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Characterization of the Effects of Sex and Estrogen Receptor Signaling on Antigen-Specific T Cells for Immunotherapy

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CHARACTERIZATION OF THE EFFECTS OF SEX AND ESTROGEN RECEPTOR SIGNALING ON ANTIGEN-SPECIFIC T CELLS FOR IMMUNOTHERAPY

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To my friends and family

Not all those who wander are lost.

J.R.R. Tolkien

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LIST OF ABREVIATIONS

℃	Degree Centigrade/Celsius
ACK	Ammonium-chloride-potassium
ACT	Adoptive T cell therapy
AEC	3-Amino-9-ethylcarbazole
AF	Activating function
Ag	Antigen
AICD	Activation induced cell death
AP-1	Activating protein-1
APC	Antigen presenting cell
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAR	Chimeric antigen receptor
CBP	CREB-binding protein
CCL	Chemokine C-C motif ligand
CD34t	Truncated CD34
CEA	Carcinoembryonic antigen
ChIP	Chromatin immunoprecipitation
cm	Centimeter

CMV	Cytomegalovirus
COOH	Carboxyl terminus
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DAG	Diacylglycerol
DBD	DNA binding domain
DC	Dendritic cell
DMSO	Dimethyl sufoxide
DNMT	DNA (cytosine-5)-methyltransferase
E1	Estrone
E2	17β-estradiol, estrogen
E3	Estriol
EGFR	Epithelial growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ELSIPOT	Enzyme-linked immune absorbent spot
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen response element
FBS	Fetal bovine serum
FDA	Food and Drug Administration

FoxP3	Forkhead box P3
FSC	Forward scatter
FSH	Follicle-stimulating hormone
GM-CSF	Granulocyte macrophage-colony-stimulating factor
GPER	G-protein coupled estrogen receptor
HAT	Histone acetylase
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HDAC	Histone deacetylase
HIV	Human Immunodeficiency virus
HPV	Human papilloma virus
HRP	Horseradish peroxidase
HRT	Hormone replacement Therapy
IBD	Inflammatory bowel disease
IFNγ	Interferon γ
Ig	Immunoglobulin
IGFR	Insulin growth factor receptor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP3	Inositol-3-phosphate
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	Janus Kinase

kg	Kilogram
KO	Knockout
L	Liter
LBD	Ligand binding domain
lck	Lymphocyte-specific protein tyrosine kinase
LH	Luteinizing hormone
MAPK	Mitogen activated protein kinase
MCL	Myeloid cell leukemia
MCM	Minichromosome maintenance component
MCP-1	monocyte chemoattractant protein 1
MDSCs	Myeloid derived suppressor cells
MFI	Mean fluorescence intensity
μg	Microgram
mg	Milligram
MHC	Major histocompatibility complex
mL	Milliliter
μL	Microliter
μm	Micrometer
mm	Millimeter
mM	Millimolar
μM	Micromolar
MMP	Matrix metallopeptidase 9

MS	Multiple sclerosis
NAFLD	Non-alcoholic fatty liver disease
NFAT	Nuclear factor of activated T cells
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH2	Amino terminus
NK	Natural killer cell
nM	Nanomolar
NO	Nitric oxide
NSCLC	Non-small cell lung cancer
ORR	Objective response rate
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PDGFR	Platelet-derived growth factor receptor
PD-L1	Programmed cell death ligand 1
PDX	Patient derived xenograft
PI3K	Phosphoinositide-3 kinase
PI-9	Proteinase inhibitor 9
PIP2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospho lipase C
PTPRO	Protein tyrosine phosphatase receptor type O

RA	Rheumatoid arthritis
RT	Room temperature
RT-PCR	Real time polymerase chain reaction
SDS	Sodium dodecyl sulfate
SLE	Systemic lupus erythematosus
SR	Progression free survival rate
SRC	Steroid receptor co-activators
SSC	Side scatter
TADCs	Tumor associated dendritic cells
TAMs	Tumor associated macrophages
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th	Helper T cell
TIL	Tumor infiltrating lymphocyte
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TME	Tumor microenvironment
TNFα	Tumor necrosis factor α
T_{regs}	Regulatory T cells
VEGF	Vascular endothelial growth factor
Zap70	ζ-chain associated protein

ABSTRACT

Adoptive cell transfer (ACT) immunotherapy using genetically modified antigen (Ag)-specific T cells is a rapidly evolving field. Although evidence from pre-clinical and clinical studies suggests the use of Ag-specific T cells can be effective treating different malignancies, several challenges remain in order to improve these therapeutics. Host factors that can affect Ag-specific T cells during ACT immunotherapy remain understudied. Evidence of this lack in research includes characterizing the effects of sex and sex hormone receptor signaling on Ag-specific T cell function. Males and females have great differences in their circulating T cell populations and subset phenotypes. While some of these sex-differences in T cell phenotype are genetically and environmentally mediated, many of these differences heighten or decrease with puberty and reproductive senescence indicating a sex hormone receptor involvement. Estrogen was shown to modulate the development and differentiation of T cells. The effect of estrogen on T cell function remains controversial and incompletely understood. With most of the important aspects concerning the role of estrogen on T cells previously investigated in autoimmunity models and at hormone concentrations limited to physiological and pregnancy estrogen levels, the role of estrogen signaling through its canonical receptors, estrogen receptor α and β (ERα and ERβ) at other ranges of estrogen dosage need to be investigated. Knowing that sex and estrogen have direct effects on T cell differentiation and function, it is important to carefully characterize their effects on Ag-specific T cells for immunotherapy in order to identify possible

mechanisms that can be targeted to enhance T cell anti-tumor immune responses and immunotherapy efficacy.

The effects of sex and estrogen signaling on T cells and other immune cells result in differences on pathogenesis of malignancies including hepatocellular carcinoma (HCC). HCC is significantly more prevalent in males and post-menopausal females not undergoing estrogen hormone replacement therapy compared to pre-menopausal females, demonstrating a protective role of estrogen against HCC. The role of estrogen signaling was investigated in transformed hepatocytes and infiltrating innate immune cells but was not reported in tumor infiltrating T cells. ACT immunotherapy using genetically modified T cells showed some success on treating HCC in pre-clinical and clinical studies but the efficacy of ACT fighting HCC remains unsatisfactory. In this study, it was hypothesized that the protective role of estrogen against HCC is partially due to the estrogen-mediated enhancement of the T cell anti-tumor immune responses. Thus, if the T cell anti-tumor immune response is enhanced by estrogen, then estrogen signaling can be used to increase the efficacy of adoptive T cell transfer immunotherapy.

The work performed in this dissertation was aimed to characterize the role of estrogen signaling on T cell function *in vitro,* and during ACT immunotherapy against HCC *in vivo*. Using human male and female Ag-specific T cells, the effect of estrogen receptor signaling on overall T cell function and anti-tumor immunity was determined. Estrogen signaling through ERα was shown to enhance the expression and secretion of Type I effector cytokines including interferon γ (IFNγ), tumor necrosis factor α (TNFα), and Granzyme B in male and female Ag-specific T cells activated with their cognate tumor Ag. Estrogen signaling through ERα was also shown to modulate the overall expression of the Type 2 cytokine interleukin 4 (IL-4) in male and

female Ag-specific T cells. These results indicated that estrogen signaling through ERα mediates the balance between Type I and Type II Ag-specific T cell responses. Estrogen signaling through ERβ enhanced the polyfunctionality, or the ability of a T cell to express several markers simultaneously upon activation, of male and female Ag-specific T cells activated with their cognate Ag. T cell polyfunctionality is correlated with enhanced T cell receptor (TCR) signaling, indicating that estrogen signaling through ERβ enhances TCR downstream signaling pathways. These results demonstrated for the first time that estrogen signaling through ERα and ERβ can enhance the function of human Ag-specific T cells. Using an HCC mouse model treated using ACT immunotherapy, the effect of estrogen on the Ag-specific T cell anti-tumor immune response was measured. The presence of estrogen resulted in reduced tumor burden through higher Ag-specific T cell tumor infiltration, survival, activation state, and cytokine expression. Removal of physiological estrogen during ACT immunotherapy reduced the survival of $CD4^+$ Ag-specific T cells resulting in reduced tumor infiltration. Lack of physiological estrogen during ACT also caused hindered cytokine production and polyfunctionality of $CD4^+$ Ag-specific T cells. These results revealed for the first time that estrogen signaling can enhance the survival and function of $CD4^+$ Ag-specific T cells which results in enhanced anti-tumor responses and reduced tumor burden.

In summary, estrogen signaling enhances male and female Ag-specific T cell cytokine expression and secretion, and polyfunctionality which lead to enhanced tumor infiltration, survival, activation state, and function during ACT immunotherapy. This indicates that inducing estrogen signaling on Ag-specific T cells can enhance the efficacy and therapeutic outcome of ACT immunotherapy.

CHAPTER I

LITERATURE REVIEW

Immunotherapy

The immune system has the ability to recognize and destroy tumor cells without affecting normal cells through a mechanism called immune surveillance, and thus functions as a primary anti-cancer defense mechanism [1]. The immune system can also prevent cancer through generating long-term memory T cell responses [1]. Immunotherapy involves the enhancement of immune responses in cancer patients to increase tumor recognition and initiation of anti-tumor responses [2]. Because of the success obtained in recent years treating specific cancers with this therapeutic approach, immunotherapy has become the fourth pillar of cancer treatment together with surgery, radiation, and traditional chemotherapy. Most clinically approved immunotherapies have T cells central to their mechanism and fall broadly into two categories: first, agents that directly target and modulate endogenous T cell responses; and, second, cellular therapies where genetically modified T cells are used as treatment. T cells are immune cells that can be activated by tumor antigens, they can specifically destroy tumor cells in response to tumor antigen stimulation, and further generate memory responses that last long periods of time. While immunotherapy enhances the anti-tumor immune response, tumors can avoid it and still develop in the presence of an active immune system through a mechanism called immune tolerance. Therefore, the focus of ongoing immunotherapy research is on not only the recognition and destruction of specific tumor cells but also toward overcoming immune tolerance posed by the

tumor microenvironment (TME), and finally in discovering new host pathways that can be targeted to enhance its efficacy. The work performed in this dissertation was aimed at identifying novel roles of host factors present in the TME, like the sex hormone estrogen, that can be modulated in order to enhance T cell-based immunotherapy. In the upcoming paragraphs, T cell function and subsets are described as well as the different current types of T cell-based immunotherapies and the challenges that immunotherapy is faced with in the clinic.

T cell Signaling and Function

T cells play a central role in mediating cellular immunity. T cells are multi-functional effector cells that protect humans from disease throughout their entire lives with their ability to recognize bacterial, viral, and cancer antigens. The specificity of T cells is mediated by the T cell receptor (TCR), a surface receptor that facilitates target antigen recognition in the context of a major histocompatibility complex (MHC) molecule. Cell surface expression of the TCR requires its association with the CD3 complex (Figure 1) [3]. The CD4 and CD8 co-receptors enhance the binding of the TCR to MHC class II and I respectively and promote downstream signaling by localizing the lymphocyte-specific protein tyrosine kinase (lck) to the TCR/CD3 complex (Figure 1) [3, 4]. Upon recognition and binding to the antigen-bearing MHC, a signaling cascade begins downstream from the TCR consisting on phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by lck in the CD3ζ chain and recruitment of ζ-chain associated protein (Zap70), which induces assembly of the remaining signaling components (Figure 1) [5]. Several signaling pathways are triggered downstream from the TCR including the activation of phospholipase C γ (PLC) which hydrolyzes membrane-bound phosphatidylinositol 4, 5-biphosphate (PIP_2) into inositol-3-phosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers the

activation of the calcium (Ca^{+2})-dependent nuclear factor of activated T cells (NFAT) signaling pathway [6]. DAG activates several major signaling pathways including the mitogen activated kinase (MAP kinase) pathway, and the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway induced by protein kinase C (PKC) activation [6]. Activation of these signaling pathways induce intracellular Ca^{2+} release, actin polymerization, integrin activation, T cell proliferation, cytokine secretion, and degranulation [7]. This T cell functional response varies depending on the subset of activated T cell. Naïve T cells can differentiate into various subsets depending on the activating stimulus and the cocktail of cytokines present during Ag recognition [8, 9]. T cell subsets are identified by cell surface markers, transcription factors expression, and cytokines secreted.

A well characterized example of T cell subsets being distinguished by cytokine secretion and transcription factor expression is the Th1 and Th2 $CD4^+$ helper T cell subsets. Th1 $CD4^+$ T cells express the transcription factor T-Bet and secrete mainly interferon γ (IFNγ), interleukin-2 (IL-2), and tumor necrosis factor α (TNFα) upon activation [9]. Th1 mediate pro-inflammatory cell mediated immunity and were shown to induce delayed-type hypersensitivity and mediate the response to some protozoa. On the other hand, Th2 $CD4^+$ T cells express the transcription factor GATA3 and secrete mainly IL-4, -5, -6, -10, and -13 [9]. Th2 cells promote non-inflammatory immediate immune responses and are essential in B cell production of immunoglobulin G (IgG), IgA, and IgE [9]. Th1 and Th2 development routes are mutually antagonistic, giving rise to the model of polarization of the T cell immune response. Other $CD4^+$ helper T cells subsets that differentiate based on transcription factor expression and cytokine production are the Th17 subset which expresses RORγt and produces IL-17a and IL-22, the regulatory T cells (T_{regs})

subset which expresses forkhead box P3 (FoxP3) and secretes IL-10 and transforming growth factor β (TGFβ), and the Th9 subset which produces IL-9 [9].

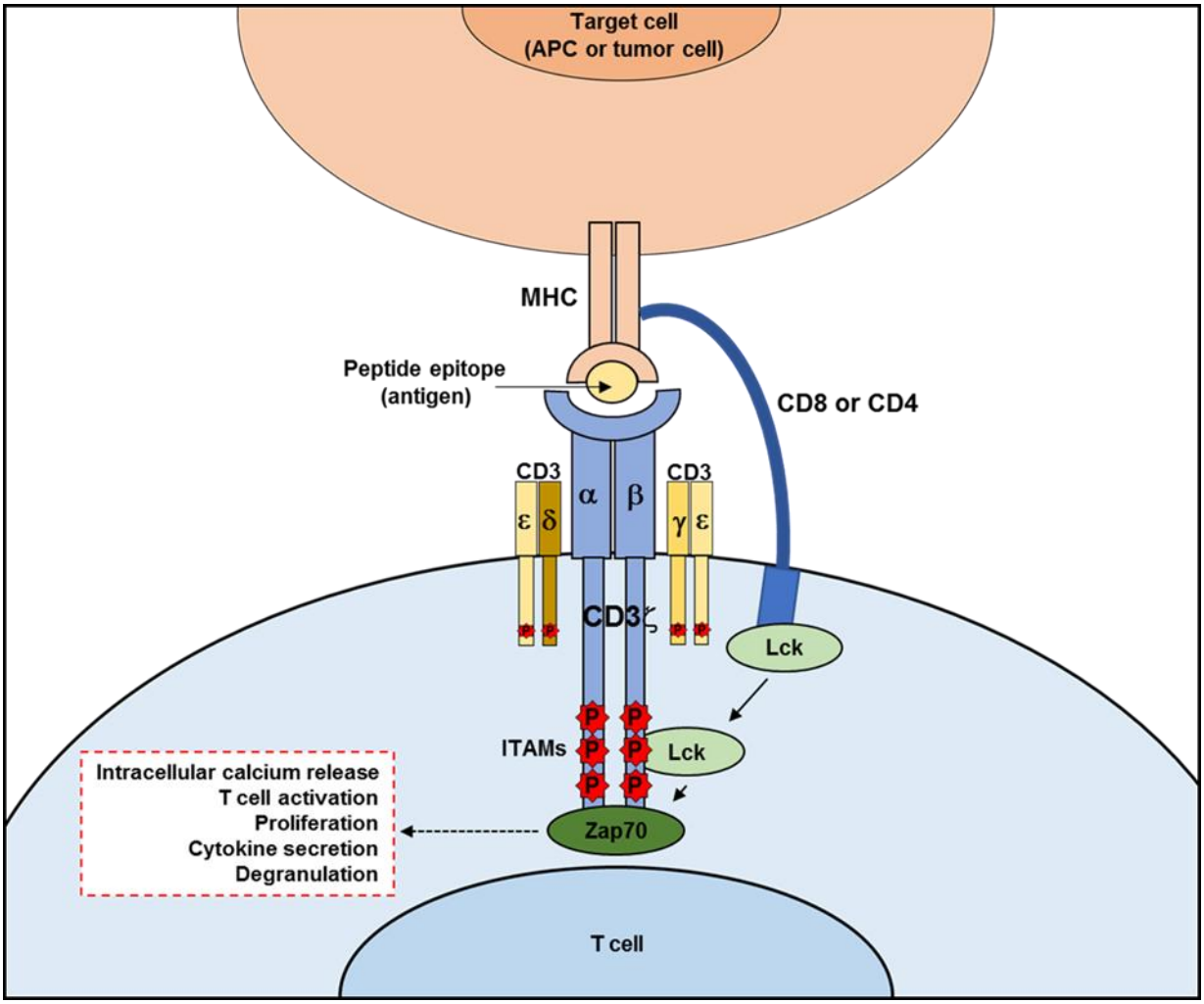


Figure 1. T Cell Receptor (TCR) Mediated Target Recognition and T Cell Activation. Depicted is a target cell, such as a tumor cell, presenting an antigen in the context of the MHC to a T cell's TCR. The TCR α and β chains form a complex with CD3 components and the CD8 or CD4 co-receptors stabilize the TCR-MHC interaction. Upon antigen recognition and TCR ligation, Lck phosphorylates the ITAMs on the CD3ζ chains which recruit Zap70 which facilitates TCR downstream signaling which results in T cell activation. MHC: major histocompatibility complex, Lck: lymphocyte-specific protein tyrosine kinase, ITAM: immunoreceptor tyrosine-based activation motif, Zap70: ζ-chain associated protein.

$CD8^+$ cytotoxic T lymphocytes (CTLs) are derived from naïve $CD8^+$ T cells, proliferate in the presence of IL-2, and can expand their number by a thousand-fold upon activation at the peak of a primary immune response [9]. Rapid expansion and the ability of $CD8^+$ CTLs to destroy more than one target while sparing bystander cells, make CTLs very efficient Ag-specific effector cells [9]. Destruction of target cells by CTLs requires Ag recognition which initiates the release of cytotoxic granules such as perforin and Granzyme B into the immunological synapse between CTL and target cells, or the initiation of Fas/Fas-ligand-mediated apoptosis. Similarly to $CD4^+$ T cells, cytotoxic $CD8^+$ effector T cells fall into two subpopulations based on cytokine secretion. Type I $CD8^+$ T cells secrete IFNγ, whereas Type II $CD8^+$ T cells secrete IL-4, IL-5, and IL-10 [10]. Both Type I and Type II $CD8^+$ T cells were shown to provide strong immunity against tumors and differentiate into $CD8^+$ memory T cells. Long-lasting protection by Type I and Type II $CD8^+$ effector cells was dependent on IL-4, IL-2, and INFγ production indicating both subsets are required for the generation of superior anti-tumor responses [10]. Overall, the immunology field generally accepts that T cells are functionally restricted by the subset into which they differentiate, each $CD4^+$ and $CD8^+$ T cell subset has different functions based on the cytokines and factors they secrete upon TCR activation [9].

Types of Immunotherapy

Immune Checkpoint Blockade

Upon Ag encounter, to ensure robust T cell activation, two independent signaling pathways are involved. The first signal requires recognition of the Ag-bearing MHC on the surface of antigen presenting cells (APCs) or tumor cells by the corresponding TCR. The second signal, which is Ag independent, is delivered by the engagement of co-stimulatory molecules [11, 12]. Positive co-stimulation is mainly mediated by CD28 receptors expressed on T cells binding to B7.1 (CD80) and B7.2 (CD86) on APCs [13]. This interaction leads to a cascade of intracellular signal transducers and regulators, and results in T cell activation and proliferation, as well as the production of various cytokines, including IL-2, thus avoiding anergy [11, 14]. $CD4^+$ and $CD8^+$ T cells have severely impaired proliferation and produce reduced levels of effector cytokines in the absence of CD28 [15, 16]. T cells can also express several inhibitory co-stimulatory factors, such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1) which are denominated immune checkpoints. Binding of these negative co-stimulatory factors to their ligands antagonizes T cell activation. T cell immunosppression by immune checkpoints is relevant in order to avoid T cell responses against self Ag which can promote autoimmunity. In the TME, the expression of immune checkpoints by TILs results in T cell hyporesponsiveness and immune exhaustion contributing to tumor immune scape. Blocking the interactions of TIL inhibitory co-stimulatory molecules results in sustained activation of the anti-tumor immune response thereby making checkpoint blockade an efficacious immunotherapy.

Activated T cells express CTLA-4 which binds to ligands B7.1 (CD80) and B7.2 (CD86) with much higher affinity than CD28 [17]. Binding of CTLA-4 to B7.1 or -2 induces powerful

inhibitory signaling that dampens T cell activation, leading to decreased cell cycle progression, proliferation, and IL-2 secretion [17]. CTLA-4 inhibition with monoclonal antibodies prevents the binding of CTLA-4 to B7.1, 2, thereby promoting T cell activation and successful anti-tumor immune responses. Several CTLA-4 blocking monoclonal antibodies such as Ipilimumab and Tremelimumab were approved by the Food and Drug Administration (FDA) to treat melanoma. Although CTLA-4 blockade was successful for some patients, the objective response rates (ORR) for stage III or IV melanoma patients treated with Ipilimumab was 15-20%, and only 11% for those treated with Tremelimumab [18-21]. Progression free survival rates (SR) for melanoma patients treated with CTLA-4 inhibitors were around 2.8 months.

The surface receptor PD-1, which is homologous to CD28, is also expressed in activated T cells and it is primarily involved in inhibitory immune signaling. PD-1 binds to the programmed cell death ligand 1 (PD-L1) and elicits intracellular signaling that results on the dephosphorylation of TCR proximal components such as CD28 [22-24]. Interfering with CD28 signaling, PD-1 signaling therefore results in reduced cytokine production, reduced T cell proliferation and survival, and reduced expression of T cell effector functions such as T-Bet and Eomes [23-25]. PD-L1 expression is observed in both cancer cells and tumor-infiltrating immune cells such as macrophages. PD-L1 expression on the cells present in the TME is upregulated in response to IFNγ signaling [26]. PD-L1 expression therefore indicates an active anti-tumor immune response and it is a way for the tumor cells to escape it through immunosuppression. Blockade of PD-1 and PD-L1 using monoclonal antibodies was approved by the FDA as a therapy for cancers including melanoma, renal cell carcinoma, squamous cell carcinoma, and non-small cell lung cancer (NSCLC). PD-1 inhibitors such as Nivolumab and Pembrolizumab

showed encouraging ORR of over 40% on stage III or IV melanoma patients with progression free survival rates of over 7 months [18, 21]. PD-1 inhibitors were also used to treat NSCLC (ORR of 20% and SR of 3 months), renal carcinoma (ORR of 25% and SR of 5 months), Hodgkin's lymphoma (ORR of 87%), ovarian cancer (ORR of 15% and SR of 4 months), and metastatic colorectal cancer (ORR of 40% and SR of 5 months) [27-31]. PD-L1 inhibitor Atezolizumab was found to be successful in solid tumors with ORR of 16% and 23% in NSCLC and urothelial carcinoma respectively [32, 33]. Better outcome was observed when both Nivolumab and Ipilimumab were used to treat stage III and IV melanoma (ORR of 57% and SR of over 11 months) indicating that blocking both PD-1 and CTLA-4 simultaneously can induce stronger anti-tumor immune responses [21, 34].

Immune checkpoint blockade treatment proves to be initially effective but unfortunately many patients eventually relapse. Due to the selection pressure caused by checkpoint blockade monoclonal antibody therapy, resistant tumor cells can arise. These checkpoint blockade immunotherapy resistant tumor cells upregulate or downregulate pathways that allow them to evade immune recognition and T cell anti-tumor immune responses. Tumor cells from relapsing anti-PD-1 treated patients were shown to downregulate IFNγ response elements including MHC class I through deactivating mutations to avoid T cell recognition [35]. Also, immune cells in anti-PD-1 treated melanoma or prostate cancer patients exhibited upregulation of other immune checkpoints like TIM-3 [36]. Tumor-associated macrophages (TAMs) present in the TME of relapsing patients were shown to be able to phagocytize the therapeutic monoclonal antibody bound to the surface of T cells, rendering them again susceptible to PD-1 inhibitory signaling [37]. A better understanding of these immune escape mechanisms in the TME limiting the

effectiveness of immune checkpoint blockade immunotherapy will therefore allow for improvement of cancer treatment.

Adoptive T cell Transfer Immunotherapy

Adoptive T cell therapy (ACT) consists on generating robust T cell anti-tumor immune responses by the *ex vivo* manipulation of patients' endogenous T cells. ACT has multiple advantages compared to other forms of immunotherapy that rely on generating enough numbers of functionally active and tumor reactive T cells *in vivo*. For use in ACT, large numbers of anti-tumor T cells can be readily generated and selected for high-avidity recognition of the tumor, as well as for the effector functions required to mediate tumor destruction. In addition, these anti-tumor T cells are generated from the patient's own circulating T cells so there is no risk of graft-versus-host disease. The first type of ACT was generated by isolating autologous Ag-specific tumor infiltrating lymphocytes (TILs) from resected melanomas [38]. Melanoma Ag-specific TILs were expanded *in vitro* and transferred into the patient in combination with IL-2 to enhance T cell activation and function. Using TILs for ACT immunotherapy, the ORR of metastatic melanoma patients reached 50% and durable remissions were achieved [38-41]. Even if TILs can be grown *in vitro* from many tumor types, only melanomas consistently give rise to TILs with effective tumor reactivity [42]. In order to develop ACT for other tumor types, techniques were developed to genetically introduce anti-tumor receptors into normal T cells that could be used for therapy. The specificity of T cells can be redirected by the integration of genes encoding either conventional $\alpha\beta$ TCRs or CARs.

The first successful clinical application of ACT using genetically engineered T cells treated melanoma patients using autologous T cells transduced with a human TCR recognizing

the MART-1 melanoma-melanocyte differentiation antigen [43]. These initial studies demonstrated that TCR gene-modified T cells were generally tolerated by patients and safe to use as cancer therapeutics. Then, this approach was expanded to larger metastatic melanoma patient numbers who received T cells modified with high-avidity TCRs recognizing MART-1 (ORR of 30%) and gp100 (ORR of 19%) [44]. Even if this treatment was efficacious it showed severe off-tumor on-target toxicity in the skin, eyes and ears due to melanocyte presence in these organs. Fortunately, in this case such toxicities resolved naturally or with administration of topical steroids. These earliest studies suggested TCR gene-modified T cells could reach clinical benefit, but that the choice of TCR was important in order to limit toxicities. Since the early studies, numerous TCR genes capable of recognizing tumor antigens were identified, and improvements in TCR gene transfer have allowed for recognition of a variety of other antigens and malignancies. These include, but are not limited to, carcinoembryonic antigen (CEA) expressed in epithelial cancers like colorectal cancer [45], cancer-testis antigens such as NY-ESO-1 and MAGE expressed in breast, bladder, colon, lung, ovarian, thyroid cancers and myeloma [46-48], and viral proteins expressed in cancers derived from viral infections. The use of these TCR engineered T cells to treat patients showed some limitations especially regarding severe side effects. Colorectal cancer patients treated with CEA-specific T cells experienced life threatening colitis and colonic hemorrhage which prevented further use of this TCR even if partial response on liver metastases was achieved [45]. Unexpected brain toxicity and gray matter destruction were observed when using MAGE-specific T cells to treat melanoma patients [43]. TCR engineered T cells that are specific for viral proteins include targets such as cytomegalovirus (CMV) [49], human immunodeficiency virus (HIV) [50], hepatitis C virus (HCV) [51, 52], and human papilloma virus (HPV) [53]. No clinical reports have yet been

published testing virus-specific TCR expressing T cells in humans, however this is a good approach to avoid off-tumor on-target effects since viral proteins are not expressed by non-infected human tissues.

Chimeric antigen receptors (CARs) are assembled by linking the variable regions of the antibody heavy and light chains, specifically reactive to a tumor antigen, to intracellular signaling molecules such as CD3ζ. CARs often include the sequences of co-stimulatory domains including but not limited to CD28 or CD137 (4-1BB) in order to fully activate T cells [54-56]. The antibody part of the CAR is specific for an antigen expressed in the tumor providing non-MHC-restricted recognition of cell surface components. CARs can be introduced into T cells with high efficiency using viral vectors [42]. Tumor antigens that are shared with nonessential organs represent potential targets for ACT immunotherapy. A prominent example is the molecule CD19 expressed in more than 90% of B cell malignancies and differentiating B cells excluding plasma cells. Administration of autologous T cells expressing the anti-CD19 CAR to patients with follicular lymphoma, large-cell lymphomas, chronic lymphocytic leukemia, and acute lymphocytic leukemia was shown to successfully reject the tumors with ORRs from 80 to 100% [55, 57-59]. Anti-CD19 CAR ACT immunotherapy not only elicits dramatic regression of lymphomas and leukemia but also provides patients lifelong protection since CAR T cells can become memory T cells detectable on circulation years after ACT [60]. Due to its success, anti-CD19 CAR T cell immunotherapy was approved by the FDA to treat B cell lymphoblastic leukemia and certain types of non-Hodgkin's lymphoma in 2017. A wide variety of other CARs were designed to target several antigens showing promise as potential cancer immunotherapies. Other targets that were pre-clinically evaluated include but are not limited to CD33 and CD123

for myeloid leukemia [61, 62], GD2 for neuroblastoma (ORR of 27%) [63], HER-2 and MUC-1 for breast cancer [64, 65], and MUC16 for ovarian cancer [66].

There are some limitations using CAR T cells for immunotherapy. For example, CARs target surface antigens, rendering them ineffective against intracellular antigens that would otherwise be processed and presented by MHC. A search for tumor-specific surface antigens expressed on tissues that are not essential for survival remains in progress. Also, the antibody-antigen interaction is much stronger than the TCR-antigen interaction which negatively impacts T cell function since T cells are more likely to undergo activation induced cell death (AICD) [67, 68]. Also, there were several reports of adverse events after CAR T cell immunotherapy due to tumor lysis syndrome and cytokine storm which are deadly side effects resulting from massive T cell activation, cytokine production, and tumor cell killing [69-71].

