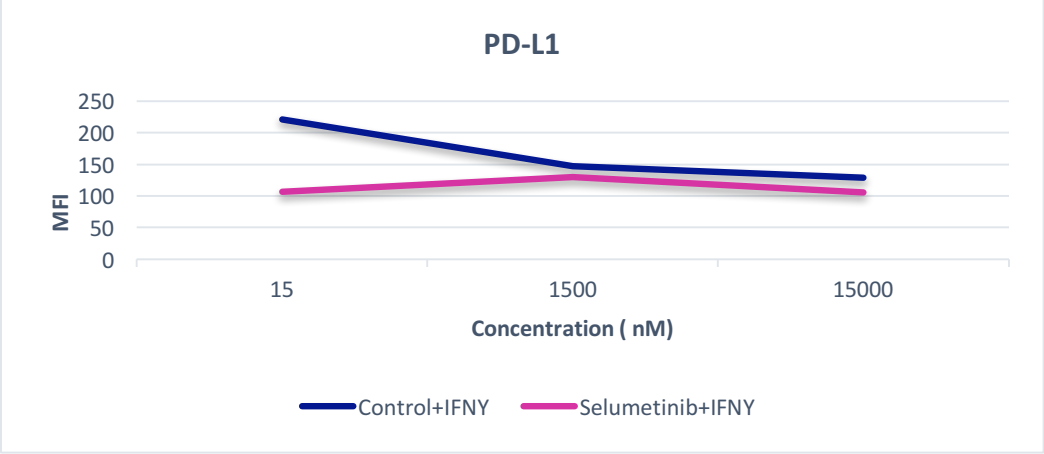
A.


HLA-DR
100
50
0
MFI
15
1500
15000
Concentration (nM)
Control+IFNY
Selumetinib+IFNY

B.


HLA class I
1500
1000
500
0
MFI
15
1500
15000
Concentration (nM)
Control+IFNY
Selumetinib+IFNY

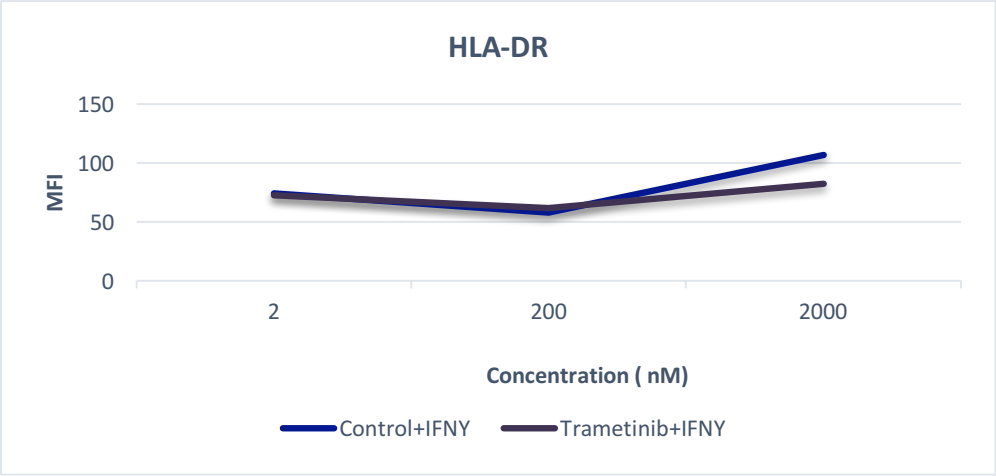
C.


PD-L1
250
200
150
100
50
0
MFI
15
1500
15000
Concentration (nM)
Control+IFNY
Selumetinib+IFNY