An important question concerning the use of genetically engineered cells for the treatment of cancer involves selection of the ideal T cell population unto which the gene should be introduced. T cells can be categorized into distinct memory subsets based on their differentiation states. $CD8^+$ T cells follow a progressive pathway of differentiation from naïve T cells into central memory and effector memory T cell populations [72]. Preclinical studies strongly suggest that improved anti-tumor responses are seen when T cells in early stages of differentiation (such as naïve or central memory cells) are transduced, a result supported by studies in monkeys showing improved *in vivo* persistence of infused central memory compared with effector memory cells [73, 74]. Also, the differentiation state of $CD8^+$ T cells is inversely correlated with their ability to undergo homeostatic proliferation. Homeostatic proliferation of adoptively transferred cells and the presence of homeostatic cytokines such as IL-7 and IL-15

were shown to increase ACT efficacy [75, 76]. Adoptively transferred younger $CD8^+$ T cells need to get activated upon antigen stimulation, lyse the tumor target cells by secreting factors like Granzyme, and secrete cytokines such as IFNγ and TNFα [77]. $CD4^+$ T cells are also critical for tumor rejection and success of immunotherapy. Adoptively transferred $CD4^+$ T cells were shown to be required for supporting the $CD8^+$ cytotoxic anti-tumor response [78-80]. $CD4^+$ T cells do not merely enhance $CD8^+$ T cell function, but they also play a more direct role in tumor elimination during immunotherapy [81]. The roles that $CD4^+$ T cells play in the antitumor immune response depend on their polarization, which is determined by their expression of key transcription factors. Adoptively transferred Th1, Th2 and Th17 $CD4^+$ T cells can directly clear the tumor and promote long-lived antitumor immunity [82-85]. On the other hand, T_{regs} are immunosuppressive and were shown to inhibit T cell anti-tumor responses and promote immune escape [86, 87]. Overall, in order to achieve ACT immunotherapy success, not fully differentiated cytotoxic $CD8^+$ and $CD4^+$ T cells need to be genetically modified to recognize a suitable tumor antigen that is not expressed in an essential organ.

Immune Escape

Once adoptively transferred T cells reach the tumor, the major challenge they face is to overcome the multiple mechanisms the tumor can elicit to avoid immune-mediated elimination. The ability of T cells to recognize and destroy tumors relies on the antigenicity of tumor cells. Tumors can express a variety of mutated and endogenous antigens that can be recognized by T cells. However, to avoid anti-tumor responses the tumor cells can lose antigenicity [88]. Cancer cells can lose antigenicity due to lack or mutation of tumor antigens, as well as through the acquirement of defects and deficiencies in antigen presentation [89]. Downregulation of the cell

surface expression of MHC class I molecules was found in approximately 20% to 60% of solid tumors, including melanoma, lung, breast, renal, prostate, and bladder cancer [90, 91]. Tumors can not only lose antigenicity but also immunogenicity in order to escape immune responses. IFNγ produced by TILs can induce the upregulation of immunoinhibitory molecules on malignant cells, surrounding stromal cells, and other immunosuppressive infiltrating immune cells [36, 92]. Some of this immunoinhibitory molecules include but are not limited to ligands of the PD-1, CTLA-4, LAG-3, TIM-3, and VISTA inhibitory receptors [93]. As described before, these inhibitory pathways alter the balance between activation and inhibitory signals received by T cells and lead to dampened antitumor T cell responses. In addition to these mechanisms, tumor cells can also metabolically restrict T cell function via glucose and oxygen restriction. High glucose intake by tumor cells creates a hypoglycemic TME and dampen TIL mTOR activity, glycolytic capacity, and IFNγ production [94]. Solid tumors have large hypoxic areas which can also interfere with immunity and cause T cell dysfunction [95, 96].

Non-neoplastic infiltrating cells can contribute to tumor progression and metastasis by hindering the anti-tumor function of T cells and other immune cells. T_{regs} are immunosuppressive $CD4^+$ T cells that contribute to tumor immune evasion by secreting TGFβ and IL-10 which are cytokines that help create an immunosuppressive environment that blunts the anti-tumor functions of $CD4^+$ and $CD8^+$ effector T cells, and NK cells [97, 98]. T_{regs} also express an IL-2 receptor that has 100-fold higher affinity for IL-2 than the receptor form expressed in effector T cells resulting in T_{regs} acting as competitive sinks for IL-2 in the TME [99]. In addition, T_{regs} can secrete copious levels of the nucleoside adenosine [100]. Adenosine binds to its receptor ($A_{2A}R$) in effector T cells and downregulates the intracellular levels of 3', 5'-cyclic AMP which result in

T cells function inhibition and subsequent immunosuppression [101, 102]. In addition, the adenosine pathways promotes further polarization, proliferation and expansion of T_{regs} and myeloid derived suppressor cells (MDSCs) [103]. MDSCs are myeloid cells found in the TME that can also mediate immune escape through several mechanisms. MDSCs express high levels of nitric oxide synthase arginase 1 which results in the metabolism of arginine and the production of nitric oxide (NO) [104, 105]. The depletion of arginine from the TME impairs the local proliferative capacity of T cells. NO has a direct suppressive role on effector T cells by the induction of apoptosis, inhibition of STAT5 signaling, and formation of peroxynitrite, a potent oxidant of amino acids that are essential for T cell function [104, 105]. Other mechanisms of MDSC-mediated immune suppression include the sequestration of cysteine leading to the limited availability of this essential amino acid for T cells [106], the secretion of suppressive cytokines including IL-10 [107], and the overproduction of reactive oxygen species [108]. Other myeloid cells with immunosuppressive abilities that play a role in immune escape are TAMS and tumor associated dendritic cells (TADCs). TAMs produce lower levels of proinflammatory cytokines, such as IL-1β, TNFα, and IL-12, and higher levels of immunosuppressive mediators IL-10, TGFβ, and vascular endothelial growth factor (VEGF) indicating that TAMs not only suppress T cell function but promote tumor angiogenesis [109]. TAMs are also stunted in their ability to mediate direct lysis of malignant cells. TADCs express no or low levels of costimulatory molecules CD40, CD80, and CD86, and express indoleamine 2, 3-dioxygenase, an enzyme that degrades the essential amino acid tryptophan that leads to the suppression of T cell functions [110, 111]. TADCs also possess defects in the machinery to effectively present antigen to T cells and downregulate MHC class I and II molecules in addition to other proteins important for this process like transporter associated with antigen processing (TAP) [112].

Overall, tumors are complex structures composed of both malignant and nonmalignant cells that support cancer growth and prevent immune destruction. The understanding of the cellular constituents of the tumor microenvironment has helped guide the design of powerful T cell therapies that can cause the regression of large tumor burdens. Finding appropriate tumor antigens, overcoming the immunosuppressive and immunotolerant tumor microenvironment, and reducing are major obstacles being investigated today in order to improve the efficacy of cancer immunotherapy.

Sexual Dimorphism in Immune Responses: The role of Estrogen

Other examples of host specific factors that can affect the efficacy of immunotherapy are the patient's age, sex, and reproductive stage in life. While the lethal side-effects of immunotherapy are markedly exacerbated with aging [113, 114], age was shown to not significantly affect the outcome or clinical efficacy of checkpoint blockade immunotherapy [115]. Elucidating the effect of patient's sex on the outcome and efficacy of immunotherapy was proven to be controversial and there are very few studies investigating this. Meta-analysis of checkpoint blockade immunotherapy treated melanoma and NSCLC patients showed that men had overall better survival rates than women indicating a sex-difference in immunotherapy efficacy [116]. This conclusion cannot be generalized to all patient populations since other group found no sex-effect on the efficacy of Nivolumab in melanoma patients [117]. On the other hand, a preclinical model of melanoma treated with checkpoint blockade immunotherapy showed increased tumor rejection and overall survival in females compared to males [118]. This was found to be through mechanisms involving estrogen signaling in T_{regs} and Ag-specific CTLs [118]. While all these studies investigated the role of sex in checkpoint blockade

immunotherapy, there are no reports on the effect of sex and hormone signaling on ACT immunotherapy due to the lack of adequate sample size and statistical power. It is well known, though, that there are marked physiological differences in T cell immunological responses and tumor incidence between males and females [119-122]. While some of these sex-specific tumor and immunological differences are genetically and environmentally mediated, many of them accentuate with puberty and reproductive senescence indicating an involvement of sex hormones, androgen and estrogen. This indicates that sex and sex hormone signaling could be an important factor to consider in order to improve efficacy of anticancer immunotherapies and further research in this field is highly necessary. In this dissertation, the role of sex and estrogen receptor signaling on Ag-specific T cells for ACT immunotherapy is studied utilizing novel approaches to characterize the role of estrogen receptor signaling on T cell cytokine production and anti-tumor function.

Circulating estrogen is present physiologically in females, and in lower concentrations in males. Additionally, adult women are exposed to exogenous estrogen in therapeutic forms as oral contraceptives and hormone replacement therapy (HRT). The next sections focus on describing the signaling and downstream effects of physiological estrogen (17β-estradiol or E2), which is the most common form of active estrogen in the body, and the principal hormone employed in the studies for this dissertation. Then, the effect of estrogen signaling on main immune cell types which account for the observed sex-specific differences in immunity is described as well.

Sources of Estrogen

Physiologically available estrogens are predominantly produced in the ovaries, the corpus luteum and the placenta [123]. There are three major forms of physiological estrogens in

females, estrone (E1), estradiol (E2 or 17β-estradiol), and estriol (E3), and they are all synthesized from cholesterol by a series of reactions called estrogen biosynthesis [123]. E2 is the major estrogen biosynthesis product and plays a major role during women's pre-menopausal period. In the ovaries, E2 is synthesized in theca and granulosa cells. The luteinizing hormone (LH) stimulates cholesterol uptake by theca cells where it is converted into progesterone and later into androstenedione [124, 125]. In response to LH, androstenedione is then diffused into granulosa cells. Follicle-stimulating hormone (FSH) stimulates the enzyme aromatase in granulosa cells to convert androstenedione into testosterone and testosterone into E2 by aromatization of the A-rings of androgens [125]. Then, E2 is released into general circulation and targets distal estrogen-responsive tissues including reproductive and non-reproductive organs [123]. Circulating E2 reaches highest concentrations immediately before ovulation. During the follicular phase, pre-ovulatory phase, and luteal phase serum E2 concentrations are 50-140 pg/mL, 110-410 pg/mL, and 50-160 pg/mL respectively (ranging from 0.5-1 nmol/L or nM) [124]. During the menopausal transition, serum estrogen concentrations decrease by 85-90% [123]. Circulating E2 concentrations in menopausal women and men are below 35 pg/mL (under 0.1 nmol/L or nM) [124]. E1 can also be synthesized in the ovaries but plays a major role after menopause when it is synthesized by adipose tissue [123]. E3 is the least potent form of estrogen and it is synthesized in the placenta from E1 hydroxylation during pregnancy [123].

In addition to the ovary, extra gonadal estrogen biosynthesis takes place in astrocytes in the hypothalamus and hippocampus regions of the brain, in stromal cells and adipocytes in the breast, in osteoblasts and chondrocytes of the bone, in adrenocortical cells of the adrenal glands, in skin fibroblasts and in hepatocytes [126]. These peripheral sites biosynthesize estrogen which

tends to act locally at high concentrations, and which signaling is especially important in menopausal women and men [127]. High concentrations of locally produced estrogen can also be found in the TME of some cancers including breast cancer and hepatocellular carcinoma (HCC). These high estrogen concentrations found in the TME are partially due to aromatase expression dysregulation in tumor and stromal cells [128, 129]. Overexpression of aromatase in breast cancer was found in tumor, stromal and parenchymal cells and it was upregulated by factors including inflammatory cytokines and secreted factors like prostaglandins [130, 131]. Elevated aromatase expression was detected in cirrhotic and malignant HCC tissue [128, 132-134]. This elevated aromatase expression results on high local estrogen concentrations in the liver. In addition, estrogen metabolism is abnormal in patients with cirrhosis and HCC which show decreased conversion of E2 into E1 and E3 via hydroxylation, and subsequent accumulation of E2 in the liver or tumor tissue [135, 136].

Estrogen Receptors (ERs)

Estrogen exerts its physiological functions on target tissues through ER-dependent and ER-independent mechanisms [137]. Estrogen can bind three specific but distinct receptors including the nuclear receptors estrogen receptor alpha and beta (ERα and ERβ) and the G-protein coupled estrogen receptor (GPER). ERα and ERβ are ligand activated transcription factors which are located intracellularly and to some degree on the cell membrane [138]. Apart from the gonads, each ER is differentially expressed in various tissues. ERα is expressed in high concentrations in the mammary glands, the pituitary, the kidney, the epididymis, the liver, bone and the adrenal gland but it has very low or undetectable expression in the prostate, the pineal gland, the thyroid gland, the urinary tract, and erythroid tissue [126, 139]. ERβ is expressed

predominantly in the prostate, the lungs, the bladder, and the hypothalamus [139, 140]. GPER is ubiquitously expressed in the membrane of cells throughout the body including the heart, brain, pancreas, skeletal muscle, kidney, vessels, and reproductive organs [141].

Structure of Estrogen Receptors

ERα and ERβ belong to the steroid nuclear receptor family, which is composed of proteins with four independent but interacting protein domains [142]. The A/B domain in the amino (NH_2) terminal, the C or DNA-binding domain, the D domain which is a hinge region, and the E/F or the ligand binding domain in the carboxyl (COOH) terminal [143, 144] (Figure 2). The NH_2 terminal domain encodes a ligand-independent activation function (AF-1) which is a region of the receptor involved in protein-protein interactions with other transcription factors and co-activators of target-gene expression [144]. In ERα this region was shown to be very active in stimulating target-gene expression, and AF-1 was shown to be active even in the absence of estrogen ligand [145]. In ERβ the activity of AF-1 is negligible and most of the protein-protein interactions happen through AF-2 found in the ligand binding domain (LBD) (Figure 2) [146]. The DNA binding domain (DBD) is 97% homologous between ERα and ERβ and contains a zinc finger structure necessary for receptor dimerization and binding of the receptors to specific DNA sequences denominated estrogen response elements (EREs) [144, 147]. The hinge region contains the nuclear localization signal and links the C domain to the multi-functional COOH-terminus E/F domain [142] (Figure 2). The hormone dependent AF-2 region in the E/F LBD is important in estrogen ligand binding dependent transcriptional activity and co-activator protein-protein interactions [148]. Besides ligand binding, the E/F region is necessary for nuclear translocation, heat shock proteins interactions, and transactivation of target gene expression

[144]. Heat shock protein such as Hsp90 act as ER chaperones in the absence of ligands, binding to unliganded ERs and maintaining the receptors in an inactive yet functional state [149]. The LBD of ERα and ERβ are highly homologous in both primary amino acid sequence and tertiary structure which results in similar affinity of both receptors for E2 and other antagonistic and agonistic compounds [139, 150] (Figure 2).

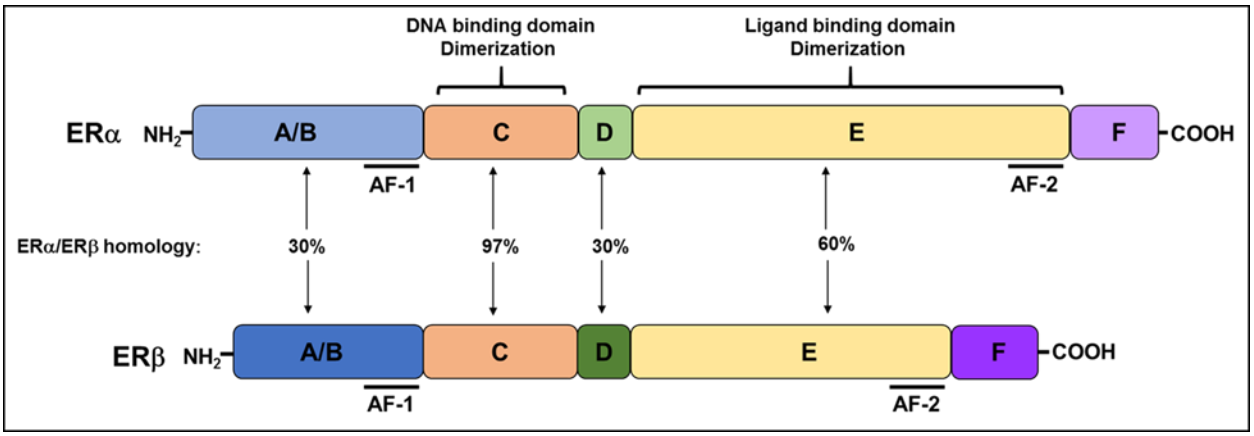


Figure 2. Structure of Estrogen Receptor α and β and Percent Homology between Them. Estrogen receptor α and β (ERα and β) are nuclear receptors that can act as transcription factors upon ligand binding. ERα and β are highly homologous and contain four different functional domains: the A/B domain in the amino (NH_2) terminus which contains the AF-1, the C or DNA-binding domain, the D domain or hinge region, and the E/F carboxyl (COOH) terminus domain which contains the ligand binding domain and the AF-2 important for receptor dimerization. AF: activating function.

GPER is a membrane bound G_s-protein coupled receptor. GPER associates with a $G\alpha_s/G\beta\gamma$ complex in its inactive state. Upon estrogen ligand binding and GPER activation, $G\alpha_s$ and $G\beta\gamma$ quickly dissociate from GPER. $G\alpha s$ and $G\beta\gamma$ can then activate several signaling pathways some of them which result in gene expression regulation [151]. GPER was also found

to be expressed intracellularly in some specific tissues including the mammary glands, the ovarian epithelium, and breast and ovarian cancer cells [141]. This indicates that GPER expression and signaling is not restricted to the plasma membrane but can also signal from the intracellular membranes of the endoplasmic reticulum (ER) and the Golgi apparatus [151].

Estrogen Receptor Signaling

Ligand-bound ERα and ERβ can regulate the transcription rates and overall expression of target genes by binding to EREs on their promoters or by assembling transcription regulation complexes with other transcription factors. It was also previously shown that ERs can regulate target gene expression in a ligand-independent manner. Membrane bound GPER signals upon estrogen ligand binding and can activate several signaling pathways that regulate many cell processes and can ultimately result in gene expression modulation.

Ligand-Dependent Estrogen Signaling

The classical pathway: the ERE-dependent genomic pathway (Figure 3A).

Transcription activation by ligand-bound ER is a multistep process that occurs in a sequential order and requires the interaction of the ER with several enzymatic activities in order to obtain a productive interaction with the transcription complexes [152]. Ligand-operated transcription by ERs is initiated when the estrogen ligand binds to the inactive ER-chaperone complex [152]. The estrogen ligand binds to the ER C-terminal LBD causing a conformational change which mediates the dissociation of the ER from its chaperones [152]. Shedding the chaperones exposes the dimerization, nuclear localization, and DNA binding domains. Dimerization of the ER strengthens the stability of the receptors and ensures binding to the EREs on target-gene promoters [152]. EREs are direct or indirect palindromic sequences separated by three non-

specific nucleotides (TCCAGTnnnACTGGA or AGGTCAnnnTGACCT) [153]. Once bound to the DNA, ER transcriptional ability depends on the AF-1 and AF-2 activating functions. Although AF-1 and AF-2 can function independently, maximal ER transcriptional activity is achieved when there is synergy between them [154]. AF-1 and AF-2 serve as sites for interaction with several co-activator or co-repressor proteins. Co-activator proteins enhance gene expression by remodeling chromatin and allowing interactions with the transcription machinery. The AF-1 region of the ER interacts with co-activators from the p160 family which include the steroid receptor co-activators 1, 2 and 3 (SRC-1, 2, 3) [155]. p160/SRC function predominantly by recruiting chromatin modifying enzymes such as histone acetylases (HATs) to the ER-DNA complex [156]. Histone deacetylation results in chromatin de-condensation and increased transcription of target genes. p160/SRC can also recruit other co-activators such as CREB binding protein (CBP) and p300 [157, 158]. The hydrophobic region of the AF-2 domain binds co-activator proteins such as GRIP1 and TIF2 [159, 160].

AF-1 and AF-2 can also recruit negative co-repressors to the ER-DNA complex which inhibit gene activation or turn off activated genes. Negative coregulatory factors RIP140 and LCoR compete with the p160/SRC co-activators for AF-1 and AF-2 binding [161, 162]. Repression by RIP140 and LCoR occurs through the recruitment of histone deacetylases (HDACs) which results in chromatin condensation and suppression of the estrogen target genes expression [156]. Other molecules found in the ER-DNA transcription complex can also recruit co-repressors. While p300 is considered a co-activator of the ER due to its activity enhancing transcription initiation when recruited to ERα and ERβ, it was shown that p300 can also recruit the co-repressor C-terminal-binding protein 1 (CtBP1) to the ER transcription factor complex.

CtBP1 can stop and inhibit the transcription of target genes by recruiting HDACs which deacetylate histone 3 lysine 9 (H3K9) and H3K13 [163]. Overall, transcription of estrogen target genes is predominantly enhanced by the binding of the ligand-bound ER homo or heterodimer to EREs on gene promoters and the recruitment of co-activators but in some cases, co-repressors compete with co-activators for ER complex binding which results in target gene expression downregulation.

Both ERα and ERβ can signal this way in order to regulate target gene expression. Interestingly, ERα and ERβ can form homo or heterodimers upon ligand binding (ERαERα, ERβERβ or ERαERβ) [164]. These homo or heterodimers show differential transcriptional activities and could explain the selective actions of estrogen in different cell types and target genes. Although both ERα and ERβ are expressed in tissues and form functional heterodimers; when co-expressed, ERβ can inhibit the transcriptional ability of ERα and vice versa [165, 166]. Therefore, overall estrogen responsiveness in specific tissues depends on the ERα versus ERβ ratio.

The ERE-independent genomic pathway (Figure 3B)

In addition to the classical mechanism of ER function, ERs can regulate gene expression of genes that lack EREs. Estrogen-bound ER dimers can bind to Jun and Fos dimers located on gene activating protein-1 (AP-1) binding sites [167]. The co-activators (p160/SRC) recruited by the ERs to Jun/Fos subunits of the AP-1 transcription factor which can then activate transcription of the AP-1 site containing genes [167, 168]. Another way that ERs can affect transcription of genes is by physically interacting with the Sp1 transcription factor. Ligand-bound ERs enhance

Sp1 DNA binding which upregulates target gene expression [169]. ERs can also repress the transcription of genes in an ERE-independent manner. ERα can interact with and sequester the c-Rel subunit of the NFκB transcription factor complex [170]. This interaction prevents NFκB from binding and stimulating expression of NFκB response element containing genes [171].

Ligand-independent estrogen signaling (Figure 3C)

Another mechanism of ER action involves the ligand-independent ER activation through ER phosphorylation by protein kinases or growth factor receptors. The epithelial growth factor receptor (EGFR) and the insulin-like growth factor receptor (IGFR) are able to bind the AF-1 domain but not the AF-2 domain and phosphorylate ER via the MAP kinase or the PI3 kinase (PI3K)-Akt pathways [172, 173]. ERs can also be directly phosphorylated by protein kinase A (PKA) or PKC [174, 175]. Phosphorylated non-ligand bound ERs can then homo or heterodimerize and translocate into the nucleus where they regulate expression of ERE-containing and non-ERE containing genes even in the absence of estrogen ligand.

Non-genomic membrane bound estrogen signaling (Figure 3D)

In addition to the nuclear genomic actions, estrogen signaling can exert rapid effects non-accounted for by the transcriptional mechanisms. These non-genomic changes by rapid signaling are provoked by membrane-bound ERα and GPER. Palmitoylation of ERα anchors a pool of ERα to the plasma membrane where they can interact with other signaling proteins such as Src and p85, the regulatory subunit of PI3K [176]. Estrogen signaling through GPER occurs through transactivation of EGFR and involves activation of non-receptor tyrosine kinases of the Src family [177]. Estrogen binding to GPER induces activation of metalloproteinases which induce

the release of heparin-binding EGF, which binds and activates EGFR leading to rapid activation of downstream signaling molecules such as the MAP kinase pathway including ERK1/2 [177]. Estradiol activation of GPER also stimulates production of intracellular cyclic adenosine monophosphate (cAMP), induces intracellular Ca^{2+} mobilization, and PI3K activation. In addition to these rapid signaling events, GPER was also shown to be internalized from the plasma membrane and was demonstrated to regulate gene transcriptional activity of genes such as c-Fos, one of the monomers of the AP-1 transcription factor complex [178]. Overall, signaling though membrane-bound ERs is more rapid than genomic ER signaling and leads to rapid activation of signaling pathways that result in cellular processes such as increase DNA synthesis and cell proliferation, and further gene transcription regulation.

Estrogen Effect on Key Cells of the Immune System

Estrogen receptors are expressed ubiquitously on immune cells and estrogen is known to modulate the survival, development, differentiation, and function of key cells of the immune system such as T and B cells, macrophages, dendritic cells (DCs) and natural killer cells (NK) [179]. In the following paragraphs, the effects of estrogen signaling on different cells of the immune system are described.

T cells & T cell cytokines

In the peripheral blood of humans, around 30% of white blood cells are lymphocytes and around 85-90% of these lymphocytes are T cells [180]. Although the total lymphocyte count in males is equivalent to females, the percentage of T cells within the total lymphocyte population is higher in females compared to males and post-menopausal females [181]. Females have

higher $CD4^+$ T cell counts and higher $CD8^+/CD4^+$ ratios than age-matched males and post-menopausal females; whereas males have higher $CD8^+$ T cell frequencies [182-184].

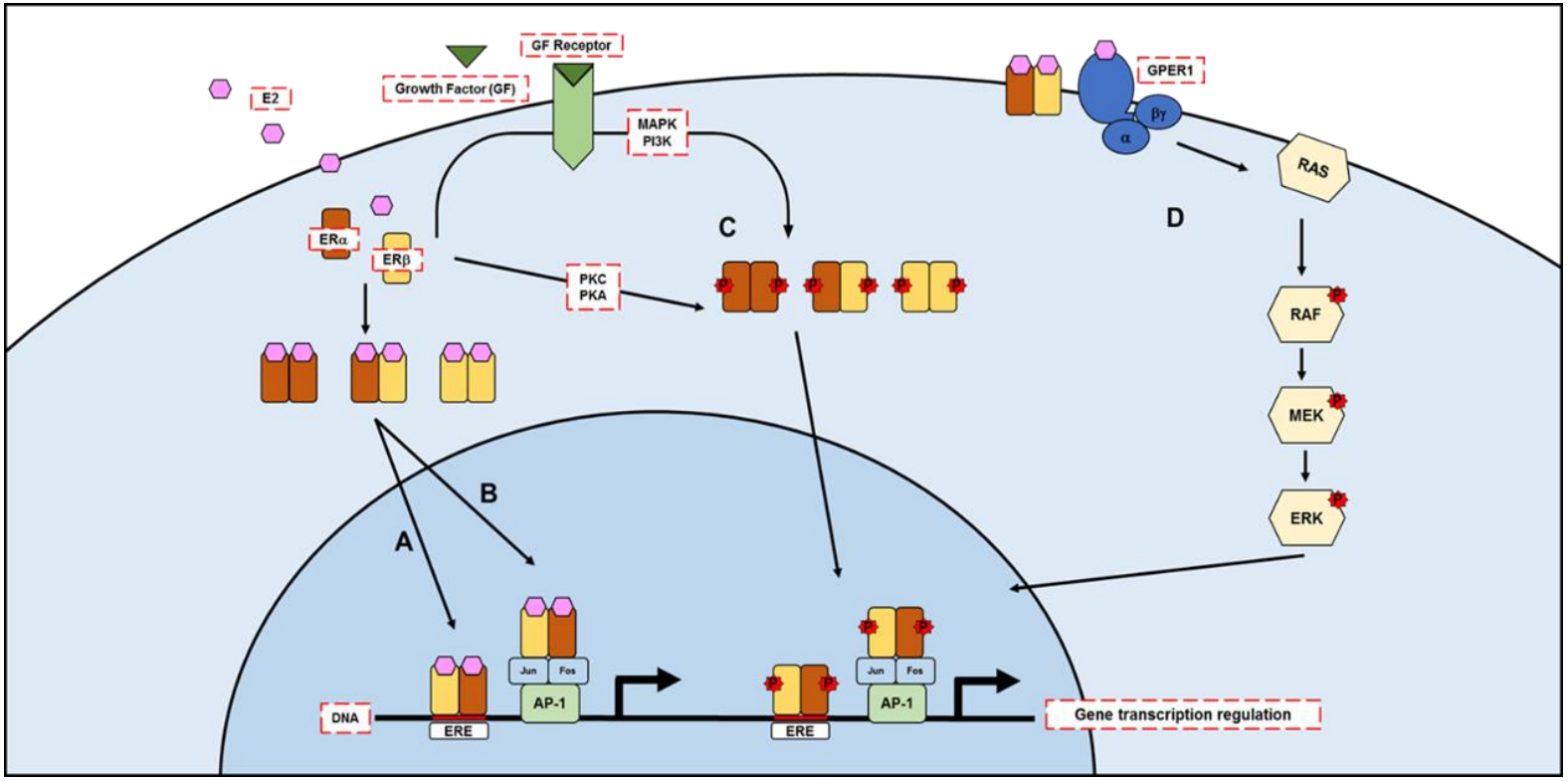


Figure 3. The Different Types of Estrogen Signaling in the Cell. The ligand-dependent and ERE-dependent or ERE-independent classical genomic estrogen receptor pathway consists on ERs binding to the estrogen ligand in the cytoplasm of the cell, dimerizing, and translocating into the nucleus where they bind **A)** EREs in gene promoters, or **B)** form complexes with other transcription factors such as AP-1 and regulate gene expression. **C)** The ligand-independent genomic estrogen receptor pathway happens when growth factor tyrosine kinase receptors or protein kinases phosphorylate the ERs promoting dimerization and translocation into the nucleus where they regulate gene expression in a ERE-dependent or independent manner. **D)** The non-genomic G-protein coupled estrogen receptor signaling happens through GPER which activated the MAP kinase pathway upon estrogen ligand binding promoting downstream signaling and gene expression regulation. E2: 17β-estradiol, ERE: estrogen response element, AP-1: activator protein-1, PKC/A: protein kinase C/A, MAPK: mitogen activated kinase, PI3K: phosphoinositide-3 kinase.

Following *in vitro* T cell stimulation, females have higher numbers of proliferating T cells and higher numbers of $CD8^+$ and $CD4^+$ activated T cells compared to males [181]. Transcriptional analysis of these activated T cells showed greater cytotoxic activity in T cells from adult females, with stimulated female T cells upregulating more antiviral and pro-inflammatory genes compared to male activated T cells [185]. Notably, over half of these activated genes have EREs in their promoters indicating direct involvement of estrogen signaling on T cell function [185]. Both $CD8^+$ and $CD4^+$ T cell subsets express ERα and ERβ and estrogen signaling was shown to also affect T cell development and differentiation.

Estrogen stimulation was shown to cause structural changes in the thymus and to affect immature T cell development. Ovariectomized mice that were treated with estrogen showed dose dependent thymic atrophy with a decrease proportion of early T cell progenitors, and an increased proportion of mature T cell populations [186]. Estrogen inhibits thymic development by decreasing the number of triple negative immature T cells ($CD3^-CD4^-CD8^-$) and depleting the rest of the maturation stages such as $CD44^+$ $CD25^+$, $CD44^-CD25^+$, and $CD44^-$ $CD25^-$ T cells [186]. On the other hand, estrogen treatment increased the numbers of thymic $CD8^+$ and $CD4^+$ single positive mature T cells. All these effects were mediated by ERα AF-1 and AF-2 functions simultaneously since the deletion of the receptor or any of these functions abolished the inhibitory effects of estrogen on the thymus [187]. Ovariectomized mice showed reduced levels of $CD8^+$ splenic T cells and the $CD8^+/CD4^+$ single positive cell ratio was increased after physiological estrogen stimulation agreeing with the human observation on pre-menopausal females [188]. Overall these data indicated that estrogen signaling though ERα induces differentiation of mature $CD8^+$ and $CD4^+$ T cells.

Besides affecting the development of T cells, estrogen is capable of affecting T cell activation and T cell function. T cells stimulated with post-menopausal estrogen levels showed decreased activation-induced phosphorylation of the CD3/TCR complex and reduced expression of the Janus kinases 2 and 3 (JAK2 and 3 kinases) compared to T cells treated with physiological estrogen [189]. JAK2 and 3 are non-receptor tyrosine kinases that mediate cytokine-mediated signals via the JAK-STAT pathway in response to IL-2 which secretion was also downregulated in T cells treated with low estrogen [189]. In a model of colitis, it was observed that ERα-deficient mice showed decreased T cell proliferation and activation state, and increased FoxP3 expression compared to WT mice indicating that T cells in ERα-deficient mouse were skewed towards a regulatory phenotype decreasing the severity of the autoimmune inflammation [190]. In addition to enhancing T cell activation, physiological estrogen stimulation was shown to induce Th1 effector T cell responses. IFNγ is a major cytokine involved in cytotoxic and effector Th1 T cells responses and it is sensitive to estrogen stimulation in mice and humans. Estrogen can modulate IFNγ secretion by enhancing *Ifnγ* gene expression in $CD4^+$ and $CD8^+$ T cells [190]. This happens through direct interaction of ERα with an ERE in the promoter of the *Ifnγ* gene [191, 192]. Administration of physiological estrogen to ovariectomized mice resulted in an increase of IFNγ producing cells as well as an increase of Th1 $CD4^+$ antigen-specific T cell responses [193]. Overall this indicates that physiological estrogen signaling through ERα promotes effector and Th1 responses which are reduced in the presence of low estrogen concentrations like the ones found in post-menopausal females and males.

Most of the important aspects concerning the role of estrogen signaling on T cell function were studied using T cells from autoimmune disease patients, and autoimmunity mouse models

including multiple sclerosis (MS) and colitis. Using primary T cells from MS patients that were treated with estrogen, it was shown that estrogen signaling mediated a dose dependent enhancement on the secretion of IL-10 by $CD4^+$ T cells [194]. The secretion of IFNγ by T cells was also increased by estrogen in a dose dependent manner confirming the genomic regulation of IFNγ through the ERE on its promoter [194]. The effects of estrogen on expression of TNFα expression were shown to be controversial. Physiological estrogen concentrations were shown to enhance TNFα expression, while pregnancy estrogen levels inhibited TNFα expression [194]. On the other hand, in bone research studies, ovariectomy of mice enhanced the production of TNFα by $CD8^+$ and $CD4^+$ T cells, and the subsequent bone loss which was abolished by estradiol replacement therapy [195].

Estrogen-induced changes in IL-4 mRNA or protein expression were not observed in response to estrogen stimulation in MS patient's primary T cells [194]. Interestingly, studies characterizing the role of estrogen during pregnancy, a state where estrogen levels are higher than physiological, demonstrated that the T cell immune response skewed toward the Th2 type compared to Th1 type response [189]. Estrogen at pregnancy levels increased IL-4 secretion and GATA3 expression in $ER\alpha^+CD4^+$ cells but not on $ER\alpha^-$ ones [190]. IL-4 is a common cytokine secreted by Th2 cells and its expression is induced by GATA3 which is the transcription factor that drives Th2 T cell differentiation. Peripheral mononuclear blood cells (PBMCs) stimulated with different estrogen levels showed that those cells treated with physiological estrogen enhanced production of IFNγ, IL-12, and IL-10 which is a Th1 skewed immune response [191]. On the other hand, cells treated with pregnancy estrogen levels had decreased IL-12 and IFNγ ratio while IL-10 production was enhanced [191]. Overall, the estrogen mediated enhancement

of cytokines such as IL-10 and IL-4 by T cells switches the T cell immune response from Th1 to Th2.

Estrogen signaling has also been shown to affect immunosuppressive T_{regs}. Pregnancy estrogen levels were reported to increase the expression of FoxP3 expression on $CD4^+CD25^+$ cells [196-199]. Pregnancy estrogen levels were able to enhance FoxP3 expression *in vivo* and *in vitro* and increase $CD25^+$ cell number, which are reduced in $ER\alpha^-CD4^+$ cells [198]. Mouse studies also showed that physiological estrogen concentration could induce expression of FoxP3 and enhance the number of $CD25^+$ regulatory $CD4^+$ T cells [188]. The role of estrogen modulating T_{reg} differentiation proves to also be controversial since post-menopausal women were shown to have enhanced numbers of circulating T_{regs} compared to pre-menopausal women [189].

While many studies have reported the effect of estrogen signaling on T cell function and cytokine production, many of the reported results are contradicting and indicate opposite roles of estrogen signaling on T cell function especially in cytokine expression and production. Overall the studies summarized in this section indicate that the effect of estrogen on T cell function and cytokine production is highly concentration dependent. Different effects were observed when T cells were exposed to physiological, post-menopausal and pregnancy estrogen concentrations. The effects of estrogen on T cell function and cytokine production were also affected by the species in which they were tested with contradicting results obtained when testing the role of estrogen signaling on human compared to mouse T cells. This indicates differences in T cell biology and estrogen signaling between these species. Regarding T cell differentiation, estrogen was reported to promote Th1, Th2, or T_{reg} $CD4^+$ T cell differentiation depending on the

concentration of the hormone, again indicating concentration dependent effects. While these studies demonstrated the effect of estrogen in signaling in T cell function and differentiation, not many of them reported through which receptor estrogen is signaling through on each case. Most of the time, estrogen signaling through ERα was investigated while ERβ and GPER were not mentioned. This indicates there is a high need for research characterizing the effects of estrogen signaling at different concentrations through each of its receptors and signaling pathways on T cell function, including the expression of several cytokines, as well as T cell differentiation.

B cells and Antibody Production

Regardless of age, females show greater B cell numbers, higher antibody responses, and higher basal immunoglobulin levels than males [181]. Estrogen affects the development and activity of B cells at different stages of differentiation and function, which can also vary among lymphoid organs. For example, in the bone marrow, estrogen was shown to decrease the number of B cells by negatively affecting the viability, proliferation and differentiation of early B cell precursors [200]. On the other hand, estrogen treated ovariectomized mice showed increase splenic weight and enlarged volumes of white and red pulp [201]. Even if estrogen treatment promoted a decrease in $B220^{+}$ splenic lymphocytes, there was a 10-fold increase in plasma cell numbers and this effect required signaling though both ERα and ERβ [202, 203]. Estrogen was also shown to increase splenic B cell activation and plasma cell antibody secretion [204, 205]. It was also shown that estrogen increases the expression of the anti-apoptotic gene *bcl-2*, and other genes such as *cd22*, *shp-1*, and *vcam-1* in B cells that result in increased survival and activation [206, 207]. Estrogen signaling also modulates B cell function by increasing immunoglobulin secretion such as IgG and IgM through ERα signaling but not ERβ [208]. This overall indicates

that while estrogen signaling inhibits B cell lymphopoiesis in the bone marrow, it enhances mature B cell survival and differentiation into plasma cells as well as antibody production upon activation.

Macrophages and Dendritic Cells

Antigen presenting cells, such as macrophages and dendritic cells, which are crucial for induction of immune responses are another target of estrogen. Macrophages express ERα, ERβ, and GPER and estrogen signaling was shown to modulate macrophage polarization, activation, and cytokine production [209]. Macrophages can be classified into classically (inflammatory) or alternatively (anti-inflammatory) activated macrophages depending on the activation stimuli. Classically activated macrophages result from activation in the presence of Th1 cytokines including IFNγ or TNFα, while alternatively activated macrophages are activated in presence of Th2 cytokines including IL-4 or IL-13 [210]. Classically activated macrophages downregulate ERα expression while they maintain ERβ and GPER expression, while alternatively activated macrophages retain ERα and ERβ expression [210]. Many studies have described how physiological estrogen can upregulate inducible nitric oxide synthase (iNOS) and NO production, increased production of proinflammatory cytokines and increased cell surface expression of Toll-like receptor 4 (TLR4) in human and mice classically activated macrophages probably through mechanisms involving ERβ or GPER [211-215]. This leads to increased macrophage activation and killing thus to increased resistance to extracellular bacteria and infections in the presence of estrogen [216]. On the other hand, high dose estrogen stimulation reduced the production of inflammatory cytokines such as IL-1, IL-6, and TNFα in activated macrophages indicating a dose dependent effect of estrogen [217].

Dendritic cells express ERα and ERβ and estrogen signaling was shown to affect functional DC differentiation from bone marrow progenitors. Differentiation of DC bone marrow progenitors stimulated with granulocyte macrophage-colony-stimulating factors (GM-CSF) was inhibited when cells were cultured in estrogen-deficient media or *in vivo* in ovariectomized mice [218-220]. DC differentiation was restored when physiological estrogen concentrations were introduced back. The estrogen-mediated DC bone marrow progenitor differentiation was found to be mediated by ERα and not ERβ signaling. ERα signaling increases the expression of IRF4, a key transcription factor induced by GM-CSF and critical for DC development [218, 219]. These estrogen-treated mature DCs were $CD11c^{+}CD11b^{intermediate}Ly6C^{-}$ and expressed high levels of MHC class II and co-stimulatory molecules B7.1, B7.2 and CD40 [220, 221]. These DCs were able to stimulate the proliferation of $CD4^{+}$ T cells and were able to produce higher levels of the inflammatory cytokines IL-16 and IL-12 [221]. Short-term estrogen stimulation had no effect on DC survival or surface marker expression. However, estrogen stimulation enhanced activated DC IL-6, IL-8, and monocyte chemoattractant protein 1 (MCP-1) production and it enhanced the migration of mature DCs towards chemokine C-C motif ligand 19 (CCL19) [222]. Overall, these data indicate that estrogen signaling enhances the differentiation and function of DCs which can further result on more potent immune responses from DC-activated B cells and T cells.

NK cells

NK cells are large granular lymphocytes from the innate immune compartment which attributed with the ability to directly lyse virus-infected cells or tumor cells, and to produce and release type I cytokines such as IL-2 or IFNγ. Surprisingly, the number and activity of NK cell

changes during the menstrual cycle of human females [223]. NK cell activity increases during the first trimester of pregnancy, but it significantly decreases during the second and third trimesters indicating an important role of estrogen in regulating NK cell activity [224]. NK cells express both ERα and ERβ and estrogen signaling was shown to affect NK cell function. Prolonged high concentration estrogen stimulation was shown to increase overall NK cell numbers but to reduce NK cell activity in a dose dependent manner [224-226]. NK cell numbers were increased by the estrogen mediated upregulation of minichromosome maintenance component 7 and 10 (MCM7 and MCM10) expression which are proteins required for DNA replication and NK cell proliferation [226]. On the other hand, the suppressed cytotoxicity of NK cells was attributed to the estrogen down-regulation of activating receptors NKp46, NKG2D, and CD244 which resulted in reduced secretion of Granzyme B and Fas ligand [226]. This suppression of NK cell activity was observed in several mouse strains and human cells when the duration of the estrogen treatment was prolonged over one month, and it was mediated by ERβ signaling since no differences were observed when ERα knockout (KO) mice were used [224]. This overall indicates that while estrogen signaling enhances NK cell proliferation, it decreases NK cell activation and cytotoxic function especially when NK cells are exposed to high dose estrogen for long periods of time like in the case of pregnancy.

Estrogen and Disease

As described in the previous section, estrogen signaling has profound effects on the development, differentiation, and function of key immune cells indicative that estrogen signaling also affects those diseases that arise from immune cell function dysregulation, like

autoimmunity, or those that are heavily controlled by immune responses, like infections and cancer. In the next paragraphs the known effects of estrogen on these diseases are described.

Estrogen and autoimmunity

About 8% of the world's population suffers from autoimmune diseases, of which 78% of all autoimmunity cases are women [227]. The female to male autoimmune disease ratio ranges from 2:1 in rheumatoid arthritis (RA) to 3:1 in MS, and to 9:1 in systemic lupus erythematosus (SLE) [227, 228]. The effects of estrogen on autoimmune diseases cannot be generalized since estrogen signaling differs depending on concentration, the estrogen receptor, and the target cell type. Estrogen was shown to enhance the severity of some autoimmune diseases. Estrogen signaling promotes systemic inflammation and induces B cell activation and anti-double stranded DNA antibody production which increases reactivity to endogenous antigens that are expressed in SLE [208, 229]. Altered expression of ER was shown in immune cells of SLE patients which have upregulated expression of ERα and decreased expression of ERβ compared to healthy controls [230]. Increased ERα expression in $CD4^+$ and $CD8^+$ T cells, macrophages, and DCs in SLE enhances Th1 type autoimmune responses by the production of cytokines including IFNγ and IL-12 [231]. T cells from SLE patients also have upregulated expression of the co-stimulatory ligand molecule CD40L which is shown to be important for activated Th1 T cells priming and IFNγ production [232]. In a T cell dependent inflammatory bowel disease (IBD) model, estrogen signaling through ERα worsens prognosis by promoting the accumulation of Th1 and Th17 T cells, and increasing T cell activation and proliferative state [233].

Contrary to other autoimmune disorders, estrogen has a protective role against MS, a disease characterized by the presence of myelin reactive $CD4^+$ T cells in the central nervous

system which leads to demyelination of axons and neuronal death [234-236]. Estrogen signaling through ERα decreases autoantigen-specific proinflammatory molecules such as TNFα, IL-17, iNOS, and MCP-1 and inhibits autoimmune inflammation [236]. ERβ signaling was also shown to be necessary for the estrogen-mediated protection against MS and for enhancing endogenous myelination [237]. Pregnant mice showed reduced CNS pathology when compared to non-pregnant animals, and the rates of relapse increases post-partum at a phase where there is a marked decrease in estrogen levels compared to pregnancy [238].

Estrogen was shown to have different effects in various autoimmune disorders. Given that estrogen affects all cells of the immune system as well as non-lymphoid tissue that is in proximity of target tissue, it is understandable that estrogen has different effects dependent on the context of each autoimmune disorder. While estrogen plays a role in sex differences in autoimmune diseases, clearly estrogen alone does not exclusively contribute to this sex differential susceptibility.

Estrogen and infection

Females and males differ in the severity, prevalence, and pathogenesis of infections caused by bacteria, virus, fungi and parasites, with males being generally more susceptible to these infections than females [239]. Males exhibit higher incidence rates for leptospirosis (4-fold increased incidence compared to females), schistosomiasis (1.5-fold), brucellosis, rabies, leishmaniasis (3-fold), pulmonary tuberculosis (2.5-fold), and hepatitis A, B and C (1.5 to 3-fold) [119, 240-243]. Similarly, premenopausal women are significantly less likely to develop meningococcal or pneumococcal infections [119]. Estrogen is thought be protective against infections due to the pro-inflammatory effects of estrogen signaling on immune cells.

Physiological estrogen concentrations were used to treat different kinds of infections in murine models and humans. Physiological estrogen administration was shown to reduce infection in males and females that suffered viral infections with encephalomyocarditis or Friend virus [244, 245], bacterial infections with *Salmonella typhimurium* [246], fungal infections with *Candida albicans* and *Paracoccidioides brasiliensis* [247, 248], and even infections with parasitic protozoa like *L. Mexicana* [249]. Overall, these data indicate that estrogen signaling has a protective role against some infectious diseases through the enhancement of immune cell function against pathogens. Heightened immunity to pathogens among females contributes to lower intensity, such as lower viral load found in the serum of hepatitis or HIV female patients [250], and prevalence of infected individuals within a population compared to males, but it may increase disease symptoms and severity among females compared with males as seen in infections including malaria or HIV [251].

Estrogen and cancer

Sex is an important factor in the pathogenesis and prognosis of cancers that occur outside of the reproductive tract [121]. For the majority of cancers throughout life, males have a higher risk of malignancy compared to females [252, 253]. Males have two-fold greater rates of mortality from malignant cancers compared to females, with sex-dependent outcomes being greatest for larynx, esophagus, lung and bronchus, skin, and liver cancers [253]. This male-biased incidence and mortality is hypothesized to reflect a combination of factors including but not limited to sex-differences in genomic predisposition, infection, immune function, gene expression, and hormonal regulation. Estrogen is thought to have a protective role against cancer which is supported by the observation that cancer incidence and mortality rates increase after

menopause in women not undergoing hormone replacement therapy [254]. In some specific cancers such as melanoma or liver cancer, estrogen signaling has an antitumor role by enhancing antitumor immune responses [255-257]. Despite the normal and beneficial actions of physiological estrogen in women, estrogen was classified as a carcinogen after the Women's Health Initiative research program discovered that postmenopausal women treated with HRT had a significant increase in the incidence of breast cancer after at least 5 years of treatment [258]. The effects of estrogen are tissue-specific and depend on which ER is predominantly expressed and further which downstream signaling pathways become activated.

Estrogen receptor signaling is well known to be cancer promoting in ER-positive (ER^+) breast tumors. In breast cancer cells, ERα regulates various genes that play key roles in cell cycle progression and proliferation. ERα can transcriptionally upregulate c-Myc (as early as 15 minutes after estrogen stimulation) and cyclin D1 which are necessary for the G1-to-S phase transition [259, 260]. On the other hand, ERβ signaling can induce G2 cell cycle arrest and inhibit the estrogen-induced cancer cell proliferation [261-263]. Expression of ERα and ERβ in human NSCLC is correlated with worse prognosis and more aggressive disease [264, 265]. ERα and ERβ are found in the nucleus and cytoplasm of NSCLS cells and are phosphorylated in residues commonly modulated by growth factors indicating ligand-independent ER signaling [266, 267]. ER signaling promotes the early activation of the MAP kinase pathway which is oncogenic in NSCLC [266]. In ovarian cancer, estrogen signaling was correlated with both worse and better disease prognosis. Estrogen levels are often observed in ovarian cancer patients and ERα signaling promotes metastasis by inhibiting cell-cell adhesion and cell survival and proliferation [268, 269]. On the other hand, ERα expression in ovarian cancer predicts longer

overall survival [270-272]. There is also contradicting evidence about the role of ERβ in ovarian cancer. Some early studies correlate ERβ expression with worse ovarian cancer prognosis [273], but ovarian cancer meta-analyses showed that ERβ expression is downregulated as disease progresses and ERα is predominantly expressed in advanced and metastatic disease [272, 274, 275].

In some cancers including endometrial, prostate, liver cancer and melanoma estrogen signaling contributes to decreased tumor risk and better disease prognosis. Meta-analyses revealed that ER expression of both ERα and ERβ simultaneously correlate with better overall survival of endometrial carcinoma [276]. Even if meta-analyses were not able to correlate prostate cancer prognosis with ER expression, prostate cancer cells express ERα, ERβ and GPER and decreasing estrogen concentrations were observed to be associated with increased prostate cancer risk [277, 278]. Melanoma tumors express both ERα and ERβ, and ERβ expression decreases as the melanoma tumor develops. Although the mechanisms remain unclear, it is thought that ERβ acts as a tumor suppressor but it is downregulated as the tumor progresses [257, 279]. Estrogen can suppress the growth of human melanoma by inhibiting IL-8 production and subsequent chronic inflammation through ERα signaling [280]. Estrogen is a key molecule in cellular development and cell cycle regulation and therefore impacts many varieties of cancer cells directly. In summary, ERβ expression is correlated with better prognosis and survival, and it can be concluded that ERβ acts as a tumor suppressor in most cancers. ERα acts as an oncogene in breast, lung and ovarian cancers, even if ERα expression is correlated with better prognosis on melanoma, prostate, and endometrial cancer.

Another cancer that is heavily affected by sex and estrogen receptor signaling is HCC. HCC is the main disease model used in the studies of this dissertation. The next paragraphs describe current knowledge on the causes and treatments of HCC, as well as the role of estrogen on tumorigenesis.

Hepatocellular Carcinoma

Primary liver cancer consists of a heterogeneous group of malignant tumors not including liver metastases from other cancer sites [281]. Primary liver cancer is the second leading cause of cancer-related deaths worldwide, and it accounts for approximately 800,000 deaths each year [282, 283]. Hepatocellular carcinoma (HCC) is the most common primary liver cancer and it accounts for 80-90% of all cases [284]. The incidence of HCC is highest in East and Southeast Asia, and East and Western Africa but HCC incidence has risen in areas with historically low rates in the last decades, such as Western Europe and North America making it a major global health burden [285]. Life expectancy of HCC patients depends on the stage of the cancer at the time of diagnosis. In advanced stage, survival of only few months is expected, however, if diagnosis is early and effective treatment is performed, then five-year survival rate can be achieved [286].

Causes

Around 90% of HCC tumors develop from the induction of liver fibrosis and/or cirrhosis resultant from chronic inflammation of the liver caused by diseases such as chronic hepatitis B virus (HBV) and HCV, autoimmune hepatitis, excessive alcohol consumption, non-alcoholic fatty liver disease (NAFLD), diabetes mellitus, and excessive tobacco use [283, 287-289]. The

liver has the unique ability to repair itself after acute damage. Differentiated hepatocytes have the ability to re-enter the cell cycle and replace themselves [290]. However, under chronic inflammatory conditions, the altered immune response and constant cell death of hepatocytes promote liver fibrosis and tumorigenesis [284, 291]. Altered survival and proliferative signals during chronic inflammation, cellular stress, epigenetic modifications, and senescence promote tumorigenesis [283]. Chronic inflammation also promotes DNA damage, replication stress, and genomic instability which are detectable before transformation fully occurs [292]. Cytokines such as IL-6 and TNF, which activate the STAT3 and NF-κB pathways respectively, were reported to be responsible for chronic inflammatory conditions and development of HCC [293, 294]. Chronic inflammation of the liver can result on activation of oncogenic pathways and suppression of tumor-suppressive mechanisms. The main oncogenic pathways involved in HCC are PI3K-Akt and MAPK pathways, the Wnt/β-Catenin pathway, c-myc, and the sonic hedgehog (HH) pathway.

Activation by ligand binding and phosphorylation of growth factor tyrosine kinase receptors such as c-MET and EGFR lead to activation of the MAPK and PI3K pathways [295]. MAPK pathway activates the oncogene cFos and transcription factor AP-1/c-Jun which induce transcription of genes that drive cell proliferation of tumor cells [296]. Activation of the PI3K-Akt pathway results on activation of the mammalian target of rapamycin (mTOR) pathway promoting carcinogenesis [297]. This pathway can also be dysregulated by constitutive activation of PI3K due to loss of function of the tumor suppressor PTEN by either mutations or epigenetic silencing [298]. C-myc amplification is observed in around 33% of all HCCs and it normally corresponds to large, less differentiated tumors in younger patients [299]. C-myc

overexpression in HCC induces tumor cell stemness, cell proliferation, and increases glutamine metabolism and cell growth [300-302]. Mutations on the Wnt/β-Catenin pathway are observed in around 50% of HCCs [303]. Activating mutations result in stabilization and hyperactivation of β-Catenin signaling [304]. Additionally, inactivation mutations in negative regulators of the Wnt pathway and the tumor suppressor gene adenomatous polyposis coli (APC) further contribute to Wnt pathway activation [304]. While mature hepatocytes show no HH signaling activity, hyperactivation of HH and its pathway components were observed in 50% of HCCs [295, 305]. In HCC cells, reactivation of the HH pathway maintains tumor cell stemness and induces epithelial-to-mesenchymal transition which increases risk of metastasis [305, 306].

Overall, several conditions including hepatitis infection, NAFLD, and excessive alcohol consumption can result in chronic inflammation of the liver. Chronic inflammation of the liver induces cell stress and cell death which can result in liver fibrosis and cirrhosis and subsequent oncogenic transformation into HCC. Many signaling pathways can induce HCC including those that enhance liver cell proliferation, stemness and metastatic capacity.

Estrogen and HCC

The age-adjusted incidence ratios of HCC in men and women are extremely consistent in 157 registries worldwide [253, 307]. Significantly higher incidence and mortality ratios of HCC in men compared to women are well documented. Worldwide, male-to-female HCC incidence rates range from 2 to 4, being greatest for age-matched males and females under 53 years of age [308]. Women have significantly longer survival rates than age-matched men. While the overall survival for women between 18-45 years old is 25 months, it is only 10 months for age-matched men [309, 310]. The incidence and mortality of HCC significantly increase in post-menopausal

women over 55 years old indicating that reproductive senescence increases the risk of HCC. The HCC overall survival in women between 45-55 years old decreases to between 10 and 15 months which almost matches the overall survival of age-matched men (8-9 months) [309, 310]. Interestingly, those menopausal women using estrogen hormone replacement therapy showed decreased incidence of developing HCC compared to the non-estrogen users [311]. This indicates that estrogen has a protective role against HCC and estrogen signaling inhibits tumor formation and development making HCC significantly more prevalent in males compared to females. Thus, male sex, menopause, and female therapeutic ovariectomies are considered risk factors for HCC [312].

Estrogen signaling is protective against HCC in several ways. Estrogen signaling inhibits chronic inflammation of the liver, the main cause of HCC. Estrogen signaling through ERα directly inhibits transcription and expression of IL-6 by binding to its promoter and impairing c-rel and RelA binding to the NF-κB promoter site [170, 313]. This mechanism was observed upon estrogen treatment in hepatocytes and Kupffer cells, the resident macrophages of the liver [293]. ERα signaling also inhibits STAT3 activity, a critical factor regulating liver chronic inflammation in hepatocytes [294]. ERα transcriptionally activates protein tyrosine phosphatase receptor type O (PTPRO) which dephosphorylates STAT3 and subsequently attenuates STAT3 signaling [314].

HCV infection, a cause for chronic inflammation of the liver that results in cirrhosis and HCC, was shown to be inhibited by estrogen signaling through GPER in hepatocytes [315]. GPER signaling promotes the cellular export of matrix metallopeptidase 9 (MMP-9) which leads to cleavage of occluding, which is a tight junction protein and HCV receptor, overall reducing

HCV hepatocyte infection and viral RNA production [315]. Estrogen signaling through ERα in hepatocytes was also shown to transcriptionally activate microRNA 34a (miR-34a) which enhances activation of p53, a well-known apoptosis inducer and tumor suppressor [316]. These data indicate that estrogen signaling not only reduces HCV infection of hepatocytes but also reduces overall inflammation and induces apoptosis in a p53 dependent manner.

Estrogen signaling was also shown to affect HCC tumor infiltrating immune cells like macrophages. During HCC progression, TAMS replace Kupffer cells as the main modulators of chronic inflammation of the liver by secreting IL-6 and TNFα. TAMs not only induce chronic inflammation but also secrete immunosuppressive molecules such as TGFβ and IL-10 that suppress the anti-tumor immune response and increase the fibrotic, hard to infiltrate, tumor microenvironment [317]. Estrogen signaling functions as suppressor for macrophage alternative activation into TAMs by inhibiting the JAK1-STAT6 pathway though ERβ [318]. Overall these data indicate that estrogen signaling suppresses tumor growth partially via regulating the polarization of macrophages. Although this is promising in order to develop new therapies based on estrogen, the role of estrogen signaling on other HCC tumor infiltrating immune cells remains unclear and needs further investigation.

Treatments for HCC

Treatments for early stage HCC

According to the Barcelona Clinic Liver Cancer (BCLC) staging classification, HCC patients are categorized into four different stages according to the tumor burden, the number of tumors, the presence of vascular invasion, and the spread of nodules or presence of extrahepatic metastases. According to these parameters, HCC patients are considered stage 0 or very early

stage, stage A or early stage, stage B or intermediate stage, stage C or advanced stage, or stage D or terminal stage [319, 320]. Resection, ablation, and transplantation are considered for patients in the early HCC stages (0 and A). Surgical resection or radiofrequency ablation are indicated to treat patients with preserved liver function, single nodule occurrence, and absence of cirrhosis. Even if the five-year survival rate is high after these therapies (50-75%), the tumor recurrence rate can reach 50% making resection and ablation not appropriate for patients with vascular invasion or metastasis [281, 321]. In early stage HCC patients with cirrhosis, liver transplantation is performed. Liver transplantation ensures reduced tumor recurrence rates and increased survival however, there is a great lack of organs available for transplantation [322, 323]. Surgical resection, tumor ablation, and liver transplantation are efficacious treatments for HCC but due to problematic HCC diagnosis that reveals high levels of false negatives and high percentage of asymptomatic patients, these treatments can only be performed in under 20% of all HCC cases [324].

Sorafenib

Intermediated or advanced stage (B and C) HCC patients with preserved liver function are treated with Sorafenib. Sorafenib is an oral multi-kinase inhibitor with anti-angiogenic and anti-proliferative properties [325]. In vascular endothelial cells, Sorafenib inhibits the receptor tyrosine kinases VEGFR1, 2 and 3 and platelet-derived growth factor receptor-β (PDGFR-β) which are involved in promoting tumor angiogenesis [325-327]. In tumor cells, Sorafenib targets the MAP kinase pathway and induces apoptosis by inhibiting RAF isoform signaling [326, 327]. Sorafenib can also induce apoptosis in a MAP-kinase independent way by inhibiting the phosphorylation of the translation initiation factor eIF4E which promotes the translation of anti-

apoptotic protein myeloid cell leukemia-1 (Mcl-1) [327, 328]. Sorafenib increases overall survival by 2.3-2.8 months and it is the current standard of care for patients with advanced HCC [329]. Overall however, the median survival for Sorafenib treated patients with advanced stage, unresectable HCC is less than one year (11 months) [330]. Sorafenib treatment in advanced HCC patients also results in major side effects such as diarrhea, fatigue, and hand-foot syndrome, a troublesome condition characterized by painful, erythematous, blistering patches on palms and soles of the feet [330]. Overall, even if treatment with the multi-kinase inhibitor Sorafenib provides some therapeutic benefit for advanced HCC cases, a great unmet need remains for patients indicating that the development of new therapies to increase the overall survival of advanced HCC patients is highly necessary.

Immunotherapy for HCC

Immunotherapy is an emerging treatment modality that could become a promising treatment option for HCC as, first, it is an inflammation-associated cancer which makes immunotherapy more likely to be effective [331]. Second, it was observed that HCC patients whose tumors contain increased lymphocytic infiltration including $CD8^+$ and $CD4^+$ T cells show longer overall survival and lower risk of recurrence than those patients with no immune tumor infiltrates [332-334]. Third, the liver is an 'immune privileged' organ and it is tolerogenic to immune responses to antigens by various immune-suppressive mechanisms, including immune checkpoint expression, T_{reg} recruitment, cytokine secretion dysregulation, and changes in the local TME that suppress APCs and T cells [335-338]. Considering all these facts, the blockade of immune checkpoints and the enhancement of the T cell anti-tumor immune response are treatment possibilities that can be highly effective against HCC.