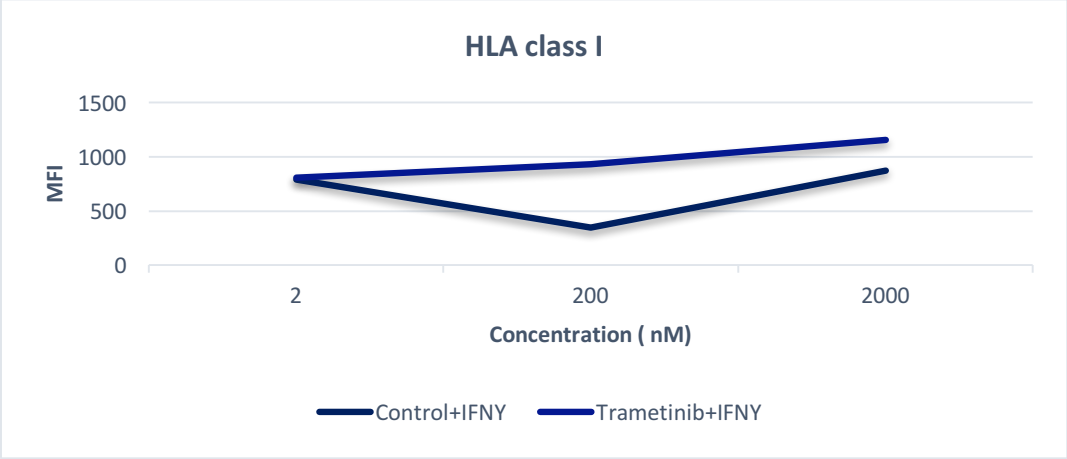
Appendix 8: Flow cytometric analysis of log dosage experiment with Selumetinib-treated MDA-231 c10A cells.

Cells were treated with Selumetinib (15 nM, 1500 nM or 15000 nM), or DMSO (vehicle control) for 72 hours and then with IFN-γ (100 u/ml) 1hr after MEK*i* treatment to determine A) Surface HLA-DR (L243) expression. B) Surface HLA class I (W6/32) expression. C) Surface PD-L1 (MIH2) expression. Experiment shown was done once.

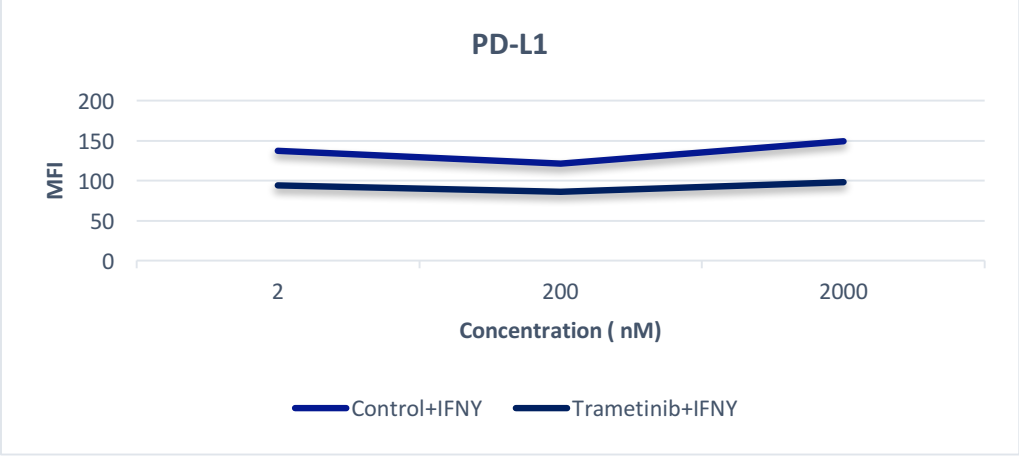
A.


HLA-DR
150
100
50
0
MFI
2
200
2000
Concentration (nM)
Control+IFNY
Trametinib+IFNY

B.


HLA class I
1500
1000
500
0
MFI
2
200
2000
Concentration (nM)
Control+IFNY
Trametinib+IFNY

C.


PD-L1
200
150
100
50
0
MFI
2
200
2000
Concentration (nM)
Control+IFNY
Trametinib+IFNY

Appendix 9: Flow cytometric analysis of log dosage experiment with Trametinib-treated MDA-231 c10A.

Cells were treated with Trametinib (2 nM, 200 nM or 2000 nM), or DMSO (vehicle control) for 72 hours and then with IFN-γ (100 u/ml) 1hr after MEK*i* treatment to determine A) Surface HLA-DR (L243) expression. B) Surface HLA class I (W6/32) expression. C) Surface PD-L1 (MIH2) expression. Experiment shown was done once.