Treatment of HCV-induced HCC patients with CTLA-4 inhibitor Tremelimumab showed 20% partial response rates and increased overall survival by around 7 months [339]. Moreover, viral loads of HCV were significantly decreased indicating anti-viral immune responses, and no patients experienced immune-related adverse events or hepatotoxicity [339]. High PD-L1 and 2 expression was observed in HCC tissue [340]. Several clinical trials were conducted treating advanced HCC patients with PD-1 inhibitors Nivolumab and Pembrolizumab. Nivolumab treated patients showed manageable adverse events and 5% of them showed complete response rate while 20% showed partial response rate [341, 342]. The overall survival rate at 6 months was 72% indicating that Nivolumab did activate sustained tumor-specific immune responses [341, 342]. Furthermore, Sorafenib resistance in HCC was shown to be mediated by enhanced DNA (cytosine-5)-methyltransferase 1 (DNMT1) expression induced by PD-L1 [343]. These data indicate that PD-L1 blockade and Sorafenib combinatorial treatment could not only enhance the anti-tumor immune response but also target the tumor cells themselves.

ACT immunotherapy for HCC consists on the use of genetically modified autologous T cells that express a TCR specific for a HCC tumor antigen (Figure 4). ACT immunotherapy was shown to be partially successful in treating HCC in preclinical models and clinical trials. ACT shows unique advantages over Sorafenib and checkpoint blockade immunotherapy in treating HCC since it improves the quality and quantity of cells destroying the tumor in order to overcome immune tolerance [330]. ACT immunotherapy using the patient's autologous TILs expanded with IL-2 *in vitro* after liver surgical resection showed 68% overall survival rates [344, 345]. However, due to small tumor burden collected during biopsies and low T cell survival after extraction, it is hard to isolate TILs from HCC patients [346]. Genetically modified T cells including CAR T cell and TCR transduced T cells are being tested for ACT immunotherapy for

HCC in phase I and II clinical trials targeting tumor antigens such as glypican-3 (GPC3) expressed in the majority of HCCs [347-350] (Clinical trial IDs: NCT02715362, NCT03130712, NCT03198546, NCT03146234), α-fetoprotein (AFP) expressed in 60-80% of HCCs [351, 352] (NCT03349255), mucin-1 (MUC-1) expressed in 60% of HCCs [353, 354] (NCT02587689), and epithelial cell adhesion molecule (EpCAM) (NCT03013712). ACT immunotherapy for virally-induced HCC was also clinically tested using HBV or HCV viral proteins as tumor antigens. During chronic hepatitis infection, HBV and HCV can integrate its genetic material into the hepatocyte's genome, and HBV and HCV antigens are found in HCC tumors even after viral infection clearance [355, 356]. Patients treated with HBV Ag-specific T cells showed positive ORRs including volumetric reduction of lung metastases with no new lesions observed in the liver or lungs [357]. In addition, no therapy-related adverse events were observed [357]. HCV Ag-specific T cells for ACT immunotherapy were generated and tested in preclinical models where they showed success, but they have not been tested in humans yet [51, 52, 358].

Overall, checkpoint blockade and ACT immunotherapy for HCC were proven to be a partially successful and safe antitumor and antiviral method for advanced HCC, but the efficacy of this therapies needs to be optimized to obtain higher response rates. Even if ACT immunotherapy brings hope for the treatment of HCC, there are several aspects of it that need to be further improved. These include the facilitation of greater T cell tumor infiltration, avoiding T cell exhaustion and tumor immune scape, and reducing adverse side effects such as cytokine storm.

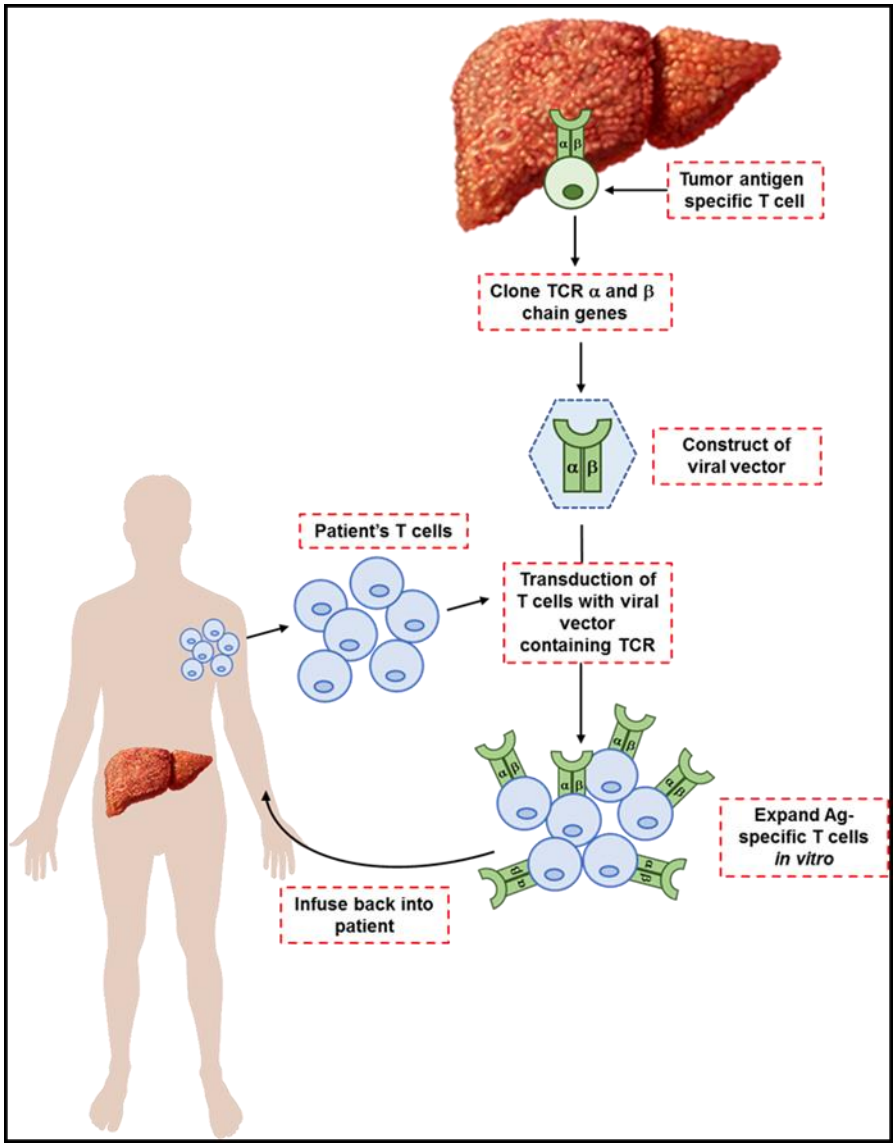


Figure 4. Adoptive T Cell Transfer Immunotherapy Using TCR Gene-Modified T Cells. **A)** Viral or cancer antigen specific T cell clones are isolated from hepatocellular carcinoma tumor infiltrating lymphocytes or peripheral blood and expanded *in vitro* to identify a therapeutic Ag-specific TCR candidate. **B)** TCR α and β genes are identified and cloned into retroviral vectors. **C)** Packing and producer cell lines are engineered to produce high titers of retrovirus containing the Ag-specific TCR. **D)** Retrovirus are used to transduce activated peripheral mononuclear blood cells from hepatocellular patients which are then expanded in vitro, and **E)** re-infused back into the patient. These autologous Ag-specific T cells have redirected specificity to provide anti-tumor immunity.

Summary

The field of immunotherapy has shown great advances for the treatment of solid tumors and hematopoietic malignancies in the last fest years. The use of genetically modified T cells that express TCRs specific for tumor antigens for ACT immunotherapy represents a promising approach to treating cancer patients [359]. Despite of the recent clinical success, there are many issues that remain in optimizing the efficacy of this type of immunotherapy. Many studies have focused on understanding the mechanisms of immune escape posed by the tumor including the role of the immunosuppressive TME. Malignant and non-malignant immune cells in the TME were characterized as well as their mechanisms to inhibit T cell anti-tumor function. On the other hand, other host factors that can affect T cell function and ACT immunotherapy efficacy remain well understudied. An example of this is the effect of sex and sex hormone signaling on the T cell anti-tumor function during immunotherapy. The sex hormone estrogen is present at physiological concentrations in females, and lower concentrations in males and post-menopausal females. Interestingly, some tumor and tumor-adjacent cells were shown to upregulate the expression of aromatase, the enzyme that synthesizes estrogen, which results in significantly increased estrogen concentrations in the TME. This indicates that adoptively transferred T cells will be exposed to different concentrations of estrogen throughout the body including the peripheral circulation depending on the sex of the patient, and then the TME.

Estrogen signaling can affect the differentiation, maturation and function of immune cells depending on various factors such as the concentration of hormone present, and the different estrogen receptor signaling pathways. Given that, one important mechanism of estrogen modulation of the immune system is by altering the differentiation, function, and cytokine

production of T cells. Estrogen signaling at physiological concentrations is generally pro-inflammatory and enhances the function of T cells. However, the effect of estrogen on T cell function at non-physiological concentrations like the ones found the TME remains unclear.

The effects of estrogen signaling on T cells and other immune cells results on differences on pathogenesis of diseases that arise from immune cell function dysregulation, like autoimmunity, or diseases that are heavily controlled by immune responses, like infections and cancer. Estrogen generally enhances the pathogenesis of autoimmunity while is protective against some infections and non-reproductive cancers. One malignancy that is heavily affected by estrogen is HCC. HCC is significantly more prevalent in males compared to pre-menopausal females [360, 361]. Post-menopausal females that are not undergoing estrogen HRT are also more prone to develop HCC than pre-menopausal females and estrogen users. The protective role of estrogen against HCC was shown to be mediated by estrogen receptor signaling inhibition of IL-6-mediated chronic inflammation and tumor infiltrating macrophage polarization. The role of estrogen signaling on other HCC tumor infiltrating immune cells remains unclear and needs further investigation.

The goal of this dissertation is to better understand the effect of estrogen signaling on genetically modified T cells used in ACT immunotherapy. The effect of estrogen signaling on T cell function, cytokine production and polyfunctionality will be investigated at physiological and non-physiological high estrogen concentrations. In addition, due to the many possibilities in estrogen receptors and different downstream signaling, the receptors through which estrogen predominantly signals in T cells from males and females, and the signaling happening downstream from the estrogen receptors will be investigated in the context of immunotherapy.

Lastly, using a novel HCC preclinical model, the effect of estrogen presence during ACT immunotherapy will be measured. Taken together, these studies will help to understand how estrogen can be beneficial or disadvantageous for T cell function in anti-tumor immune responses and will aid to design better and stronger T cells for adoptive cell transfer ACT immunotherapy.

CHAPTER II

MATERIALS AND METHODS

T Cells

All human female and male PBMCs samples used for T cell transductions were obtained from HLA-$A2^+$ buffy coats purchased from Zenbio Inc. (Research triangle Park, NC). T cells were derived from the PBMCs of healthy donors isolated from buffy coats using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation. T cells were then maintained on AIM-V serum-free media (Gibco) containing 10% pooled human male AB serum (Sigma-Aldrich), 300 IU/mL recombinant human IL-2, and 100 ng/mL recombinant human IL-15 (rhIL-2 and rhIL-15, National Institute of Health, Biological Resources Branch Bethesda, MD) at 37ºC in a humidified 5% CO_2 incubator.

HCV1406 TCR Retroviral Vector

The retroviral vector containing the TCR specific for the HCV antigen was generated by introducing the TCR chain genes and a truncated CD34 (CD34t) molecule lacking its intracellular signaling domain into an original SAMEN vector described by Treisman *et al.* [362-364]. The modified SAMEN vector, denominated HCV1406, contains the sequence for the α and β chains of the TCR specific for the HCV non-structural protein 3 (NS3) antigen (amino acids 1406-1415) separated by a P2A-self-cleaving linker, and then it contains the CD34t sequence separated by a T2A self-cleaving linker (Figure 5) [51, 358, 365]. The HCV1406 TCR retroviral

vector was used to generate a high viral titer producer PG13 cell line which retroviral supernatant was used to transduce human male and female primary T cells.

Figure 5. Structure of the HCV1406 TCR Retroviral Vector Used to Transduce Human Male and Female T Cells. TCR retroviral vector containing the HCV1406 TCR α and β chain genes fused by a P2A self-cleaving peptide linker. A truncated version of the CD34 molecule (CD34t) missing the intracellular signaling domain serves as a marker for transduction and it is fused to the 3' end of the TCR b chain via a T2A self-cleaving peptide. LTR: long terminal repeat, SD: splicing donor, SA: splicing acceptor, Ψ': packaging signal. This retroviral vector was previously described by Rosen *et al*. *J. Immunol*. 2004.

Generation of High Viral Titer Producer PG13 Cells

To generate stable virus producer cells, 3×10^6 HEK-293gp cells were plated in a 10 cm tissue culture plate in DMEM medium (Gibco) containing 10% FBS. The next day, the HEK-293gp cells were transfected with 20 μg of the HCV1406 TCR retroviral vector and 5μg of a plasmid encoding the vesicular stomatitis virus envelope gene in 50 μL of Lipofectamine 2000 (LifeTechnologies). After six hours, the supernatant was carefully removed and replaced with 10 mL of fresh DMEM containing 10% FBS. After 48-hour incubation at 37ºC in a humidified 5% CO_2 incubator the viral supernatant was collected and filtered through a 0.45 μm cellulose acetate syringe filter (VWR). 2×10^6 PG13 cells were plated in a 10 cm tissue culture plate and the viral supernatant from the HEK-293gp cells was used to transduce them over three days while incubated at 37ºC in a humidified 5% CO_2 incubator. Transduction efficiency was quantified by measuring CD34 expression via flow cytometry with an anti-human CD34-PE

antibody (BioLegend), and transduced cells were sorted for high and uniform CD34 expression using a FACS Aria Illu cell sorter (BD BioSciences).

In order to generate HCV1406 TCR retrovirus for transductions, $10x10^6$ sorted stable producer PG13 cells were plated in a T-175 flask and treated with IMDM media supplemented with 10% FBS, 1 μM sodium butyrate (Sigma-Aldrich), and 10 mM HEPES (Gibco) for 8 to 10 hours. After treatment, sodium butyrate containing media was replaced with IMDM supplemented with 10% FBS and cells were incubated overnight. Then, fresh retroviral supernatant was collected and filtered through a 0.45 μm cellulose acetate syringe filter and used for T cell transduction.

HCV1406 Retroviral T cell Transduction

Primary human male and female T cells obtained from HLA-$A2^+$ buffy coats were transduced via inoculation [366, 367]. Prior to retroviral transduction, primary human male and female T cells were activated with 50 ng/mL of anti-CD3 mAb OKT3 (BioLegend, San Diego, CA) for four to five days. 24-well flat-bottom non-tissue culture plates (VWR) were coated with 30 mg/mL Retronectin (Takara, Otsu, Japan) in 0.5 mL of DPBS (Dulbecco's modified phosphate buffered saline) (Gibco) overnight at 4ºC. Before transduction, Retronectin coated plates were blocked with 2% PBSA (bovine serum albumin in PBS) for 30 minutes at room temperature (RT) and washed with DPBS. Then, 2 mL of fresh retroviral supernatant was added to each well and plates were spun for 2 hours at 32ºC at 2,000×g. Then, viral supernatants were aspirated and 2×10^6 activated T cells were placed on each well in 1 mL of AIM-V media containing 10% human serum, 600 IU/mL rhIL-2, and 200 ng/mL rhIL-15 in combination with 1 mL of retroviral supernatant. Plates were spun for 2 hours at 32ºC at 2,000×g. Plates were then incubated

overnight at 37ºC in a 5% CO_2 humidified incubator and then cells were transferred to tissue-culture treated plates in supplemented AIM-V medium. Three or four days after, transduction efficiency was measured via flow cytometry using human anti-human CD3-APC-Cy7 and anti-CD34-AlexaFluor700 antibodies (BioLegend).

Transduced cells were sorted by adding anti-CD34 magnetic microbeads (Miltenyi) to the T cells and passing them through a magnetic column (positive selection sorting). T cells that remained magnetically attached to the column were eluted and purity of selection was confirmed via flow cytometric analysis. All experiments described in this dissertation were performed using >90% pure $CD34^+$ transduced T cell populations.

Cell Lines and Media

The PG13 cell line (stable viral producer cell line) and the T2 cell line (CD3 negative, TAP-deficient HLA-A2-expressing APC) were gifts from Dr. Nishimura, Loyola University Chicago, Maywood. PG13 cells were maintained in IMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (VWR, Radnor, PA). T2 cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 5% penicillin and streptomycin (Gibco), and 5% L-glutamine (Gibco).

Sorting of $CD4^+$ and $CD8^+$ Ag-specific T cells

Human male and female HCV1406 TCR transduced T cells were sorted into $CD4^+$ or $CD8^+$ populations using CD4 or CD8 negative selection magnetic microbead kits (Miltenyi Biotec). Non-$CD4^+$ or non-$CD8^+$ cells were labeled with a cocktail of biotin-conjugated antibodies as indicated by the manufacturer. Subsequently, non-$CD4^+$ cells or non-$CD8^+$ target

cells were magnetically labeled with beads and passed through a magnetic column that captured them. The column flow-through was collected and contained >90% pure unlabeled $CD4^+$ or $CD8^+$ T cells. Flow cytometry was used to ensure purification with antibodies anti-human CD3-APCyCy7, CD4-PECy7, and CD8-FITC (BioLegend).

Estrogen and Estrogen Receptor Antagonists and Agonists

17β-estradiol (≥98% purity) powder suitable for cell culture was obtained from Sigma-Aldrich and reconstituted in DMSO at a 10 μM concentration. Human female and male Ag-specific T cells were treated with 0.5 nM (physiological) or 50 nM (super-physiological) 17β-estradiol for 2 hours and control cells were treated with DMSO alone for 2 hours prior to co-culture with target T2 cells. The ERα antagonist 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP-dihydrochloride) (≥98% purity), and the ERβ antagonist 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) (≥99% purity) were obtained from Tocris Biotechne and were reconstituted in in DMSO at a 10 μM concentration [368, 369]. Human female and male Ag-specific T cells were treated with 100 nM of either antagonist or both simultaneously for 2 hours in combination with 0.5 or 50 nM 17β-estradiol for 2 hours prior to co-culture with target T2 cells.

Isolation of Cytoplasmic and Nuclear Extracts

As previously described in Navarro & Watkins (*Gender and the Genome*, 2017), 3×10^6 Ag-specific T cells from male and female donors were treated with none, 0.5, or 50 nM E2 for 2, 10, 30, 60, or 120 minutes and nuclear and cytoplasmic protein extracts were obtained using the NE-PER Nuclear Cytoplasmic Extraction Reagent Kit (Pierce, Rockford, IL) according to the

manufacturer's instructions. Treated cells were washed twice with ice-cold phosphate buffered saline (PBS) and centrifuged at 500×g for 5 minutes. Cell pellets were resuspended in 100 μL of cytoplasmic extraction reagent I (CER I) by vortexing and incubated on ice for 10 minutes followed by the addition of 5.5 μL of CER II. They were then vortexed for 5 seconds, incubated on ice for 1 minute, and centrifuged for 5 minutes at 16000×g. The supernatant fraction (cytoplasmic) was transferred to a pre-chilled tube and stored at -80°C. The insoluble fraction was resuspended in 50 μL of nuclear extraction reagent and vortexed every 10 minutes for a total of 40 minutes. Tubes were centrifuged at 16000×g for 10 minutes, and the resulting supernatant was collected (nuclear fraction) and stored at -80°C until further Western Blot analysis.

Western Blot Analysis

As previously described in Navarro & Watkins (*Gender and the Genome*, 2017), equal amounts of nuclear and cytoplasmic protein isolated from estrogen treated male and female Ag-specific T cells were loaded onto a sodium dodecyl sulfate (SDS)/polyacrylamide gel. After electrophoresis, proteins were transferred into a polyvinylidene fluoride membrane (Bio-Rad) and blocked for 1 hour with 5% BSA in Tris-buffered saline containing Tween-20. After blocking, membranes were incubated overnight with the anti-human ERα primary antibody clone D8H8 (1:1000) (Cell Signaling Technologies) at 4°C while shaking. After washing, membranes were incubated for 1 hour at RT with the horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:5000) (Cell Signaling Technologies). Proteins were detected using an enhanced chemiluminescence substrate kit (Thermo Fisher). All blots were stripped and re-probed with anti-human β-actin antibody (1:2000) or anti-human Lamin A/C (1:1000) (Cell

signaling Technologies). Western blots were imaged using a ChemiDoc MP imaging system (Bio-Rad) and quantified using the software ImageJ.

Peptides

HCV-NS3 (1406–1415): KLVALGINAV and tyrosinase (368–376): YMDGTMSQV peptides were obtained from Synthetic Biomolecules (San Diego, CA). Peptides were reconstituted in dimethyl sulfoxide (DMSO) and stored at -20ºC. For all the experiments described, T2 cells were pulsed with 5μg/mL of either peptide for 2 to 4 hours at 37ºC in a 5% CO_2 humidified incubator.

Cytokine Release Assays

T cell cytokine secretion was measured in cytokine release assays such as enzyme-linked immune absorbent spot (ELISPOT) and enzyme-linked immunosorbent assay (ELISA). 2.5×10^4 T2 cells loaded with peptides or HCV-expressing tumor cells were used as target cells. T2 cells were pulsed with 5 μg/mL of HCV1406 or tyrosinase peptides for 2-4 hours, and 2.5×10^4 human male and female HCV Ag-specific T cells were treated with 0, 0.5, or 50 nM estrogen for two hours. IFNγ and Granzyme B secretion was measured via ELISPOT by coating ethanol activated ELISPOT plates with anti-human IFNγ (BD Biosciences) or anti-human Granzyme B (R&D Systems) capture antibodies overnight at 4°C. Plates were blocked with RPMI containing 10% FBS for 2 hours at RT. Treated T cells were co-cultured with pulsed T2 target cells at a 1:1 ratio on coated ELISPOT plates for 18 hours at 37ºC in a 5% CO_2 humidified incubator. Co-cultures were then aspirated and the relevant biotinylated secondary and streptavidin–horseradish peroxidase antibodies (Streptavidin-HRP, BD Biosciences) were added and plates were

incubated for 2 hours or 1 hour respectively at RT. Plates were developed using 3-Amino-9-ethylcarbazole (AEC) substrate (BD Biosciences) and spots were quantified using a CTL ImmunoSpot S6 reader. After each step of this process, the plate was thoroughly washed 5 times with PBS containing 0.05% Tween-20.

TNFα and IL-4 secretion was measured via ELISA by coating 96-wells ELISA plates with anti-human TNFα or IL-4 (MabTech) capture antibodies overnight at 4°C. Plates were blocked using PBS with 10% FBS for 1 hour at RT. Then, 100 μL of co-culture supernatant and standards were added to the plate and incubated for 2 hours at RT. This was followed by incubating the plate with the relevant biotinylated secondary and streptavidin-HRP antibodies for 1 hour at RT. Plates were developed using tetramethylbenzidine (TMB) substrate and stopped by adding 1 M sulfuric acid. The absorbance was then read at 450 nm in iMark Microplate absorbance reader (Bio-Rad). After each step of this process, the plate was thoroughly washed 5 times with PBS containing 0.05% Tween-20.

Flow Cytometry

Fluorochrome-conjugated antibodies were used to detect human transduced Ag-specific T cell surface markers (CD3, CD34, CD4 and CD8) and activation markers (CD25, CD69). In functional assays, Ag-specific T cells were also stained for the lytic marker CD107a and intracellular cytokines (IFNγ, TNFα, IL-2, IL-4, IL-17a, IL-22, and Granzyme B). Intra-nuclear staining was used to detect T cell transcription factors (T-Bet, GATA3, RORγt, and FoxP3). A table summarizing all flow cytometry antibodies used can be found in Table 1.

Flow cytometry staining standard protocols were followed for measuring extra and intracellular protein expression. Cells were spun in 5 mL polystyrene tubes (VWR) and washed with PBS containing 2% FBS and 0.01% sodium azide (FACS buffer) twice. Cell suspensions were treated with anti-CD16/32 antibody (BD Pharmigen) for 10 minutes to block Fc receptors and non-specific staining. Next, cell suspensions were incubated with extracellular marker antibodies for 30 minutes at 4°C in the dark. Cells were washed to remove antibody excess and incubated with intracellular fixation buffer (BioLegend) for 20 minutes at 4°C in the dark and then washed twice with permeabilization buffer (BioLegend). Fixed and permeabilized cells were then incubated with intracellular marker antibodies for 30 minutes at 4°C in the dark. Then, cells were washed with permeabilization buffer and staining was measured in a BD Fortessa flow cytometer. Data were analyzed using FlowJo software package.

Use	Marker	Fluorochrome	Clone	Manufacturer	Catalog #
Transduced cell sorting	CD34	PE	581	BioLegend	343506
Extracellular and intracellular markers used for cytokine expression measurement	CD3	APC-Cy7	HIT3A	BioLegend	300318
	CD34	AlexaFluor 700	581	BioLegend	343526
	CD4	PE-Cy7	A161A1	BioLegend	357410
	CD8	FITC	HIT8A	BioLegend	300906
	CD107a	Brilliant Violet 510	H4A3	BioLegend	328632
	IFNγ	Brilliant Violet 421	4S.B3	BioLegend	502532
	TNFα	Brilliant Violet 711	Mab11	BioLegend	502940
	IL-2	PerCp-Cy5.5.	MQ1-17H12	eBioscience	500350
	IL-4	AlexaFluor 647	8D4-8	BioLegend	500714
	IL-17a	Brilliant Violet 570	BL168	BioLegend	512324
	IL-22	PE	22URTI	eBioscience	12-7229-42
Transcription factor staining	T-Bet	Brilliant Violet 711	4B10	BioLegend	644820
	GATA3	AlexaFluor594	16E10A23	BioLegend	653816
	RORγt	AlexaFluor647	Q21-559	BD Pharmigen	563620
	FoxP3	PE	259D	BioLegend	320208
	Granzyme B	PerCp-Cy5.5.	QA16A02	BioLegend	372212
T cell activation staining	CD25	PerCp-Cy5.5.	BC96	BioLegend	302626
	CD69	APC-Cy7	FN50	BioLegend	310910
	CD44	eFluor450	IM7	Invitrogen	75-0441

Table 1. Antibodies Used for Flow Cytometry Staining.

For transcription factor staining, cells that had been stained for extracellular markers were incubated in 1 mL of Foxp3 Fixation/Permeabilization working solution (eBioscience) for 45 minutes at RT in the dark and then washed twice with permeabilization buffer (eBioscience). Fixed and permeabilized cells were then incubated with transcription factor antibodies for 45 minutes at RT in the dark. Then, cells were washed with permeabilization buffer and staining was measured in a BD Fortessa flow cytometer. Data were analyzed using FlowJo software package.

Multi-Intracellular Cytokine Assay

2.5×10^5 human male and female sorted HCV Ag-specific T cells were treated with 0, 0.5, or 50 nM estrogen in combination with 100 nM MPP-dihydrochloride (ERα inhibitor), or 100 nM PHTPP (ERβ inhibitor), or 100 nM of both MPP-dihydrochloride and PHTPP simultaneously for 2 hours. Treated T cells were then co-cultured with 2.5×10^5 T2 cells that had been pulsed with 5μg/mL of HCV NS3 or tyrosinase peptides at 37ºC for 5 hours in the presence of protein transport inhibitors GolgiPlug and GolgiStop (BD Biosceinces). Then extracellular staining for markers CD3, CD34, CD4, CD8, and CD107a was performed as described before. Subsequently, cells were fixed and permeabilized and staining for intracellular markers IFNγ, TNFα, IL-2, IL-4, IL-17a, IL-22 was performed as described before. Samples were ran in a BD Fortessa flow cytometer and analyzed using FlowJoX.

Gating strategy to analyze these data consisted on selecting the singlet population (FSC-A vs FSC-H), and then the T cell population was gated individually from T2 cells by FSC vs SSC comparison. T cells were gated on $CD3^+CD34^+$ populations and then Ag-specific T cells were gated on $CD8^+CD4^-$ or $CD8^-CD4^+$ populations. These $CD3^+CD34^+CD8^+CD4^-$ or $CD3^+CD34^+CD8^-CD4^+$ populations were used as starting point for subsequent functional analysis. Functional parameters included CD107a, IFNγ, TNFα, IL-2, IL-4, IL-17a, and IL-22. All these parameters were gated against SSC to obtain the percent frequency of $CD3^+CD34^+CD8^+CD4^-$ or $CD3^+CD34^+CD8^-CD4^+$expressing each of them

Polyfunctional Flow Cytometry Data Analysis

In order to obtain the levels of expression of all the possible marker combinations, Boolean combinatorial gating was applied to the seven markers expressed in both the $CD3^+CD34^+CD8^+CD4^-$ and $CD3^+CD34^+CD8^-CD4^+$ population. Boolean combinatorial gating yielded a total of 128 marker combinations (2^n n=7 2^7=128). This seven-parameter functional analysis yielded a dataset way too complex to graphically depict in FlowJoX. Comparisons of expression of all these marker combination between sexes and estrogen and estrogen receptor inhibitor treatments were also too complicated to perform using FlowJoX. Subsequent analysis of these data was performed using the software Simplified Presentation of Incredibly Complex Evaluation (SPICE). Pre-processing of the data was performed using the software Pestle. After combinatorial Boolean gating, Pestle offers data formatting and background subtraction of multivariate data sets. The polyfunctionality data obtained from Ag-specific T cells stimulated with T2 cells pulsed with tyrosinase was used for background subtraction. Data sets were then imported to SPICE for graphical analysis. While background subtraction can result in below zero values, SPICE has a threshold approach which will minimize systematic bias and can maximize the amount of information that can be gained from positive measurements [370]. Visualization of data includes pie charts, bar graphs, and cool plots, a type of heat map. Heat maps were chosen to represent the data. The mean of the percent frequency expression of each cytokine combination for the 15 female and 15 male donors mean values were plotted in the heat maps.

Animal Experiments

All NOD.Cg-Mcph1$^{Tg(HLA-A2.1)1Enge}$ Prkdc^{scid} Il2^{rgtm1Wjl}/SzJ (NSG-A2) mice aged 6-8 weeks were obtained from The Jackson Laboratory. Bilateral ovariectomies or sham surgeries were performed on female and male mice. Briefly, any mouse having surgery was placed on Carprofen gel diet (MediGel) 24 hours preceding surgery and kept on that diet for 72 hours post-operation. Each animal also received Enrofloxacin (Bayer Animal Health) injected subcutaneously at 10 mg/kg as a broad-spectrum antibiotic. The animal was then anesthetized using Isoflurane (Abbott Labs) and placed on its sternum and prepared for surgery using 70% ethanol and iodine wipes. Two 1 cm skin incisions were made longitudinally over the animal's fat pads, then one at the time, the fat pads were pulled from the body cavity until the ovaries were exposed. Ovaries were dissected away from the fat pad and the remaining uterine horns were placed back into the cavity (this step was skipped during sham surgeries). Skin was closed using 9 mm skin clips on each side. Then, the animal was placed in a clean cage with access to Carprofen gel diet and Enrofloxacin treatments as indicated before. Skin clips were removed 14 days post-operation and tumor challenge was then performed.

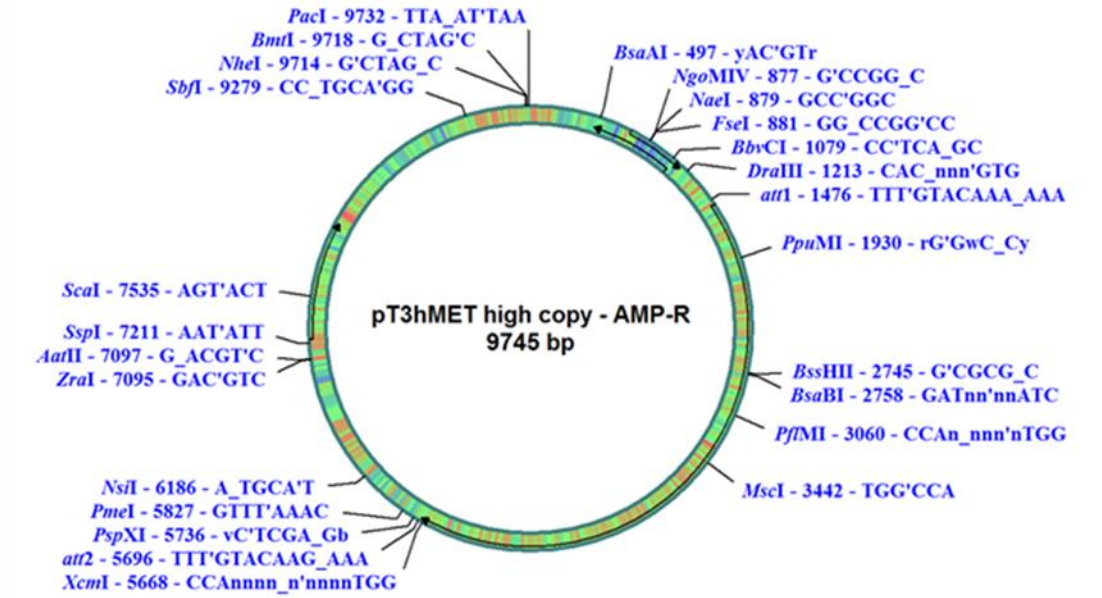
To generate HCC tumors, human oncogenes c-MET and β-catenin, and the HCV:NS3 antigen expressions vectors in combination with sleeping beauty (SB) transposons were used [371, 372]. Plasmids maps containing the expression vectors for c-Met, βcatenin and sleeping beauty transposase are described in Figure 6. The structure of the HCV NS3 expression vector is described in Figure 7. 9 μg of pT3hMET, 9 μg of pT3CAT, and 9 μg of pCDNAIII-NS3 (containing the sequence of the HCV:NS3 peptide) in combination with SB transposase at a 2:1

ratio were diluted in 2 mL of normal saline (0.9% NaCl), filtered through a 0.2 μm filter (Corning), and injected into the lateral tail vein of female ovariectomized/sham mice or male sham mice that were around 8-9 weeks old in <7 seconds. At day 20 or day 40 after tumor challenge, mice were given ACT immunotherapy consisting of 10×10^6 sex-matched human HCV Ag-specific T cells via tail vein injection. Mice that received ACT were given 2.5 μg of rhIL-15 every 3 days to ensure human Ag-specific T cell survival. 20 days after T cell transfer, mice were sacrificed, and livers and spleens were collected for analysis. All mice were housed under specific pathogen-free conditions and were treated in accordance with NIH guidelines under protocols approved by the animal care and use committee (IACUC) of Loyola University Chicago (Maywood, IL).

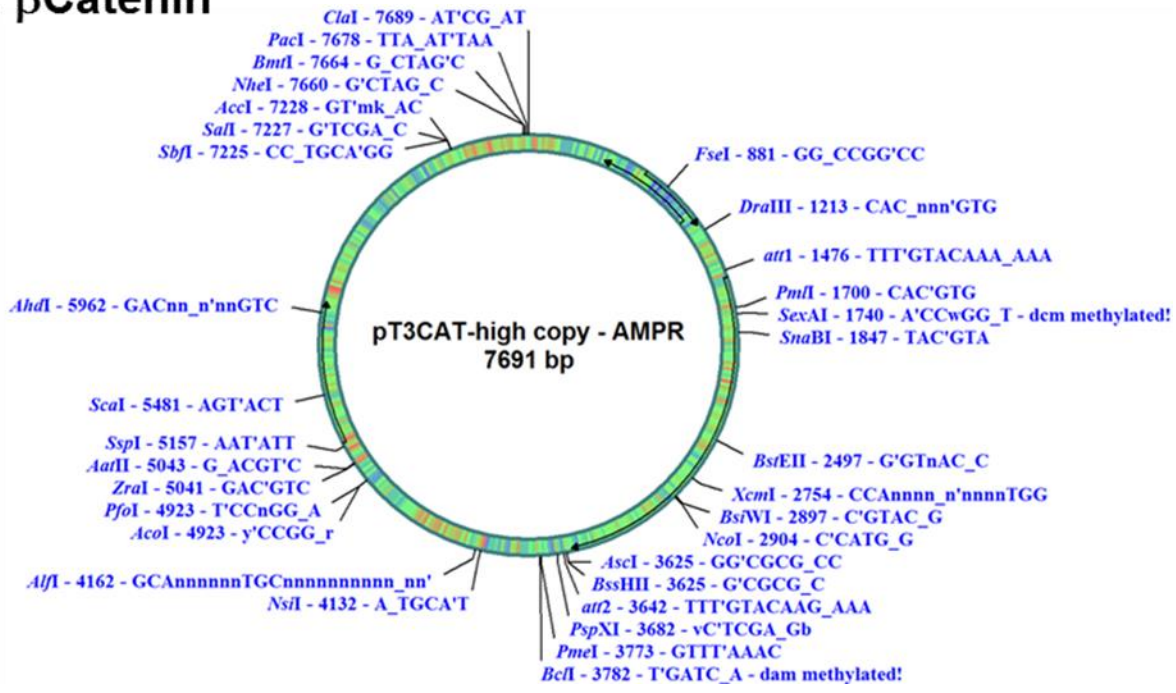
Cell Isolations

Human Ag-specific T cells were isolated from single cell suspensions of tumors obtained by digesting livers with 1 mg/mL collagenase D (Roche) for 30 minutes at 37°C while shaking and dissociating them using a Stomacher bag (Sewer). Liver single cell suspensions were then subjected to a Percoll (GE Healthcare) density gradient. Briefly, cell suspensions were prepared in 10 mL cell culture media and underlayed with 40% and subsequently 80% Percoll to separate out lymphocytes from tumor cells. Cells were collected at the 40/80 interface for further analysis. Human Ag-specific T cells were also isolated from spleen single cell suspensions obtained by pushing the tissue through a 70 μm nylon filter. Then, red blood cells were lysed using ammonium-chloride-potassium (ACK) lysis buffer (Lonza).

A) c-MET



B) βCatenin



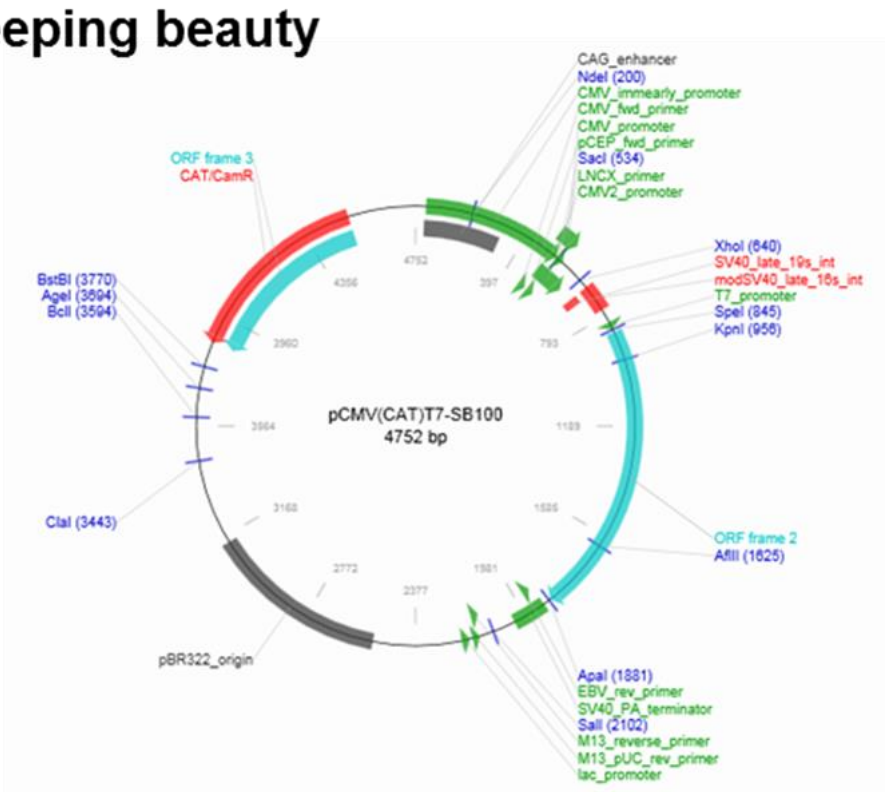


Figure 6. Vector Maps of Human c-MET and βCatenin-P3T, and Sleeping Beauty 100 Transposase-pCMV Used to Generate HCC in Mice. A) Human c-MET (9745 bp) and **B)** human βCatenin (7691 bp) are shown in the black arrows. **C)** Human SB100 (4752 bp) is shown in the cyan after the T7 promoter. These vectors were previously described by Tward *et al. PNAS*. 2007.

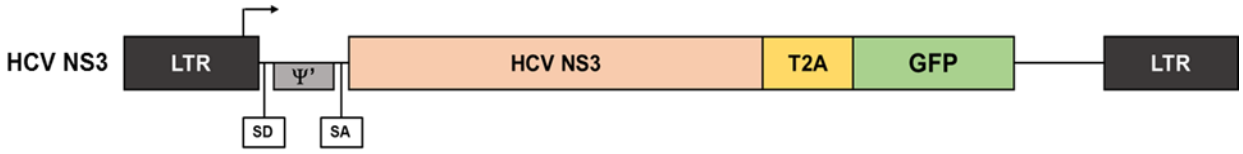


Figure 7. Structure of the pcDNAIII Vector Containing the HCV NS3 Sequence. The full sequence of the HCV non-structural protein 3 (NS3) was linked to GFP with a T2A self-cleaving peptide. LTR: long terminal repeat, SD: splicing donor, SA: splicing acceptor, Ψ': packaging signal. This pdNAIII vector was described by Spear *et al*. *Cancer Immunol Immunother*. 2016.

ELISPOT Using Tumor Cells as Targets

NSG-A2$^+$ mice were injected with MET/CAT/HCV or MET/CAT hydrodynamically and tumors were left to develop for 60 days. On day 60 tumors were extracted and digested using 1 mg/mL of collagenase D as previously described. Single cell suspensions of tumor cells were washed using RPMI containing 10% FBS and counted. 2.5×10^5 tumor cells from mice injected with MET/CAT/HCV or MET/CAT were then co-cultured with human female and male HCV Ag-specific T cells from 3 different donors in triplicates in a 1:1 ratio for 18 hours on an anti-human IFNγ coated ELISPOT plate. After 18 hour co-culture plates were developed as previously described and spots were quantified.

Statistical Analysis

All data are represented as the mean ± standard error of the mean (SEM). All the statistical analyses were conducted using GraphPad Prism (version 7). For experiments that did not require group wise comparisons, student's t test was used with significance set at $p<0.05$. For experiments that did require group wise comparisons, 1 or 2 way ANOVA were used with a Tukey's post-hoc test with significance set at $p<0.05$.

For experiments analyzing the effect of estrogen signaling on T cell polyfunctionality, data were analyzed using MANOVA with a Tukey's post-hoc test with significance set at $p<0.05$. In order to test whether the effects of estrogen inhibitor treatments on the percent expression for a given cytokine combination vary significantly based on estrogen treatment, and vice versa, Michael Wesolowski, MPH (Clinical Research Office, Loyola University Chicago, IL) performed linear regression model. Mixed-effects linear regression models were used to

estimate the expression of unique cytokine combinations for various estrogen and estrogen inhibitor treatment combinations. Models including an estrogen main effect, an estrogen inhibitor main effect, and an estrogen x estrogen inhibitor interaction term were run for each unique cytokine combination. These models featured random intercepts to account for within-donor correlations existing between cells and treatments related to the same donor, and unstructured variance-covariance structures. The estimated effects of estrogen and estrogen inhibitor treatment combinations, as well as the average estimated cytokine expression for each estrogen and estrogen inhibitor treatment combination, are reported. Post-hoc pairwise comparisons between each index estrogen treatment (50 & 0.5) and the reference estrogen treatment (0) were conducted within each estrogen inhibitor treatment stratum. Separate models were run for each of 83 unique cytokine combinations, and as a result, an experiment-wise Sidak-corrected significance level of $\alpha = 0.000618$ was used to control the Type I error rate. An additional Sidak adjustment was used to correct p values for the multiple, post-hoc pairwise comparison tests.

Mixed-effects linear regression model was described as:

Within-Donor Model:

$$Cytokine\ Combination\ Percent\ Expression_{ij} = b_{0i} + b_{1i}*Estrogen\ Inhibitor_{ij} + b_{2i}*Estrogen_{ij} + b_{3i}*Estrogen\ Inhibitor_{ij}*Estrogen_{ij} + \varepsilon_{ij}$$

Between-Donor Model:

$b_{0i} = \beta_0 + \nu_{0i}$

$b_{1i} = \beta_1$

$b_{2i} = \beta_2$

$b_{3i} = \beta_3$

CHAPTER III

ESTROGEN SIGNALING THROUGH ERα AND ERβ DIFFRENTIALLY IMPACTS HUMAN MALE AND FEMALE AG-SPECIFIC T CELL CYTOKINE PRODUCTION

Introduction and Rationale

Immunotherapy is a treatment modality that was proven to be effective in many types of difficult to treat cancer, and it is a promising therapy option for HCC. Treatment of HCC patients with checkpoint blockade immunotherapy showed some success with 20% partial response rates [342]. Many tumor specific antigens that can be used as targets of T cells for ACT immunotherapy were identified including but not limited to GPC3, α-fetoprotein, MUC-1, and the viral proteins of HCV and HBV [350, 351, 354, 357]. Even if ACT immunotherapy brings hope for the treatment of HCC, critical aspects that need to be further improved upon during immunotherapy include facilitating greater T cell tumor infiltration, avoiding T cell exhaustion, and reducing adverse side effects such as cytokine storm. The role of host factors including sex and estrogen signaling has never been investigated in ACT immunotherapy for HCC. Adoptively transferred T cells are exposed to different estrogen concentrations in the body including physiological estrogen or low estrogen depending on the sex and reproductive state of the patient, and super-physiological estrogen in the TME. Considering that estrogen has a protective role against HCC through mechanisms involving inflammation, it can be hypothesized that estrogen signaling enhances T cell function and anti-tumor response which subsequently can enhance the efficacy of ACT immunotherapy. Estrogen signaling in immune cells is highly

dependent on concentration and estrogen receptor subtype hence it is necessary to investigate these factors on immune cells mediating the anti-tumor immune response in order to optimize immunotherapy treatment against HCC. This dissertation is based in studying the effects of estrogen signaling on the function of TCR transduced T cells used for immunotherapy.

In order to characterize the effect of estrogen signaling on Ag-specific T cells used for ACT immunotherapy against HCC, HCV was chosen as a model target antigen. HCV infects approximately 140 million people worldwide and chronic HCV infection can result in associated liver disease including cirrhosis and HCC [284]. Moreover, 50 to 80% of HCC cases derive from chronic HCV infection [373]. Even if HCV protease inhibitor anti-viral drugs Telaprevir, Boceprevir, and Simeprevir were developed and recently approved for clinical use [374, 375], the rapidly mutating HCV genome generates drug resistance in hepatocyte [376, 377]. In addition, many patients treated with anti-viral drugs have already developed associated liver disease, such as cirrhosis, that cannot be treated with these drugs. Knowing all this, a TCR that recognizes the HCV NS3 (amino acids 1406 to 1415) antigen was selected. This TCR, denominated HCV1406, was isolated from a T cell clone found in an HLA-$A2^+$ HCV^+ HCC patient that received an HLA-$A2^+$ liver transplant [378]. T cells genetically modified with this TCR were previously shown to recognize HCV^+ target cells *in vitro* in an HLA-A2-dependent manner, and to be able to reject patient-derived xenografts (PDX) tumors *in vivo* [51]. In the following sections, the effect of estrogen signaling on human male and female HCV Ag-specific T cell cytokine expression and production are described.

Results

Ag-specific T cells from Male and Female Donors Express and Signal through ERα in Response to Estrogen Stimulation

To study the effect of estrogen signaling on human male and female Ag-specific T cells, peripheral blood derived T cells from 15 healthy male and 15 healthy female donors were transduced with the HCV1406 TCR (Figure 5). While 5 male and 5 female donors were of unknown age, the remaining male and female donors were between 22 and 48 years old corresponding to pre-menopausal age for female donors. The HCV1406 TCR retroviral vector codes for a truncated version of CD34 (CD34t), which was used for transduction recognition and purification of transduced T cells for further *in vitro* and *in vivo* studies. Transduction of either male or female T cells with the HCV1406 TCR typically yielded around 30 to 60% transduction efficiency (Figure 8 A). After purification by magnetic bead cell sorting using the CD34 expression as a target, studies described in this dissertation are based on the effect of estrogen on a population of Ag-specific T cells at > 90% purity (Figure 8 A). HCV 1406 transduced sorted T cells from male and female donors had very similar $CD3^+CD34^+$ mean fluoresce intensity (MFI) which is important to achieve to ensure that differences on HCV1406 TCR expression between donors did not give rise to differences on T cell activation and function (Figure 8 A). Transduced T cell populations from male and females showed significantly higher frequencies of $CD8^+$ compared to $CD4^+$ T cells but there were no significant differences on T cell subset distribution between sexes (Figure 8 B, C).

After transduction and $CD34^+$ purification, male and female HCV Ag-specific T cells were sorted into $CD4^+$ and $CD8^+$ populations and ERα expression was tested via western blot. There were no sex-differences on the expression level of ERα in the bulk population or in the

$CD4^+$ and $CD8^+$ populations of Ag-specific T cells (Figure 9 A, B). $CD4^+$ Ag-specific T cells from both sexes showed significantly higher expression of ERα compared to $CD8^+$ T cells and bulk T cell populations (Figure 9 A, B). In order to test if ERα translocates into the nucleus after estrogen ligand stimulation, male and female Ag-specific T cells were treated with no estrogen (0), physiological (0.5 nM), or super-physiological (50 nM) concentrations of E2 for 0, 2, 10, 30, 60, and 120 minutes before lysis of the cells and separation of cytoplasmic and nuclear fractions to tests for ERα expression. ERα was expressed in the cytoplasm of the cell of male and female Ag-specific T cells but its expression was significantly decreased after 2-minute estrogen treatment on female T cells (Figure 9 C, E) and after 10-minute estrogen treatment on male cells as demonstrated by normalizing expression to β-actin as a loading control (Figure 9 C, F). On the other hand, ERα expression in the nucleus was quickly increased in male T cells after 2-minute estrogen treatment **(**Figure 9 D, F), and increased overtime after estrogen stimulation in female T cells as demonstrated by normalizing expression to Lamin A/C loading control (Figure 9 D, E). The time course of E2 treatment revealed that nuclear translocation of ERα upon E2 ligation is very rapid, within 2 to 10 minutes, in male and female Ag-specific T cells. This rapid translocation was found to be transient since cytoplasmic ERα expression re-accumulated, and nuclear ERα expression diminished over time. Cytoplasmic and nuclear ERα was not significantly different during this time course between T cells treated with 0.5 nM or 50 nM estrogen concentrations indicating that ERα can quickly translocate into the nucleus of male and female Ag-specific T cells upon ligand binding in physiological and super-physiological conditions.

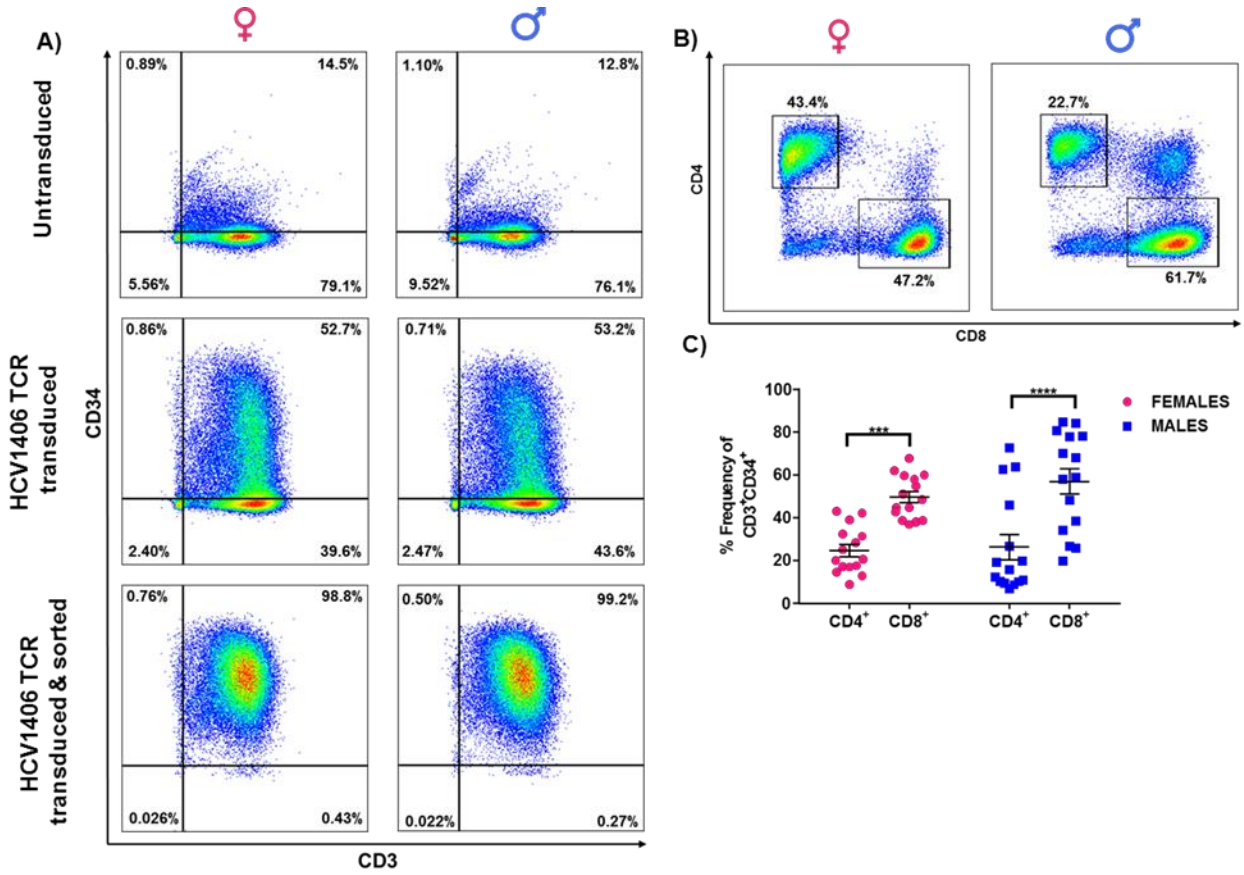


Figure 8. T Cells from Male and Female Donors Were Transduced to Express the HCV1406 TCR to Study Ag-specific T Cell Function. A) Representative flow cytometry dot plots for one female and one male donor T cells before transduction showing T cell populations with very low CD34 expression, after transduction showing T cell populations with variable expression of CD34, and after CD34 magnetic bead sorting showing T cell populations over 95% $CD3^{+}CD34^{+}$. **B)** Representative dot plots for $CD4^{+}$ and $CD8^{+}$ T cell subsets of TCR transduced and sorted T cells. **C)** $CD4^{+}$ and $CD8^{+}$ frequencies of TCR transduced and sorted T cells from 15 female and 15 male human healthy donors. Each donor plotted and SEM indicated. Analyzed using 2-way ANOVA with Tukey's post-hoc. $p<0.0001$=****, $p<0.001$=***.

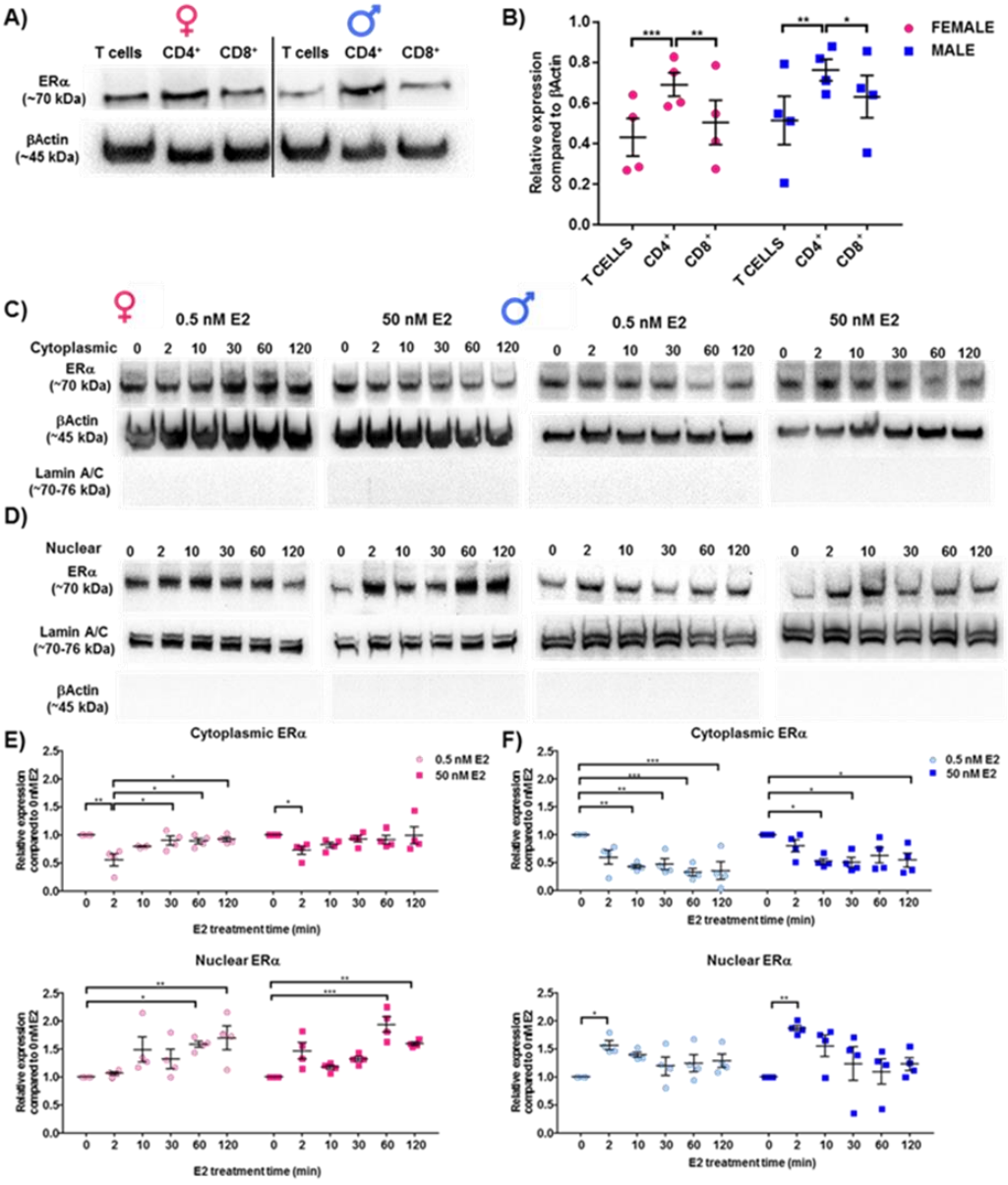


Figure 9. Female and Male HCV1406 TCR-transduced T Cells Express ERα which Rapidly Translocates into the Nucleus upon 17β-estradiol (E2) Stimulation. A) Representative western blot showing ERα expression in the bulk Ag-specific T cell population or sorted $CD4^+$ or $CD8^+$ T cells from one female and one male donor, and **B)** quantification of the relative expression of ERα compared to βActin in bulk T cells, $CD4^+$, and $CD8^+$ sorted Ag-specific T cells. TCR-transduced T cells were then treated with 0.5, or 50 nM E2 for 0, 2, 10, 30, 60, or 120 minutes and ERα expression was measured via western blot from protein extracts of **C)** the cytoplasm or **D)** the nucleus. ERα expression compared to βActin cytoplasmic loading control or Lamin A/C nuclear loading control was quantified for Ag-specific T cells from **E)** female and **F)** male donors treated with 0.5 or 50 nM E2 and normalized to T cells treated with no estrogen (0 nM). Data represents n=4 donors, each donor plotted and the SEM indicated. Analyzed using 2-way ANOVA with Tukey's post-hoc. $p<0.001$=***, $p<0.01$=**, $p<0.05$=*.

This western blot analysis was not able to be performed to test for ERβ nuclear translocation due to lack of reliable antibodies [379, 380]. ERβ was previously shown to be expressed in human $CD4^+$ and $CD8^+$ T cells even if it was expressed at significantly lower levels than ERα [381]. Knowing than ERα and ERβ are highly homologous and have similar affinity for the estrogen ligand [382], it can be hypothesized that ERβ also translocates into the nucleus of Ag-specific T cells upon estrogen ligand binding. The roles of estrogen signaling through both ERα and ERβ on male and female Ag-specific T cell function were studied throughout this dissertation.

Estrogen Signaling through ERα and ERβ Differentially Modulates T cell Cytokine Expression and Secretion

In order to determine the effect of physiological and super-physiological estrogen signaling through ERα and ERβ on T cell function, Ag-specific T cells were activated with their HCV cognate antigen in the presence of estrogen and ERα and ERβ selective inhibitors, and cytokine production was measured. The best system to assess HCV1406 TCR-transduced T cell activation and cytokine production would be to use HCV-expressing human liver cells or liver tumor cells, but these cells were not available for the experiments described. As an alternative, HLA-$A2^+$ T2 cells were pulsed with the HCV NS3 antigen and used as Ag-specific T cell targets. T2 cells pulsed with a tyrosinase irrelevant peptide were used as controls. Male and female Ag-specific T cells were treated with no estrogen, physiological (0.5 nM), or super-physiological (50 nM) estrogen in combination with 100 nM of an ERα, or ERβ selective antagonists, or both inhibitors simultaneously for two hours. The ERα and ERβ inhibitors used were MPP-dihydrochloride and PHTPP respectively. MPP-dihydrochloride is a selective, high

affinity silent antagonist of ERα that displays greater than 200-fold selectivity of ERα over ERβ (K_i values are 2.7 and 1800 nM at ERα and ERβ respectively) [383, 384]. PHTPP is a selective ERβ antagonist that exhibits 36-fold selectivity for ERβ over ERα (K_i values 139 nM and 2.22 μM at ERβ and ERα respectively) [384].

Treated T cells were then co-cultured with pulsed T2 cells. Co-cultures were maintained for 5 hours in the presence of protein transport inhibitors to avoid secretion of markers, and then cells were stained and analyzed for expression of several T cell cytokines. The cytokines assessed included IFNγ, TNFα, IL-2, IL-4, IL-17a, IL-22 and the lytic marker CD107a was assessed on the surface. These markers were chosen to represent the signature cytokines of the different effector and helper T cell subsets (effector $CD8^+$, and $CD4^+$ Th1, Th2, Th17, and Th22) excluding T_{regs} which do not mediate tumor cell killing or beneficial T helper responses for immunotherapy. $CD8^+$ effector T cells commonly secrete high amounts of cytotoxic granules (Perforin and Granzymes), as well as IFNγ and TNFα upon Ag stimulation and activation [385]. Effector CD4 Th1 cells can secrete IFNγ and TNFα as well, while Th2 cells also secrete IL-4. IL-17 and IL-22 were chosen to represent the Th17 and Th22 subsets respectively. Upon activation, Ag-specific $CD4^+$ and $CD8^+$ T cells also produce IL-2 which promotes survival of both $CD4^+$ and $CD8^+$ T cells, and induces subset differentiation of $CD4^+$ T cells 5 [385]. CD107a was chosen as an indicator of Granzyme B release [386]. Granzyme B is released by effector T cells upon antigen stimulation and activation; it is a serine protease which activates apoptosis once in the cytoplasm of the target cell [385, 386]. Gating strategy of these cytokines and CD107a for $CD4^+$ and $CD8^+$ HCV Ag-specific T cells is represented in Figure 10. Gates were selected for the $CD3^+CD34^+$ transduced T cell population and $CD8^+$ and $CD4^+$ T cell subsets

were analyzed separately. For each T cell subset each cytokine and the lytic marker CD107a were plotted against side scatter (SSC) and gated based on unstained controls. The dot plots in Figure 10 represent the data obtained for $CD8^+$ Ag-specific T cells of one donor out of the 30 analyzed. The majority of the donors showed highly increased frequency of $CD8^+$ and $CD4^+$ T cells expressing IFNγ, CD107a and TNFα upon activation which correspond to a cytotoxic $CD8^+$ or an effector Th1 $CD4^+$ T cell response. Some donors also showed increased frequency of $CD4^+$ T cells expressing IL-4 upon activation corresponding to Th2 responses. Surprisingly, $CD8^+$ Ag-specific T cells also expressed IL-4 upon antigen stimulation. IL-2 was expressed by low frequencies of activated T cells. Recombinant human IL-2 was added to Ag-specific T cell cultures during their expansion after transduction and this IL-2 supplementation could be the reason why activated Ag-specific T cells do not express high IL-2 upon antigen stimulation. Also, IL-4 is another gamma cytokine that was shown to enhance the survival of T cells and promote memory formation [387-389] indicating that T cells in these experiments could be partially surviving by IL-4 downstream signaling instead of IL-2. IL-22 and IL-17 were expressed in very low frequencies of T cells upon activation indicating low Th17 and Th22 responses (Figure 10). ELISPOT or ELISA were used to measure effector and helper T cell cytokine secretion including TNFα, IL-4, IFNγ, and Granzyme B from the co-cultures of estrogen treated Ag-specific T cells from male and female donors and T2 cells pulsed with the HCV antigen or the tyrosinase irrelevant peptide. While the expression or secretion of several of the cytokines tested were not directly affected by estrogen receptor signaling, the expression and secretion of three of the cytokines (TNFα, IL-4, and IFNγ) and the lytic molecule Granzyme B were significantly changed upon estrogen stimulation and estrogen receptor signaling as described in the following sections.

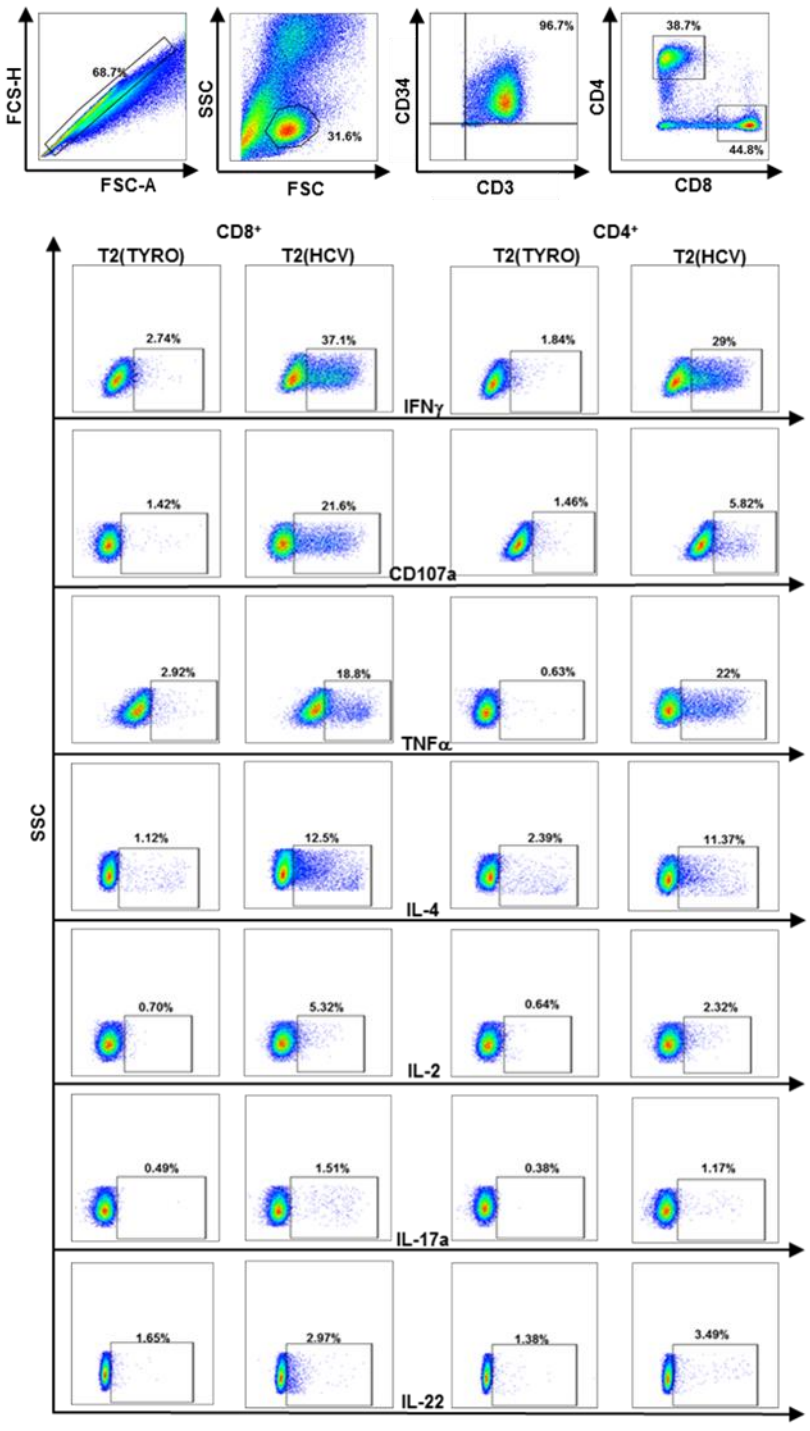


Figure 10. Representative Gating Strategy to Measure Cytokine and CD107a Expression on HCV 1406 TCR-transduced T Cells. T cells were activated with T2 target cells pulsed with the HCV NS3 relevant antigen, T2(HCV), or an tyrosinase irrelevant peptide, T2(TYRO). Singlet cells were gated in the lymphocyte population, and then $CD4^+CD34^+$ transduced T cells were gated into $CD4^+$ and $CD8^+$ populations. Cytokines and CD107a were gated against side scatter (SSC) for both $CD4^+$ and $CD8^+$ transduced T cell populations.

Estrogen Signaling Through ERα Enhances TNFα Expression and Secretion in a Dose Dependent Manner

The percent frequency of male and female transduced $CD8^+$ T cells stimulated with the HCV cognate antigen expressing TNFα was significantly increased upon super-physiological estrogen stimulation (Figure 11 B). The estrogen-mediated increase of TNFα expression was abrogated when male and female $CD8^+$ T cells were treated with estrogen in combination with an ERα inhibitor, or when T cells were treated with estrogen in combination with ERα and ERβ inhibitors simultaneously (Figure 11 B). On the other hand, the estrogen-mediated increase of TNFα expression was not affected by blocking ERβ signaling with an ERβ-specific inhibitor (Figure 11 B). A representative example of these flow cytometry results is depicted in Figure 11 **A**. These data indicate that super-physiological estrogen enhances the expression of TNFα in male and female $CD8^+$ Ag-specific T cells through ERα and not ERβ mediated signaling. The expression of TNFα in $CD4^+$ male and female Ag-specific T cells showed similar trends like the ones observed in $CD8^+$ T cells but due to variability among donors resulting in larger error bars, significance was not achieved (Figure 11 C). There were no sex differences observed in the expression of TNFα since similar frequencies of $CD8^+$ and $CD4^+$ male and female T cells expressed TNFα upon activation (Figure 11 B, C).

TNFα secretion by male and female Ag-specific T cells stimulated with cognate or irrelevant antigen was measured via ELISA. Estrogen stimulation of male and female Ag-specific T cells significantly increased TNFα secretion at physiological (0.5 nM), and super-physiological (50 nM) concentration (Figure 11 D, E).

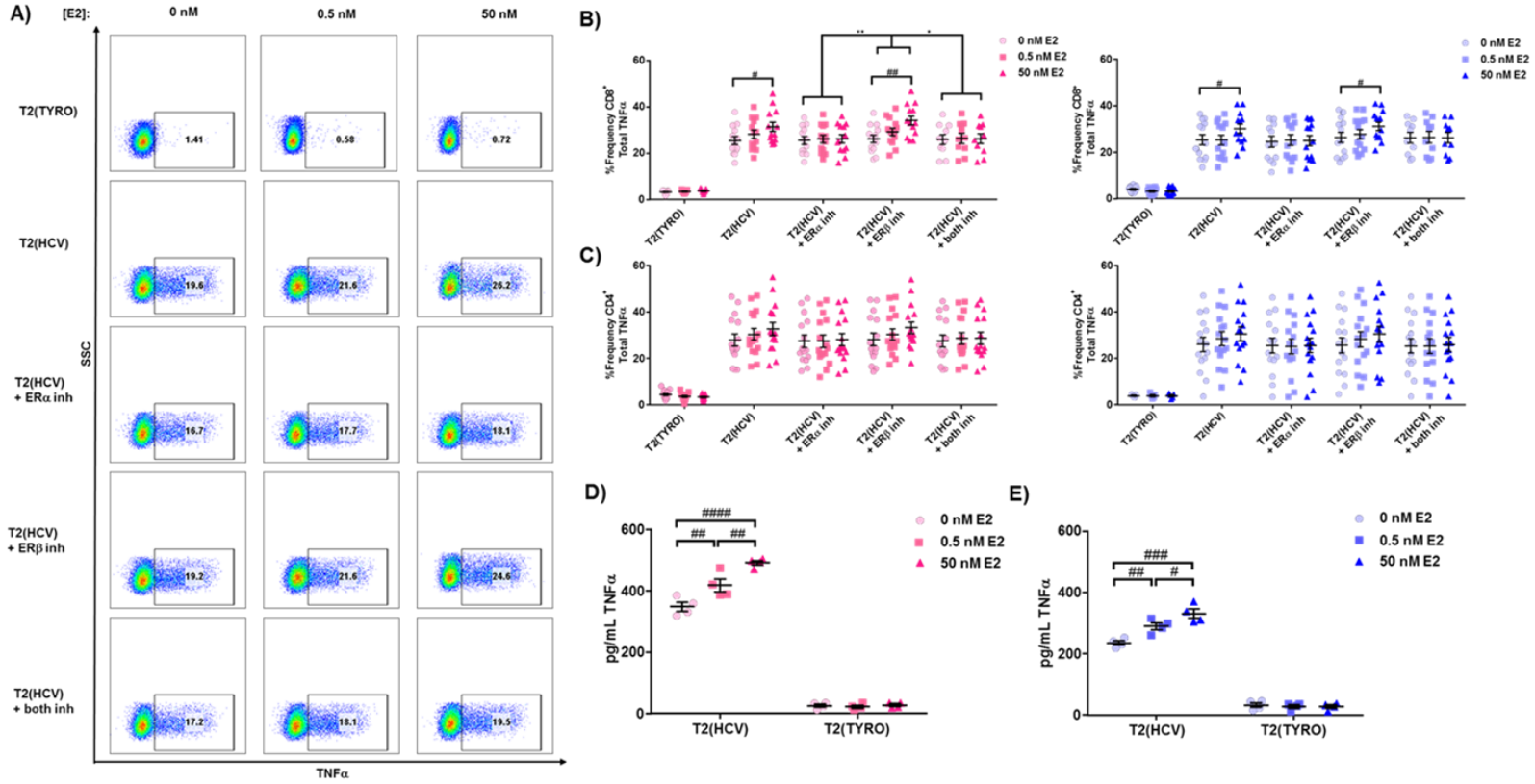


Figure 11. Estrogen Signaling through ERα Enhances TNFα Expression and Secretion in Female and Male Ag-specific T Cells upon Ag Stimulation. Male and female HCV Ag-specific T cells were treated with no estrogen, physiological (0.5 nM), or super-physiological (50 nM) estrogen in combination with 100 nM of an ERα inhibitor (MPP-dihydrochloride), an ERβ inhibitor (PHTPP), or both inhibitors simultaneously for 2 hours prior to activation with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide. After 5 hour activation in the presence of protein transport inhibitors, T cells were analyzed for TNFα expression via flow cytometry intracellular staining. **A)** Representative dot plots for the expression of TNFα in treated T cells from one donor. **B) & C)** The percent frequency of female and male activated **B)** $CD8^+$ and **C)** $CD4^+$ Ag-specific T cells expressing TNFα after being treated with 0, 0.5, or 50 nM estrogen in combination with ER inhibitors. **D) & E)** In order to measure TNFα secretion, the supernatant of 18 hour co-cultures containing equal number of estrogen treated T cells and pulsed T2 target cells were tested via anti-human TNFα ELISA. Data represents n=15 or n=4 donors, each donor plotted and the SEM indicated. Analyzed using 2-way ANOVA with Tukey's post-hoc. $p<0.01$=**, $p<0.05$=* comparing the different estrogen receptor treatment groups. $p<0.0001$=####, $p<0.001$=###, $p<0.01$=##, $p<0.05$=# comparing estrogen treatments (0, 0.5, 50 nM) within each group.

Overall these data indicate that estrogen stimulation can enhance the secretion of TNFα, and estrogen signaling through ERα can enhance the expression of TNFα on $CD8^+$ male and female Ag-specific T cells.

Estrogen Signaling Through ERα Modulates the Overall Expression of IL-4, and Further Enhances its Expression and Secretion in a Dose Dependent Manner

The percent frequency of male and female transduced $CD8^+$ T cells stimulated with their HCV cognate antigen expressing IL-4 was significantly increased upon estrogen stimulation at physiological and super-physiological concentrations (Figure 12 B). The estrogen-mediated increase in IL-4 expression was completely abrogated when male and female $CD8^+$ T cells were treated with estrogen in combination with an ERα inhibitor or with ERα and ERβ inhibitors simultaneously (Figure 12 B). Not only the estrogen-mediated increase on IL-4 expression was abrogated but IL-4 expression was decreased to the levels observed in $CD8^+$ T cells stimulated with the tyrosinase irrelevant antigen (Figure 12 B). On the other hand, blocking ERβ during estrogen stimulation showed no effect on the estrogen-mediated increase, or the overall IL-4 expression of male and female $CD8^+$ Ag-specific T cells (Figure 12 B). Similar results were obtained regarding the expression of IL-4 on estrogen stimulated $CD4^+$ male and female Ag-specific T cells. Estrogen stimulation significantly increased the frequency of $CD4^+$ T cells expressing IL-4 upon cognate antigen activation, and the blockade of ERα signaling during estrogen stimulation decreased IL-4 to the levels of Ag-specific T cells activated with tyrosinase (irrelevant peptide) (Figure 12 C). Blockade of ERβ signaling during estrogen stimulation had no effect on the estrogen-mediated increase of IL-4 expression (Figure 12 C). There were no sex differences observed in the expression of IL-4 since similar frequencies of $CD8^+$ and $CD4^+$ male and female T cells expressed IL-4 upon activation (Figure 12 B, C). These data indicate that

estrogen signaling through ERα and not ERβ modulates the overall expression, and the estrogen-mediated enhancement of expression of IL-4 in male and female Ag-specific T cells.

IL-4 secretion by male and female Ag-specific T cells stimulated with cognate or irrelevant antigen was measured via ELISA. Estrogen stimulation at physiological (0.5 nM), and super-physiological (50 nM) concentrations significantly increased IL-4 secretion of male and female Ag-specific T cells (Figure 12 D, E). Interestingly, estrogen stimulation increased the secretion of IL-4 on male T cells activated with tyrosinase irrelevant antigen (Figure 12 E). This indicates that estrogen stimulation can directly upregulate IL-4 secretion independently from TCR stimulation and T cell activation. These results demonstrate that estrogen signaling through ERα and not ERβ modulates the overall expression of IL-4, and estrogen stimulation at physiological and super-physiological concentrations enhances IL-4 expression and secretion on male and female activated Ag-specific T cells.

Ideally, in order to conclude that Estrogen signaling through ERα directly enhances expression and secretion of TNFα and IL-4, a different set of experiments need to be performed in which human Ag-specific T cells are treated with an ERα agonist instead of estrogen. The ERα selective agonist 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) which shows a 410-fold selectivity for ERα over ERβ can be used [368, 369]. If expression and/or secretion of TNFα and IL-4 are enhanced in male and female Ag-specific T cells treated with PPT after Ag stimulation to levels similar to those treated with estrogen, then ERα alone may enhance the expression/secretion of these cytokines. This can be fully concluded if the PPT-mediated enhancement on the cytokine expression/production is abrogated in T cells treated with PPT and MPP-dihydrochloride (ERα antagonist) simultaneously.

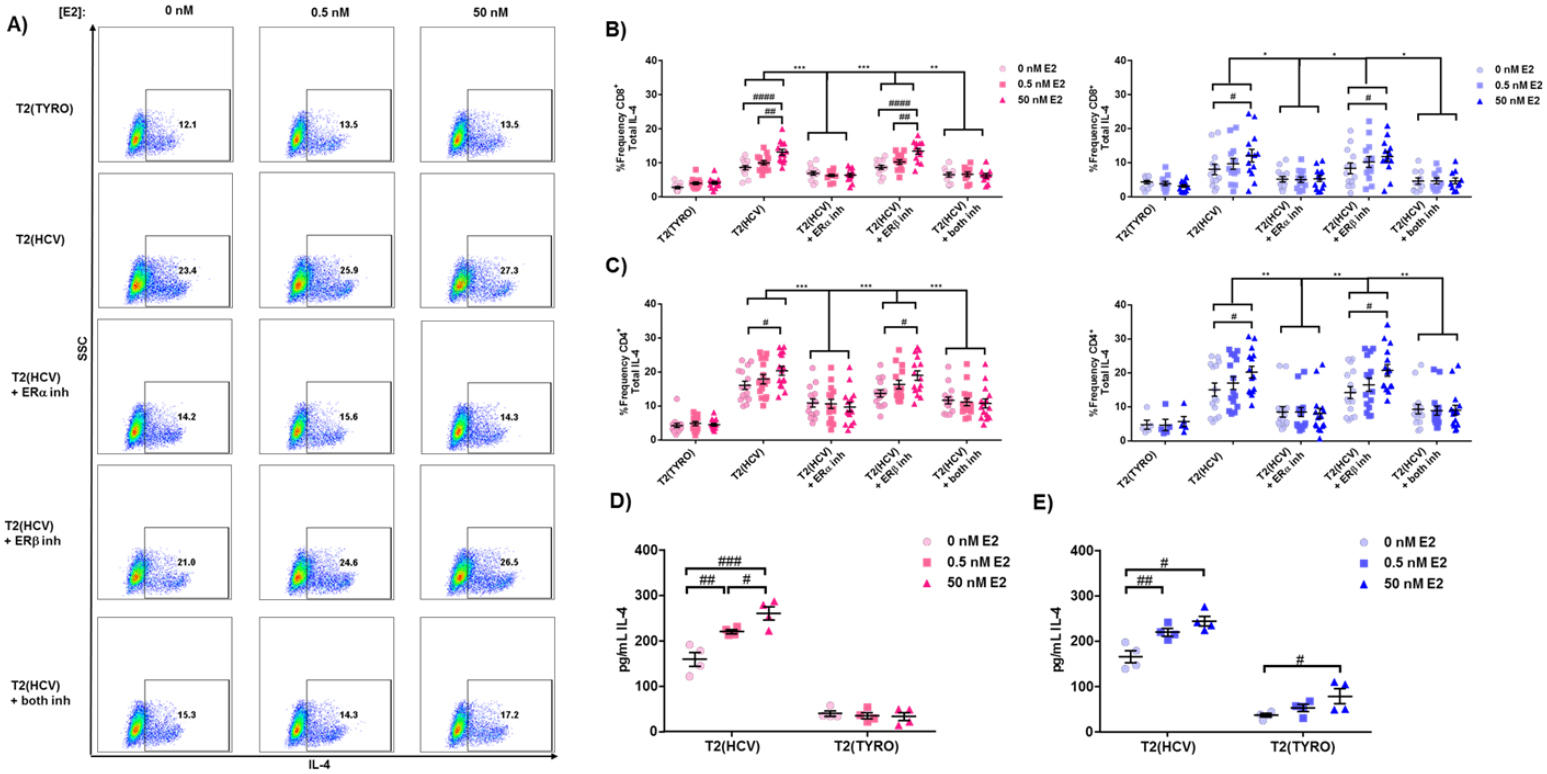


Figure 12. Estrogen Signaling through ERα Modulates Overall IL-4 Expression and Enhances IL-4 Secretion on Male and Female Ag-specific T Cells upon Ag Stimulation. Male and female HCV Ag-specific T cells were treated with no estrogen, physiological (0.5 nM), or super-physiological (50 nM) estrogen in combination with 100 nM of an ERα inhibitor (MPP-dihydrochloride), an ERβ inhibitor (PHTPP), or both inhibitors simultaneously for 2 hours prior to activation with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide. After 5 hour activation in the presence of protein transport inhibitors, T cells were analyzed for IL-4 expression via flow cytometry intracellular staining. **A)** Representative flow cytometry dot plots for the expression of IL-4 in treated T cells from one donor out of the 30 tested. **B) & C)** The percent frequency of female and male activated **B)** $CD8^+$ and **C)** $CD4^+$ Ag-specific T cells expressing IL-4 after being treated with 0, 0.5, or 50 nM estrogen in combination with ER inhibitors. **D) & E)** In order to measure IL-4 secretion, the supernatant of 18 hour co-cultures containing equal number of estrogen treated T cells and pulsed T2 target cells were tested via anti-human IL-4 ELISA. Data represents n=15 or n=4 donors, each donor plotted and the SEM indicated. Analyzed using 2-way ANOVA with Tukey's post-hoc. $P<0.001$=***, $p<0.01$=**, $p<0.05$=* Comparing the different estrogen receptor treatment groups. $p<0.0001$=####, $p<0.001$=###, $p<0.01$=##, $p<0.05$=# comparing estrogen treatments (0, 0.5, 50 nM) within each group.

On the other hand, if treating Ag-specific T cells with PPT does not enhance cytokine expression upon Ag-stimulation, then estrogen signaling through other receptor is mediating this effect. Inhibition of ERβ showed no effect on the estrogen-mediated enhancement of these cytokines' expression indicating that ERβ signaling is not required for their expression. Estrogen signaling through GPER could be enhancing T cell cytokine expression in response to Ag. To investigate that possibility, male and female Ag-specific T cells need to be treated with estrogen in combination of a GPER antagonist. If the expression of TNFα and IL-4 is decreased in T cells treated with estrogen in combination of a GPER antagonist compared to estrogen treated cells then, estrogen is enhancing TNFα and IL-4 expression through GPER. GPER signaling was shown to upregulate expression of some cytokines such as IL-10 and IL-1β [390, 391] so there is a possibility that GPER signaling controls expression of other cytokines such as TNFα and IL-4. Unfortunately, these experiments could not be performed, but these results in combination with the ones reported in this chapter, would indicate that estrogen signaling through ERα and GPER enhance Ag-specific T cell cytokine expression and secretion.

Estrogen Stimulation Enhances Ag-specific T cell IFNγ and Granzyme B Secretion

Even if some trends were observed, the frequency of male and female $CD4^+$ and $CD8^+$ Ag-specific T cells expressing IFNγ was not significantly changed by estrogen stimulation and estrogen receptor signaling during activation as measured by flow cytometry (Figure 13 A, B). The trends observed indicated that estrogen stimulation increased IFNγ expression possibly through ERα and not ERβ. Although IFNγ expression was not significantly affected by estrogen stimulation, there was a significant increase on IFNγ secretion upon super-physiological estrogen treatment in female and male $CD8^+$ and $CD4^+$ Ag-specific T cells activated with the HCV

cognate antigen as measured by ELISPOT (Figure 13 C, D, E). Physiological estrogen significantly increased the secretion of IFNγ in activated male $CD4^+$ Ag-specific T cells (Figure 13 D). Interestingly, the secretion of IFNγ was overall higher in $CD8^+$ and $CD4^+$ Ag-specific T cells from female donors compared to male donors (Figure 13 C, D, E). While female Ag-specific T cells showed an average of 600 to 800 IFNγ spots at baseline estrogen, male T cells showed an average of 300 IFNγ spots at baseline estrogen (Figure 13 C, D). Overall these data demonstrate that estrogen significantly enhances the secretion of IFNγ on male and female $CD4^+$ and $CD8^+$ T cells upon antigen stimulation even if female T cells secrete more IFNγ than male T cells in an estrogen-independent manner.

The secretion of Granzyme B was also measured via ELISPOT on estrogen treated male and female activated Ag-specific T cells. There was a significant increase on Granzyme B secretion on male and female $CD8^+$ Ag-specific T cells treated with super-physiological estrogen (Figure 14 A, B, C). The secretion of Granzyme B was also significantly increased by super-physiological estrogen treatment on female $CD4^+$ Ag-specific T cells, and a trend was observed for male $CD4^+$ T cells even if significance was not achieved (Figure 14 A). The secretion of Granzyme B by female $CD4^+$ T cells was higher than by male $CD4^+$ T cells at baseline estrogen (around 100 average Granzyme B spots compared to 50) and it was increased significantly upon estrogen treatment to around 300 Granzyme B spots compared to only around 100 spots in male $CD4^+$ T cells. Overall these results indicate that estrogen signaling enhances the secretion of Granzyme B in female and male $CD8^+$ T cells, and female $CD4^+$ T cells which secrete more Granzyme B than male T cells in an estrogen-independent manner

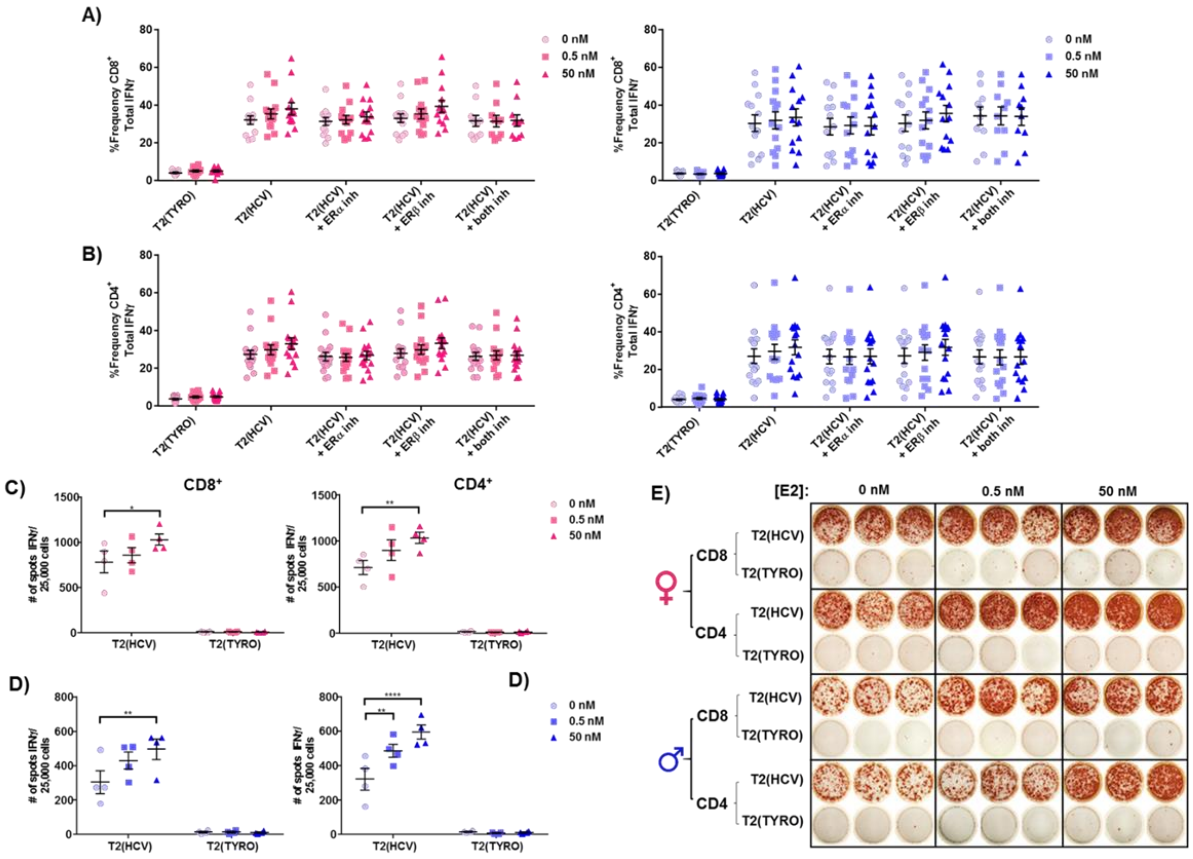


Figure 13. Estrogen Signaling Enhances Male and Female Ag-specific T Cell IFNγ Secretion upon Ag Stimulation. Male and female HCV Ag-specific T cells were treated with no estrogen, physiological (0.5 nM), or super-physiological (50 nM) estrogen in combination with 100 nM of an ERα inhibitor (MPP-dihydrochloride), an ERβ inhibitor (PHTPP), or both inhibitors simultaneously for 2 hours prior to activation with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide. After 5 hour activation in the presence of protein transport inhibitors, T cells were analyzed for IFNγ expression via flow cytometry intracellular staining. **A) & B)** The percent frequency of female and male activated **A)** $CD8^+$ and **B)** $CD4^+$ Ag-specific T cells expressing IFNγ after being treated with 0, 0.5, or 50 nM estrogen in combination with ER inhibitors. In order to measure IFNγ secretion, 18 hour co-cultures containing equal number of estrogen treated **C)** female or **D)** male sorted $CD8^+$ or $CD4^+$ T cells and pulsed T2 target cells were tested via ELISPOT. **E)** Representative ELISPOT plate image from co-cultures of $CD8^+$ or $CD4^+$ T cells from one female and male donor out of the 4 donors tested. Data represents n=15 or n=4 donors, each donor plotted and the SEM indicated. Analyzed using 2-way ANOVA with Tukey's post-hoc. P<0.0001=****, p<0.01=**, p<0.05=*.

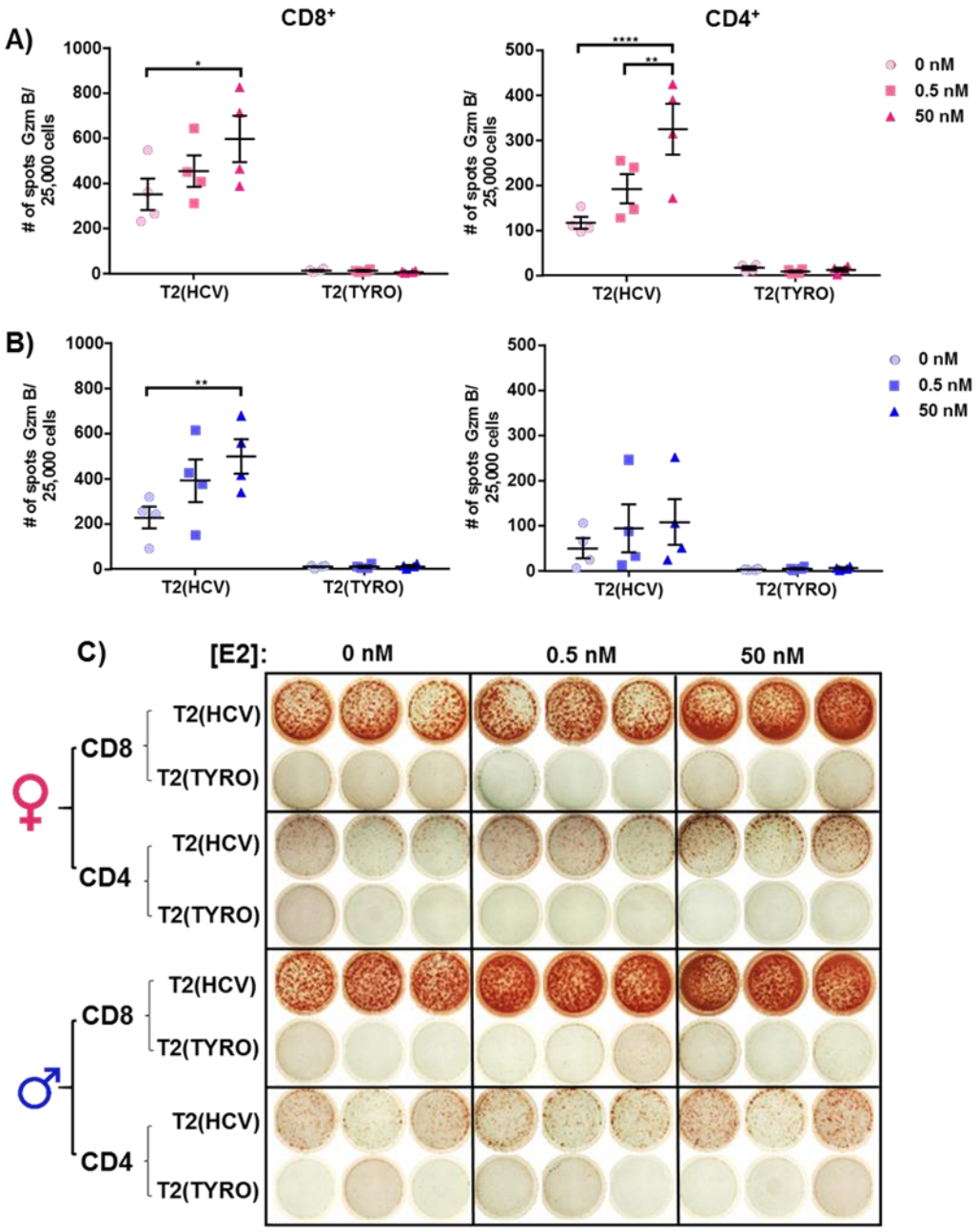


Figure 14. Estrogen Signaling Enhances Granzyme B Secretion in Female and Male CD8$^+$ Ag-specific T Cells, and Female CD4$^+$ Ag-specific T Cells upon Ag Stimulation. In order to measure Granzyme B secretion, 18 hour co-cultures containing equal number of estrogen treated **A)** female or **B)** male CD8$^+$ or CD4$^+$ T cells and pulsed T2 target cells were tested via anti-human Granzyme B ELISPOT. **C)** Representative ELISPOT plate from co-cultures of CD8$^+$ or CD4$^+$ T cells from one female and male donor out of the 4 donors tested. Data represents n=4 donors, each donor plotted and the SEM. Analyzed using 2-way ANOVA with Tukey's post-hoc. P<0.0001=****, p<0.01=**, p<0.05=*.

Chapter Discussion

This chapter of the dissertation was aimed to determine the impact of sex and estrogen signaling in regulating Ag-specific T cell function and cytokine production. For the first time, it was demonstrated that estrogen signaling and sex of the donor regulate the expression and secretion of effector, helper, and lytic T cell cytokines such as TNFα, IL-4, IFNγ, and Granzyme B in human Ag-specific T cells.

Herein it was shown that male and female Ag-specific T cells express equivalent levels of the canonical estrogen receptor, ERα. ERα is well known to bind the estrogen ligand in the cytoplasm of the cell and then to translocate into the nucleus where it can regulate target gene expression by binding to EREs on gene promoters or by binding other transcription factor complexes. ERα rapid nucleus translocation upon ligand binding was observed when both male and female Ag-specific T cells were stimulated with physiological (0.5 nM) or super-physiological (50 nM) estrogen concentrations. This rapid translocation was transient since reduced nuclear ERα expression, and increased cytoplasmic ERα expression was observed overtime after estrogen treatment. This indicates that both male and female Ag-specific T cells can signal through ERα in response to two different estrogen ligand concentrations. It is notable that estrogen can also bind to ERβ, with same affinity as ERα [382], which elicits different downstream effects and modulates different gene expression than ERα. Due to insufficient antibody validation [379, 380], the nuclear translocation of ERβ upon estrogen binding in Ag-specific T cells could not be tested at this time. Knowing that human T cells express ERβ [381], ERβ signaling effects on T cell function cannot be dismissed and are further studied in the chapters of this dissertation. Estrogen can also bind to the membrane bound GPER which

mediates rapid non-genomic responses such as the activation of the MAPK pathway [177]. The changes in cytokine secretion and expression observed in Ag-specific T cells could be the work of not only the canonical ERs but also be influenced by GPER. This needs to be further studied to better understand how to improve the beneficial signaling cascades in these T cells that will promote stronger antitumor responses.

To determine the functional consequences of ERα expression in T cells, cytokine expression and secretion was measured from antigen stimulated $CD8^+$ and $CD4^+$ T cells. Estrogen signaling through ERα was shown to enhance the expression of TNFα in male and female $CD8^+$ Ag-specific T cells, and estrogen treatment also enhanced the secretion of TNFα in male and female Ag-specific T cells in a concentration dependent manner. The promoter of TNFα contains an ERE at position -1044 bp [392] indicating that the enhancement of TNFα expression and secretion in male and female Ag-specific T cells could be mediated by canonical ligand-dependent genomic ERα signaling (Table 2).

Estrogen signaling through ERα was shown to regulate the overall expression of IL-4 in male and female $CD8^+$ and $CD4^+$ Ag-specific T cells. The estrogen-mediated enhancement of IL-4 expression and the overall IL-4 expression were abrogated when T cells were treated with estrogen in combination with an ERα inhibitor, suggesting that estrogen regulates IL-4 expression in a classical ligand-dependent genomic manner. Surprisingly, an ERE was not found on the promoter of IL-4 which could indicate ERE-independent ERα genomic signaling. It was demonstrated that a significant correlation exists between the expression of ERα and GATA3 in breast cancer cell lines and mouse T cells, and an ERE was found at position -1502 bp on the promoter of GATA3 (Table 2) [393-395]. GATA3 is an essential Th2 transcription factor that

not only drives differentiation of TCR stimulated $CD4^+$ T cells into the Th2 subset but also directly induces IL-4 gene expression [396, 397]. In fact, T cells from GATA3 KO mice fail to develop appropriate Th2 cytokine expression including IL-4 [398, 399]. Overall this indicates that ERα signaling regulates IL-4 expression and secretion in Ag-specific T cells through mechanisms that are ERE-independent possibly including the upregulation of expression of GATA3.

Estrogen signaling was shown to enhance the secretion of IFNγ in female and male Ag-specific T cells. IFNγ contains an ERE at position -8342 bp of its promoter which indicates that estrogen signaling through ERα can enhance IFNγ secretion through the ligand-bound ERE-dependent canonical pathway. In addition, an ERE was also found on the promoter of T-BET at position -1676 bp (Table 2). T-BET is a transcription factor known to mediate Th1 T cell differentiation and induce IFNγ expression [400, 401]. Estrogen signaling enhancement of T-BET expression could result in subsequent IFNγ expression and secretion enhancement in activated T cells. Estrogen was also shown to enhance the secretion of Granzyme B in female and male Ag-specific T cells. While Granzyme B does not contain an ERE in its promoter, proteinase inhibitor 9 (PI-9), which is a Granzyme B inhibitor, contains one ERE at position -200 bp of its promoter [402, 403]. This indicates that estrogen could be upregulating Granzyme B secretion by negatively regulating transcription of PI-9 through ERα ligand-bound ERE-dependent genomic pathway.

Estrogen treatment increases the expression and/or secretion of TNFα, IL-4, IFNγ, and Granzyme B in human Ag-specific T cells. TNFα, IFNγ, and Granzyme B are cytokines commonly secreted by Type I cytotoxic $CD8^+$ T cells and effector Th1 $CD4^+$ T cells in response

to antigen stimulation. On the other hand, IL-4 is secreted by Type II $CD8^+$ T cells and it is the prototypic Th2 $CD4^+$ T cell cytokine required for Th2 T cell differentiation. The Th1 and Th2 $CD4^+$ T cell subset differentiation pathways can downregulate each other and are believed to be mutually exclusive [404, 405]. Estrogen signaling through ERα can upregulate the expression and secretion of both Type I/Th1 and Type II/Th2 cytokines in male and female Ag-specific T cells. This could indicate that estrogen modulates the balance of the $CD8^+$ Type I and Type II, and the $CD4^+$ Th1 and Th2 T cell responses. Most of the effects on these cytokines were observed when T cells were stimulated with super-physiological estrogen (50 nM) indicating that the estrogen-mediated Th1/Th2 T cell response balance could happen in locally elevated estrogen environments including the HCC TME. In order to test if ERα signaling can modulate the balance between Th1 and Th2 T cell subset differentiation, first, estrogen receptor binding to the EREs present on the GATA3 and T-BET gene promoters needs to be confirmed via chromatin immunoprecipitation (ChIP); and, second, enhancement of T-BET and GATA3 expression by ER signaling needs to be confirmed by real time polymerase chain reaction (RT-PCR) or by transducing T cells with expression vectors containing the promoters of T-BET and GATA3.

Apart from their roles in Th1 and Th2 T cell differentiation, the cytokines enhanced by estrogen in Ag-specific T cells have important anti-tumor roles. IFNγ is cytotoxic to certain malignant cells, and it enhances MHC class I expression [406]. Granzyme B directly lyses malignant cells, and TNFα promotes T cell activation, co-stimulation, and promotes certain cancer cell death [406]. IL-4 promotes T cell and B cell survival, induces Ig class switch to IgE and IgG in B cells, drives long-term development of $CD8^+$ T cell memory, and in combination

with TGFβ it drives Th9 T cell differentiation which are T cells that augment anti-tumor responses in ACT models [387-389, 407, 408]. Overall these data indicate for the first time, that estrogen signaling through ERα enhances expression and secretion of cytokines that enhance the T cell anti-tumor immune response and estrogen signaling is an important factor to consider when designing ACT immunotherapies for estrogen-sensitive and estrogen-producing malignancies like HCC.

Hs Gene Name	Hs Chr #	Hs ERE Sequence	Hs ERE position	Hs Gene Start	Hs Gene End	Hs Dist. Gene-ERE
TNFA	Chr 6	AGGTCACAGTGACCT	31574523	31575567	31578336	-1044
GATA3	Chr 10	AGGTCCTTCTGACCA	8043876	8045378	8075203	-1502
IFNG	Chr12	AGGTTGCAGTGACCC	30688467	30696809	30691855	-8342
TBX21	Chr17	GGGTCATGCCCACCT	1102133	1103809	1116684	-1676
IP-9	Chr 6	GGTCAATTTGAAA	2887066	2887266	2903280	-200

Table 2. Estrogen Response Elements Found in the Promoters of TNFα, GATA3, IFNγ, TBX21(T-BET), and IP-9 Genes. Table shows the human (Hs) gene name, the chromosome location, the ERE sequence and its position in respect to the gene starting site. EREs were found in the EREfinder database described by Bourdeade *et al*. *Mol Endocrinol*. 2004.

CHAPTER IV

ESTROGEN SIGNALING THROUGH ERα AND ERβ DIFFRENTIALLY IMPACTS HUMAN MALE AND FEMALE AG-SPECIFIC T CELL POLYFUNCTIONALITY

Introduction and Rationale

The results presented in the previous chapter demonstrated that estrogen signaling enhances the expression and secretion of T cell cytokines typically classified into the $CD4^+$ Th1 or Th2 subsets, or into the $CD8^+$ Type I or Type II subsets. Despite their differences, both Type I/Th1 and Type II/Th2 T cell responses were shown to enhance anti-tumor immunity by enhancing CTL expansion and survival, and by killing tumor cells directly [409, 410]. Most importantly, ACT immunotherapy was shown to be most successful when $CD8^+$ T cells were delivered in combination with a mixture of $CD4^+$ Th1 cells and Th2 cells [82, 411]. More recently, other T cell subsets were correlated with enhanced T cell anti-tumor immune responses such as the IL-9 producing Th9 $CD4^+$ T cells [412, 413], and the IL-17a producing Th17 $CD4^+$ T cells [83]. Even if the anti-tumor or tumor-promoting role of IL-22 producing Th22 $CD4^+$ T cells remains controversial, Th22 infiltration was correlated with tumor rejection in colorectal cancer [414]. The presence of multiple T cell subsets secreting varying combinations of cytokines indicates that the evaluation of a single cytokine at one time does not accurately characterize the role of estrogen signaling on the overall T cell anti-tumor function. In fact, a previous study demonstrated that analysis of expression of only one effector T cell cytokine, such as IFNγ, in advanced stage cancer patients was not correlated with survival advantage, but once other

cytokines, such as IL-2, were analyzed simultaneously via flow cytometry with IFNγ there was an association with clinical outcome [415]. In addition to the heterogeneity of their subset phenotype, T cells show differences in their functional profile or the mixture of cytokines that they secrete upon TCR ligation. It was found that T cells can not only be subdivided based on prototypic cytokine and transcription factor expression, but also based on the distinctive patterns of cytokines secreted after stimulation [416]. Activated T cells can be monofunctional if they only express one cytokine, or polyfunctional if they expressed over two different cytokines upon TCR stimulation.

T cell polyfunctionality is described as the ability of one T cell to deploy a broad spectrum of immune programs upon antigen stimulation. These immune programs can be cytokines or lytic markers with polyfunctional T cells being able to, for example, secrete IFNγ, IL-2 and degranulate producing Granzyme B simultaneously. Polyfunctional $CD8^+$ and $CD4^+$ T cells were shown to be able to generate superior anti-tumor immune responses [417-420], and to clear viral infections better than monofunctional T cells [421, 422]. In the setting of immunotherapy, several studies have found enhanced numbers of tumor Ag-specific polyfunctional T cells in patients responding favorably to ACT, and checkpoint blockade using anti-CTLA-4 [423-427]. A study by Rossi *et al.* using CD19-targeted CAR T cells to treat acute lymphoblastic leukemia demonstrated that CAR-T cell polyfunctionality was correlated with patient clinical outcome [428]. Even if only approximately 20% of adoptively transferred CAR T cells were polyfunctional (able to produce >2 out of 32 different cytokines and chemokines simultaneously), this low frequency of polyfunctional cells was still able to produce superior anti-tumor immune responses compared to monofunctional T cells. Polyfunctional $CD8^+$ CAR-T cells in this study produced combinations of IFNγ, macrophage inflammatory protein-1α (MIP-

1α) (or CCL3), IL-8, and Granzyme B simultaneously while $CD4^+$ CAR-T cells expressed combinations of IFNγ, MIP-1α, IL-8, IL-2, IL-17a, and IL-5 simultaneously [428]. T cell polyfunctionality was also correlated with $CD8^+$ T cell memory formation. Memory $CD8^+$ T cells were shown to be highly polyfunctional expressing cytokine combinations such as $IFN\gamma^+IL\text{-}2^+TNF\alpha^+MIP\text{-}1\beta^+$ (or CCL4), $IFN\gamma^+TNF\alpha^+IL\text{-}2^+$, and $IFN\gamma^+IL\text{-}2^+CD107a^+$ during antigen stimulation [429-432]. This indicates that the use of polyfunctional T cells could increase the efficacy of ACT immunotherapy by generating superior anti-tumor responses and subsequent T cell memory development against tumor antigens.

Since estrogen signaling was able to enhance the secretion of several effector Th1 and helper Th2 cytokines in female and male Ag-specific T cells, it was hypothesized that estrogen signaling enhances the polyfunctionality or the ability of Ag-specific T cells to express several cytokines simultaneously upon antigen stimulation. In order to study the effect of estrogen receptor signaling on Ag-specific T cell polyfunctionality, male and female Ag-specific T cells were treated with physiological (0.5 nM) or super-physiological (50 nM) estrogen alone or in combination with an ERα inhibitor (100 nM), an ERβ inhibitor (100 nM), or both inhibitors simultaneously prior to activation with T2 cells pulsed with the HCV cognate antigen or a tyrosinase irrelevant peptide. After 5 hour activation in the presence of protein transport inhibitors, multi-dimensional flow cytometry was used to measure expression of IFNγ, TNFα, IL-2, IL-4, IL-17a, IL-22 and the lytic marker CD107a. This multi-functional evaluation was designed to reflect the changes in mono or polyfunctional phenotypes of T cell populations. The analysis of these seven functional markers after gating on $CD3^+CD34^+$ transduced T cells generated 128 possible combinations (2^n, n=7, 2^7=128) of markers expressed by $CD8^+$ or $CD4^+$ T

cells; from monofunctional cells that only express one marker, to highly polyfunctional cells that expressed all 7 markers simultaneously. Since they were a total number of 12 treatments, including 3 estrogen conditions (0, 0.5, and 50 nM) and 4 ER inhibitor conditions (no inhibitor, ERα inhibitor, ERβ inhibitor, and both inhibitors), an exceptionally complex dataset was generated that was difficult to analyze with basic flow cytometry software (FlowJoX).

Analysis of polyfunctionality data using FlowJo is very limited since it restricts the visualization of data to one or two parameters at a time. In this case, visualization and analysis of 7 markers requires 21 pairwise dot plots for a single Ag-stimulation condition per estrogen treatment. Figure 15 shows a representative example of pairwise dot plots in untreated female $CD8^+$ HCV Ag-specific T cells stimulated with HCV (Figure 15 A) or tyrosinase (Figure 15 B) pulsed T2 target cells. Even if these comparisons are very limited, some simple observations can be deduced by observing the percent frequency of T cells that express two markers simultaneously. The highest frequency of bifunctional $CD8^+$ cells expressed the $IFN\gamma^+TNF\alpha^+$ combination but $IFN\gamma^+$ cells also expressed CD107a, or IL-4 simultaneously. Similarly, $TNF\alpha^+$ cells also expressed IL-4, and some CD107a or IL-2 simultaneously. This was surprising since $CD8^+$ T cells could express cytokines like IFNγ and TNFα, which are believed to be restricted to the type I (Th1) phenotype, simultaneously with the type II (Th2) cytokine IL-4. These data contradicts what is normally accepted in the field of T cell immunology concerning type I and II T cell populations and function. This indicates that evaluation of several functional markers and generation of polyfunctional T cell profiles can shine light on the study of new patterns of T cell function benefigureFficial for generating superior anti-tumor immune responses.

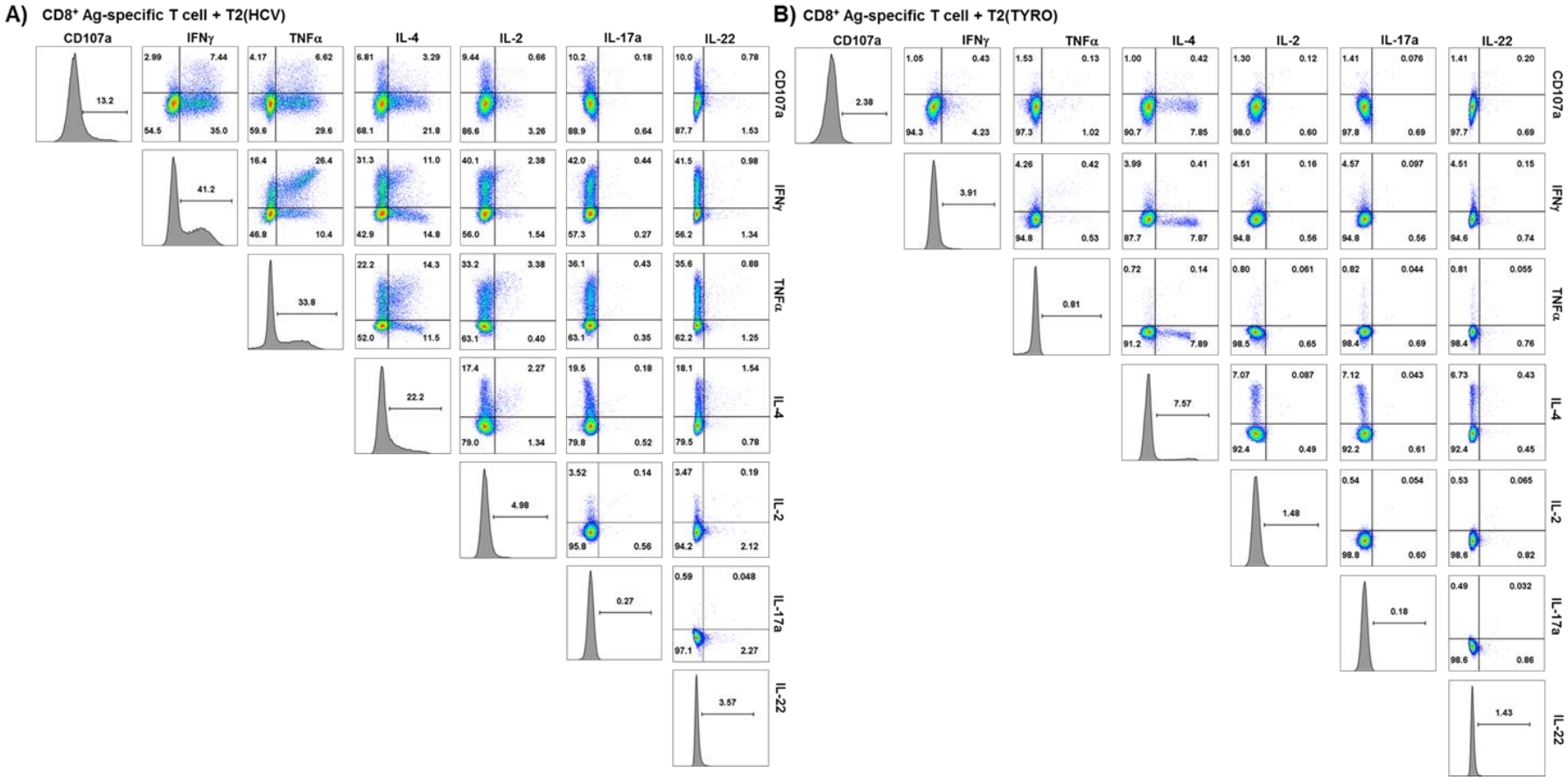


Figure 15. Pairwise Comparisons of Each Functional Marker Expressed by Activated CD8$^+$ HCV Ag-specific T Cells. HCV Ag-specific T cells from one female donor were co-cultured with T2 cells pulsed with the **A)** HCV cognate antigen or **B)** a tyrosinase irrelevant peptide for 5 hours in the presence of protein transport inhibitors. Cells were then stained for flow cytometric analysis of CD3, CD34, CD8, and CD4 and functional markers CD107a, IFNγ, TNFα, IL-4, IL-2, Il-17a, and IL-22. Gating and analysis was performed using FlowJoX. Uni-dimensional histograms represent the expression of each functional marker, and each dot plot represents pairwise comparisons between each of the 7 functional markers expressed by CD8$^+$ Ag-specific T cells

In order to generate the frequency of T cells expressing each of the 128 different cytokine combinations, higher resolution multi-dimensional analysis was performed within FlowJo using Boolean combinatorial gates for all 7 markers. The frequencies of populations were added into categories comprising cells expressing 1 through 7 markers simultaneously. Percent frequencies of T cells expressing >2 makers simultaneously were considered polyfunctional. By grouping polyfunctional phenotypes into categories according to the number of markers expressed, clear comparisons between female and male T cells and different estrogen and inhibitor treatments can be made. Statistical analysis of these data can be performed using a Multivariate Analysis of Variance (MANOVA) which is a multivariate ANOVA with two or more continuous response variables. MANOVA allows analyzing $CD8^+$ and $CD4^+$ T cells within one sex under one treatment condition (either estrogen or estrogen + ER inhibitor) and compares two or more continuous response variables, in this case 7 different categories, or groups of T cells expressing 1 through 7 markers simultaneously. Post-hoc pairwise comparisons between each index estrogen treatment (50 & 0.5) and the reference estrogen treatment (0) were conducted within each estrogen inhibitor treatment.

Despite these meaningful interpretations, analysis in FlowJo lacks the ability to graphically represent individual polyfunctional phenotypes or display how estrogen signaling impacts their relative abundance. In order to compare the levels of expression of all 128 marker combinations, Boolean gating with FlowJo was followed by analysis in Pestle and SPICE data processing software packages which allow to adequately visualize individual and polyfunctional populations. Using the data generated from Pestle and SPICE, heat maps were created containing the percent frequency of cells expressing each combination of markers. Results from these

analyses are discussed in the following sections. Statistical analysis of the percent expression of each cytokine combination can be performed using MANOVA. MANOVA is used on $CD8^+$ and $CD4^+$ T cells within one sex under one treatment condition (either estrogen or estrogen + ER inhibitor) and compares two or more continuous response variables, in this case all the different (up to 83) combinations of cytokines expressed by Ag stimulated T cells. Post-hoc pairwise comparisons between each index estrogen treatment (50 & 0.5) and the reference estrogen treatment (0) were conducted within each estrogen inhibitor treatment stratum.

Results

$CD8^+$ Ag-specific female T cells are inherently more polyfunctional than male T cells

It was observed that non-estrogen treated $CD8^+$ and $CD4^+$ Ag-specific T cells contained similar percentages of monofunctional and polyfunctional cells when stimulated with their HCV cognate antigen. Both populations exhibited around 30 to 40% of monofunctional and bifunctional cells, and around 15 to 20% of trifunctional cells. Less than 10% of $CD8^+$ and $CD4^+$ Ag-specific T cells expressed more than 4 markers simultaneously and percentages for those cells expressing 6 or 7 markers were so low that are not represented on the results (Figure 16). While there were no sex-differences on the polyfunctionality of $CD4^+$ Ag-specific T cells when marker combinations were analyzed as added groups (Figure 16 B), significantly higher percentage of male $CD8^+$ Ag-specific T cells were monofunctional (only expressed 1 marker) compared to female $CD8^+$ T cells (Figure 16 A). A trend was observed for higher percentages of female $CD8^+$ T cells expressing 3 markers simultaneously compared to male $CD8^+$ T cells. This indicates that independently from estrogen signaling, female $CD8^+$ Ag-specific T cells are inherently more polyfunctional than male $CD8^+$ T cells. While these results were shown to be

statistically significant, there was a high variability between male donors in the percentage of $CD8^+$ T cells expressing only one marker. Most of the male donors had frequencies of monofunctional cells similar to those of female donors, but some of the male donors showed very high percentage of monofunctional cells. This variability is not surprising due to genetic and environmental differences between humans, and it indicates that there are differences in polyfunctionality between same sex donors. This could be caused by differences on age (some of the donors used for the experiment were of unknown age), disease background, circulating hormone levels, and differences on T cell activation.

The female and male $CD8^+$ and $CD4^+$ Ag-specific polyfunctional responses were very diverse expressing a great amount of marker combinations that were polyfunctional, bifunctional, and monofunctional. $CD8^+$ T cells from females and males expressed 71 different functional marker combinations in response to antigen stimulation (Figure 17 A). While a lot of these marker combinations were expressed by a very low frequency of cells, some combinations including $TNF\alpha^+$ (~10-15%), $IFN\gamma^+TNF\alpha^+$ (~25-30%), $IFN\gamma^+$ (~25%), $CD107a^+IFN\gamma^+TNF\alpha^+$ (~7%), $IFN\gamma^+$ $IL\text{-}2^+TNF\alpha^+$ (~5%), and $IFN\gamma^+IL\text{-}4^+TNF\alpha^+$ (~4%) were expressed at higher levels. The only combination that shows significant differences in expression between female and male T cells was $TNF\alpha$ alone which was expressed by a significantly higher frequency of male T cells compared to female T cells (Figure 17 A). Other combinations that showed trending differences in expression included $CD107a^+IFN\gamma^+TNF\alpha^+$, $CD107a^+IFN\gamma^+$, $IFN\gamma^+IL\text{-}2^+TNF\alpha^+$, $IFN\gamma^+IL\text{-}4^+TNF\alpha^+$, and $IFN\gamma^+TNF\alpha^+$ which were all expressed by higher frequencies on female T cells compared to male T cells. Taking these data together with the ones obtained in Figure 16 A, it can be concluded that female $CD8^+$ Ag-specific T cells are inherently more polyfunctional upon

antigen stimulation than male $CD8^+$ Ag-specific T cells. In addition, higher percentages of female $CD8^+$ polyfunctional cells expressed marker combinations that are detrimental to tumors, such as the ones containing cytotoxic cytokines IFNγ, TNFα, and the marker CD107a, as well as marker combinations that include cytokines known to enhance T cell survival like IL-2 and IL-4.

Male and female $CD4^+$ Ag-specific T cells expressed up to 83 different functional marker combinations upon antigen stimulation (Figure 17 B). As observed in the $CD8^+$ T cell subset, most of the combinations were expressed by very low frequency of cells with the exception of the highest expressed functional combinations being $IFN\gamma^+TNF\alpha^+$ (~30%), $IFN\gamma^+$ (~22%), $TNF\alpha^+$ (~15%), $IFN\gamma^+$ $IL\text{-}4^+TNF\alpha^+$ (~5%), and $IFN\gamma^+IL\text{-}2^+TNF\alpha^+$ (~4%) (Figure 17 B). The only combination that showed a significant difference in expression between female and male $CD4^+$ T cells was IFNγ alone which was expressed by a significantly higher frequency of female T cells compared to male T cells (Figure 17 B). Other trending differences in expression were observed including higher frequencies of female $CD4^+$ T cells expressing $IFN\gamma^+TNF\alpha^+$, and $IFN\gamma^+IL\text{-}4^+TNF\alpha^+$ compared to male $CD4^+$ T cells. While there may be differences in expression on some polyfunctional phenotypes between female and male $CD4^+$ T cells, significance was not achieved and taken these results with the ones observed in Figure 16 B it can be concluded that there are no sex-differences in male and female $CD4^+$ T cell polyfunctionality.

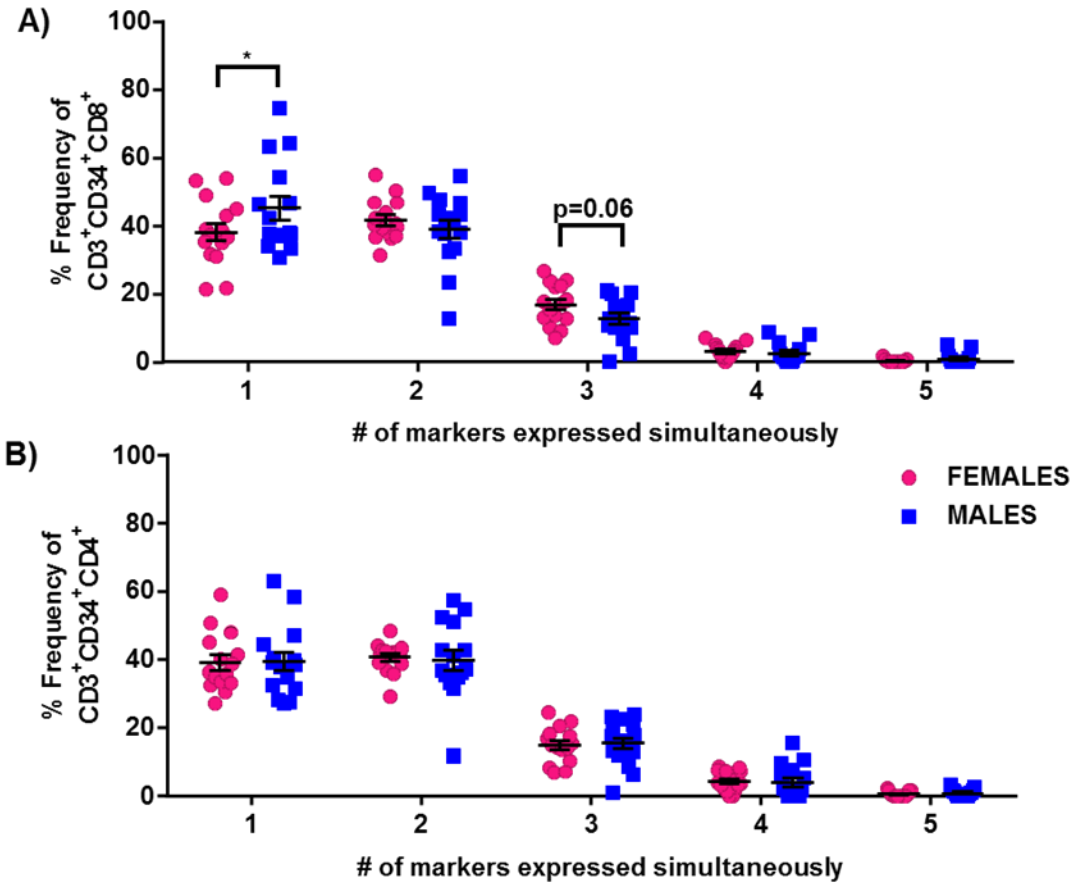


Figure 16. Female $CD8^+$ Ag-specific T Cells are More Polyfunctional than Male Counterparts upon Ag Stimulation. Male and female HCV Ag-specific T cells from 15 female and 15 male donors were co-cultured for 5 hours with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide in the presence of protein transport inhibitors. Cells were then stained for flow cytometric analysis of CD3, CD34, CD8, and CD4 and functional markers CD107a, IFNγ, TNFα, IL-4, IL-2, IL-17a, and IL-22. Using Boolean gates in FlowJoX, the frequency of $CD8^+$ and $CD4^+$ T cells expressing each combination of these 7 markers was generated and added into 7 functional categories. The frequencies of **A)** $CD8^+$, and **B)** $CD4^+$ female and male Ag-specific T cells expressing one through seven markers simultaneously was plotted. The frequencies of $CD8^+$ and $CD4^+$ T cells stimulated with the tyrosinase irrelevant peptide were used for background subtraction. Data for T cells expressing 6 or 7 markers simultaneously is not shown since the percent frequency was very low (<1%). Data represents n=15 donors, each donor plotted and SEM indicated. Data was analyzed using MANOVA and Tukey's post-hoc. $p<0.05$=*.

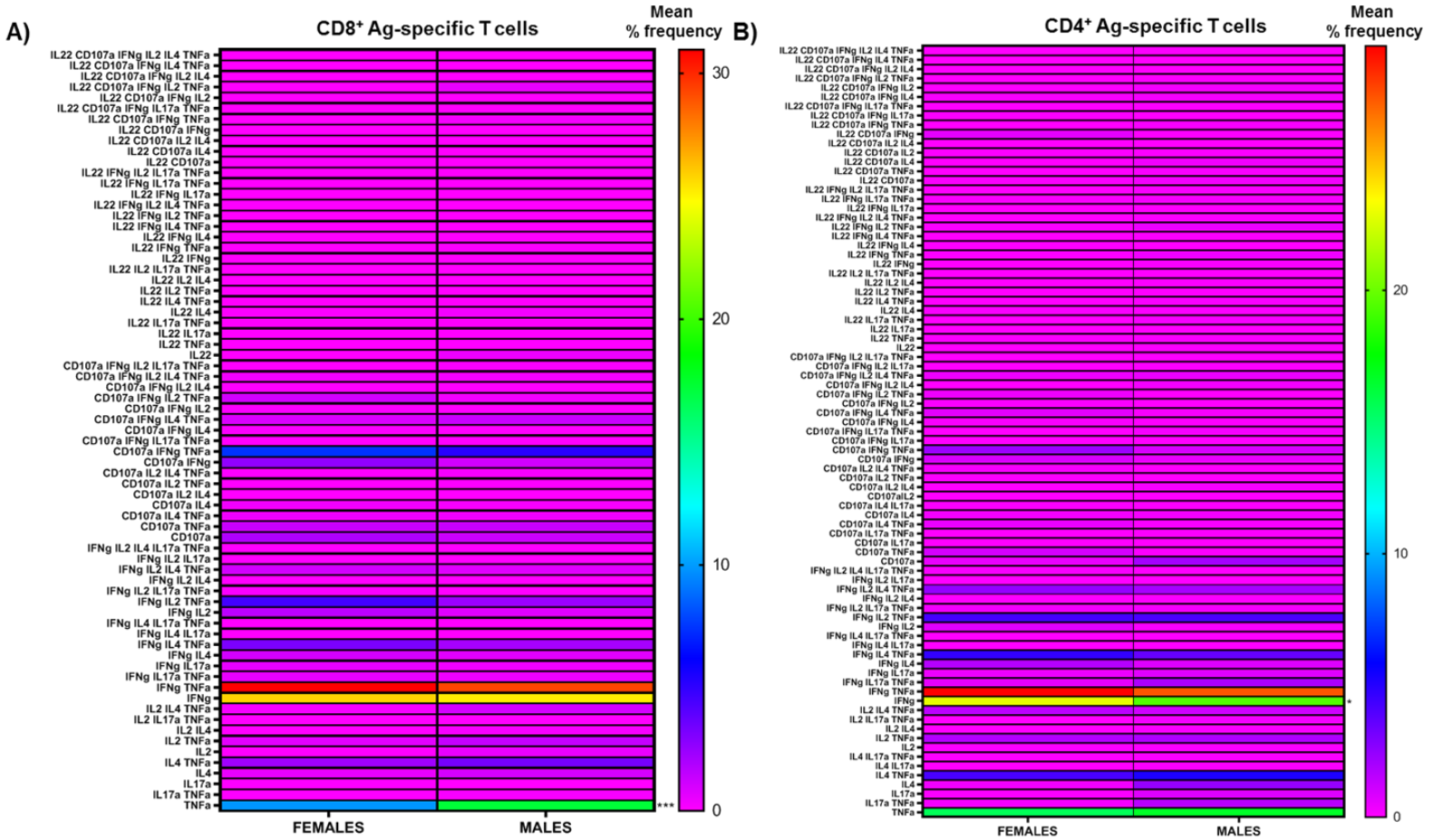


Figure 17. Significantly Higher Percentage of Male CD8$^+$ Ag-specific T Cells Express TNFα in Combination with no Other Marker Compared to Female CD8$^+$ T Cells. Significantly Higher Percentage of Female CD4$^+$ Ag-specific T Cells Express IFNγ in Combination with no Other Marker Compared to Male CD4$^+$ T Cells. Male and female HCV Ag-specific T cells from 15 female and 15 male donors were co-cultured for 5 hours with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide in the presence of protein transport inhibitors. Cells were then stained for flow cytometric analysis of CD3, CD34, CD8, and CD4 and functional markers CD107a, IFNγ, TNFα, IL-4, IL-2, IL-17a, and IL-22. Using Boolean gates in FlowJoX, and the software SPICE the frequency of CD8$^+$ and CD4$^+$ T cells expressing each combination of these 7 markers was generated. The frequencies of **A)** CD8$^+$ and **B)** CD4$^+$ female and male Ag-specific T cells expressing each of the 128 possible markers combinations was plotted. Data represents the mean of n=15 donors. Data was analyzed using MANOVA and Tukey's post-hoc. $p<0.001$=***, $p<0.05$=*.

Estrogen signaling through ERβ enhances female and male $CD8^+$ Ag-specific T cell polyfunctionality

A significant decrease on the percent frequency of monofunctional male and female $CD8^+$ Ag-specific T cells was observed upon estrogen treatment, especially at super-physiological concentrations. While the percent frequency of bifunctional cells was not significantly changed, the percent frequency of $CD8^+$ T cells expressing 3 markers simultaneously was significantly increased upon estrogen treatment (Figure 18 & 19 A). This indicated that estrogen signaling enhances the percentage of polyfunctional cells while decreasing the percentage of monofunctional cells. The estrogen-mediated enhancement of $CD8^+$ male and female T cell polyfunctionality was not affected by blocking ERα with a specific antagonist (MPP-dihydrochloride) during estrogen treatment (Figure 18 & 19 B). In fact, upon ERα blockade, the estrogen-mediated decrease on monofunctional T cell percentage and increase on trifunctional percentages was even more significant at physiological estrogen concentrations. Thus demonstrating that ERα signaling does not enhance $CD8^+$ Ag-specific polyfunctionality but rather it partially inhibits it when T cells are exposed to physiological estrogen. Surprisingly, the estrogen-mediated enhancement of $CD8^+$ T cell polyfunctionality was completely abrogated when ERβ signaling was blocked with a specific antagonist (PHTPP) (Figure 18 & 19 C). No changes in polyfunctionality were observed when male and female $CD8^+$ T cells were treated with estrogen in combination with both ERα and ERβ antagonists (Figure 18 & 19 D). Overall these data reveal that estrogen signaling through ERβ enhances $CD8^+$ male and female Ag-specific T cell polyfunctionality.

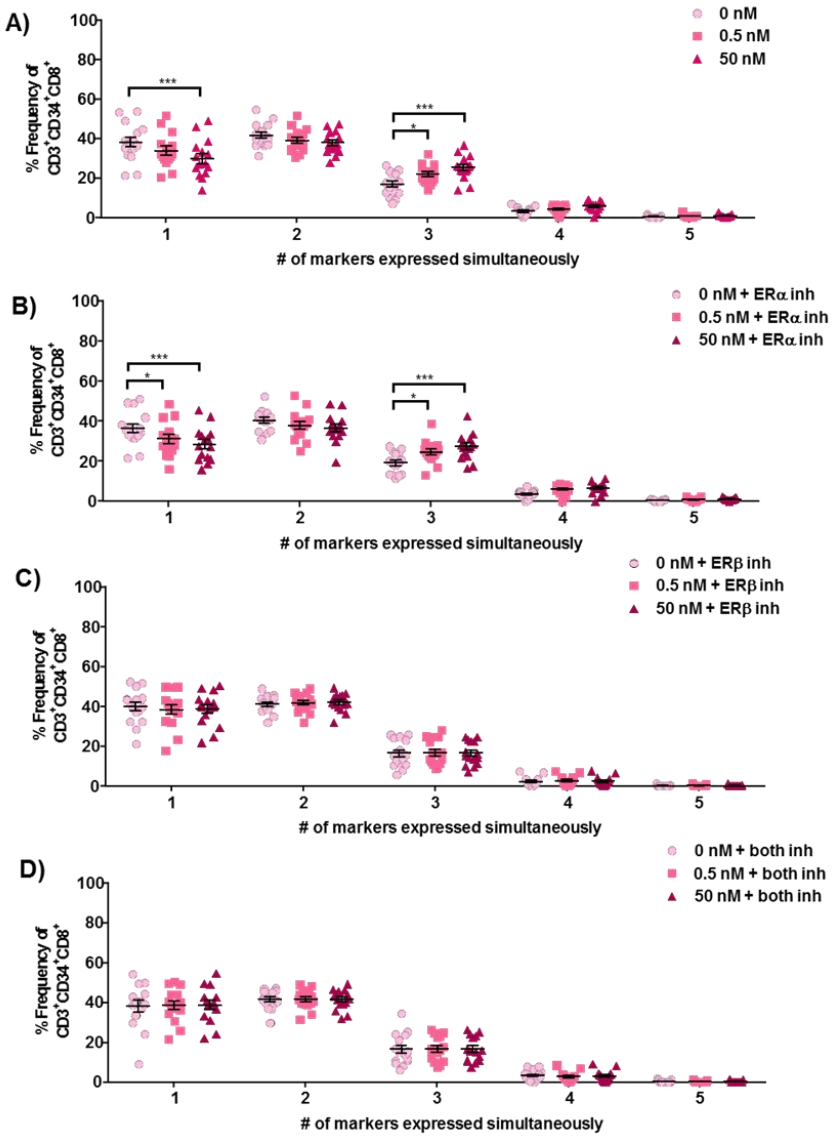


Figure 18. Estrogen Signaling through ERβ Enhances the Percentage of Polyfunctional Female CD8$^+$ Ag-specific T Cells and Decreases the Percentage of Monofunctional T Cells after Ag Stimulation. Female HCV Ag-specific T cells from 15 donors were treated with 0, 0.5, or 50 nM estrogen in combination with an ERα inhibitor, and ERβ inhibitor, or both inhibitors simultaneously for 2 hours. After treatment, Ag-specific T cells were co-cultured for 5 hours with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide in the presence of protein transport inhibitors. Cells were then stained for flow cytometric analysis of CD3, CD34, CD8, and CD4 and functional markers CD107a, IFNγ, TNFα, IL-4, IL-2, IL-17a, and IL-22. Using Boolean gates in FlowJoX, the frequency of CD8$^+$ T cells expressing each combination of these 7 markers was generated and added into 7 functional categories. The frequencies of CD8$^+$ female Ag-specific T cells expressing one through seven markers simultaneously after treatment with **A)** estrogen, **B)** estrogen in combination with an ERα inhibitor, **C)** estrogen in combination with an ERβ inhibitor, **D)** estrogen in combination of both ERα and ERβ inhibitors were plotted. Data for T cells expressing 6 or 7 markers simultaneously is not shown since the percent frequency was very low. Data represents n=15 donors, each donor plotted and SEM represented. Data was analyzed using MANOVA and Tukey's post-hoc. $p< 0.001$=***, $p<0.05$=*.

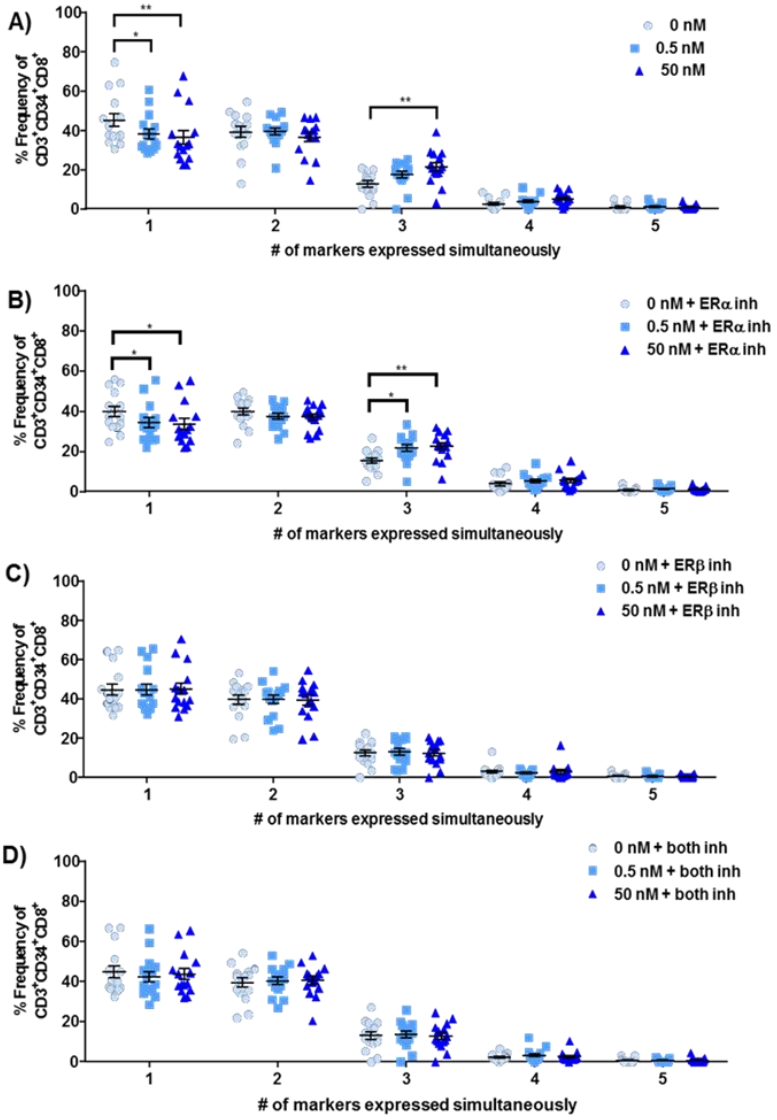
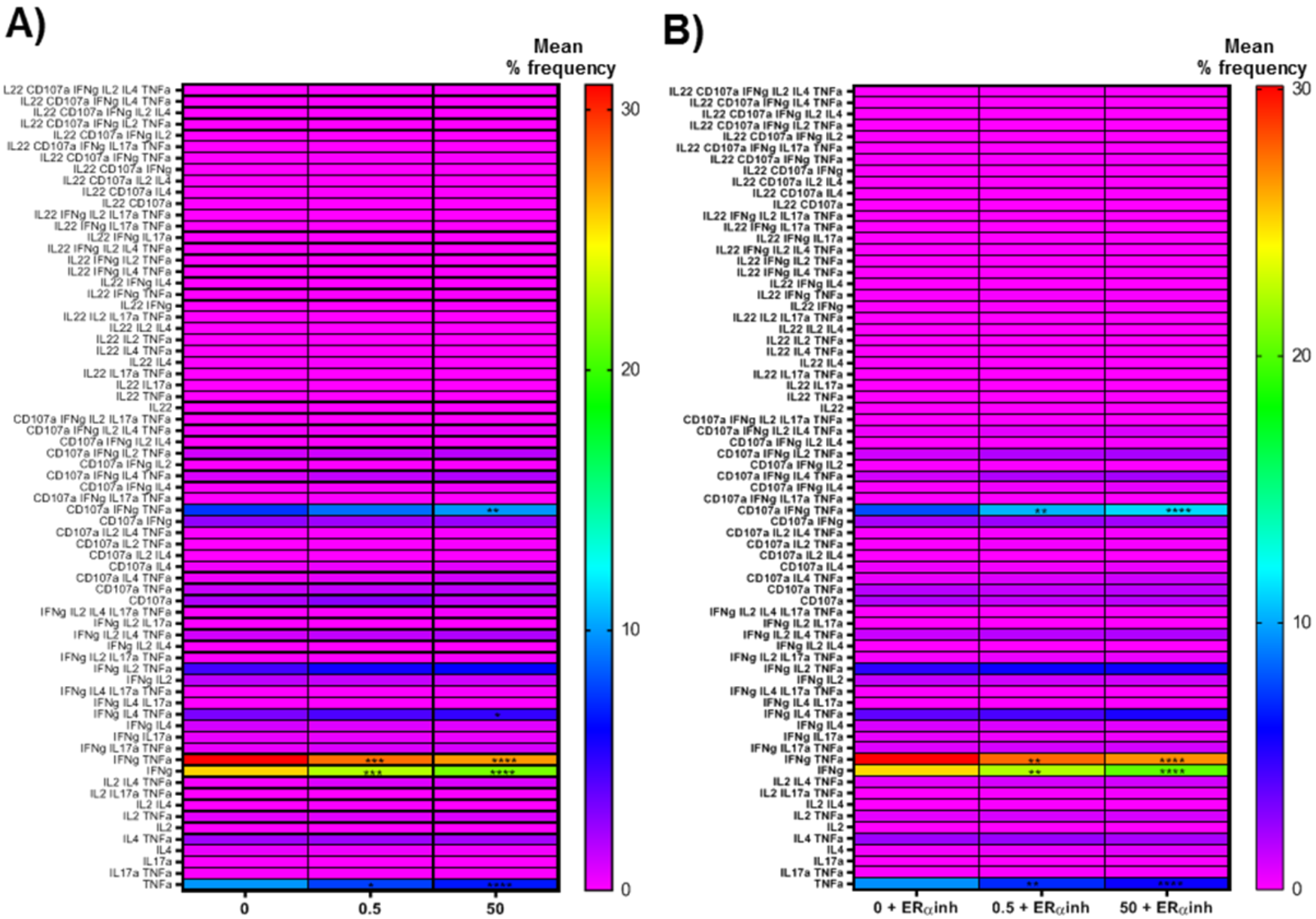


Figure 19. Estrogen Signaling through ERβ Enhances the Percentage of Polyfunctional Male CD8$^+$ Ag-specific T Cells and Decreases the Percentage of Monofunctional T Cells after Ag Stimulation. Male HCV Ag-specific T cells from 15 donors were treated with 0, 0.5, or 50 nM estrogen in combination with an ERα inhibitor, and ERβ inhibitor, or both inhibitors simultaneously for 2 hours. After treatment, Ag-specific T cells were co-cultured for 5 hours with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide in the presence of protein transport inhibitors. Cells were then stained for flow cytometric analysis of CD3, CD34, CD8, and CD4 and functional markers CD107a, IFNγ, TNFα, IL-4, IL-2, IL-17a, and IL-22. Using Boolean gates in FlowJoX, the frequency of CD8$^+$ T cells expressing each combination of these 7 markers was generated and added into 7 functional categories. The frequencies of CD8$^+$ male Ag-specific T cells expressing one through seven markers simultaneously after treatment with **A)** estrogen, **B)** estrogen in combination with an ERα inhibitor, **C)** estrogen in combination with an ERβ inhibitor, **D)** estrogen in combination of both ERα and ERβ inhibitors were plotted. Data for T cells expressing 6 or 7 markers simultaneously is not shown since the percent frequency was very low. Data represents n=15 donors, each donor plotted and SEM indicated. Data was analyzed using MANOVA and Tukey's post-hoc. $p< 0.01$=**, $p<0.05$=*.

The percent frequencies of female and male $CD8^+$ Ag-specific T cells expressing $CD107a^+$ $IFN\gamma^+TNF\alpha^+$ was significantly increased by super-physiological estrogen treatment (Figure 20 & 21 A). The percent frequency of female $CD8^+$ T cells expressing $IFN\gamma^+IL\text{-}4^+TNF\alpha^+$ was also significantly increased by super-physiological estrogen treatment (Figure 20 A). On the other hand, the frequencies of male and female $CD8^+$ T cells expressing $TNF\alpha^+IFN\gamma^+$, $IFN\gamma^+$, and $TNF\alpha^+$ were significantly decreased upon physiological and super-physiological estrogen treatments (Figure 20 & 21 A). The estrogen-induced significant changes on expression of polyfunctional ($CD107a^+IFN\gamma^+TNF\alpha^+$), bifunctional ($TNF\alpha^+IFN\gamma^+$), and monofunctional ($TNF\alpha$ or $IFN\gamma$ alone) phenotypes were maintained when $CD8^+$ Ag-specific T cells were treated with estrogen in combination with an $ER\alpha$ inhibitor (Figure 20 & 21 B). In female $CD8^+$ T cells, blockade of $ER\alpha$ during physiological estrogen treatment significantly enhanced the expression of $CD107a^+IFN\gamma^+TNF\alpha^+$ indicating that $ER\alpha$ signaling could be partially inhibiting the expression of these three markers simultaneously (Figure 20 B). When male and female Ag-specific T cells were treated with estrogen in combination with an $ER\beta$ inhibitor or with $ER\alpha$ and $ER\beta$ inhibitors simultaneously, all significant changes on expression of monofunctional or polyfunctional phenotypes were abrogated (Figure 20 & 21 C, D). Overall, taking these data together with the results obtained in Figures 18 & 19, it can be concluded that estrogen signaling through $ER\beta$ enhances $CD8^+$ Ag-specific T cell polyfunctionality by increasing expression of polyfunctional phenotypes $CD107a^+IFN\gamma^+TNF\alpha^+$ and $IFN\gamma^+IL\text{-}4^+TNF\alpha^+$, and decreasing expression of monofunctional and bifunctional phenotypes including $TNF\alpha^+IFN\gamma^+$, $TNF\alpha^+$, and $IFN\gamma^+$.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50 + ERαinh

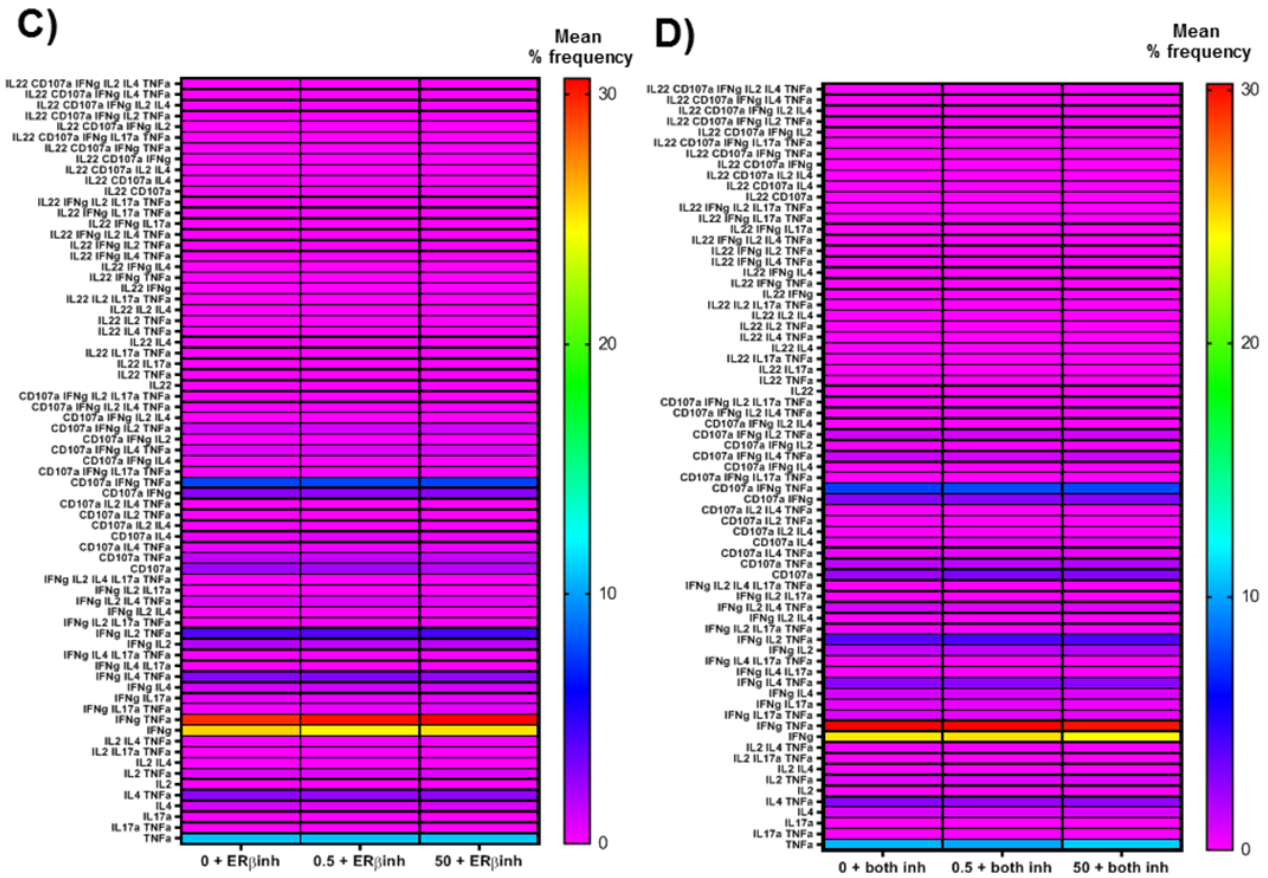
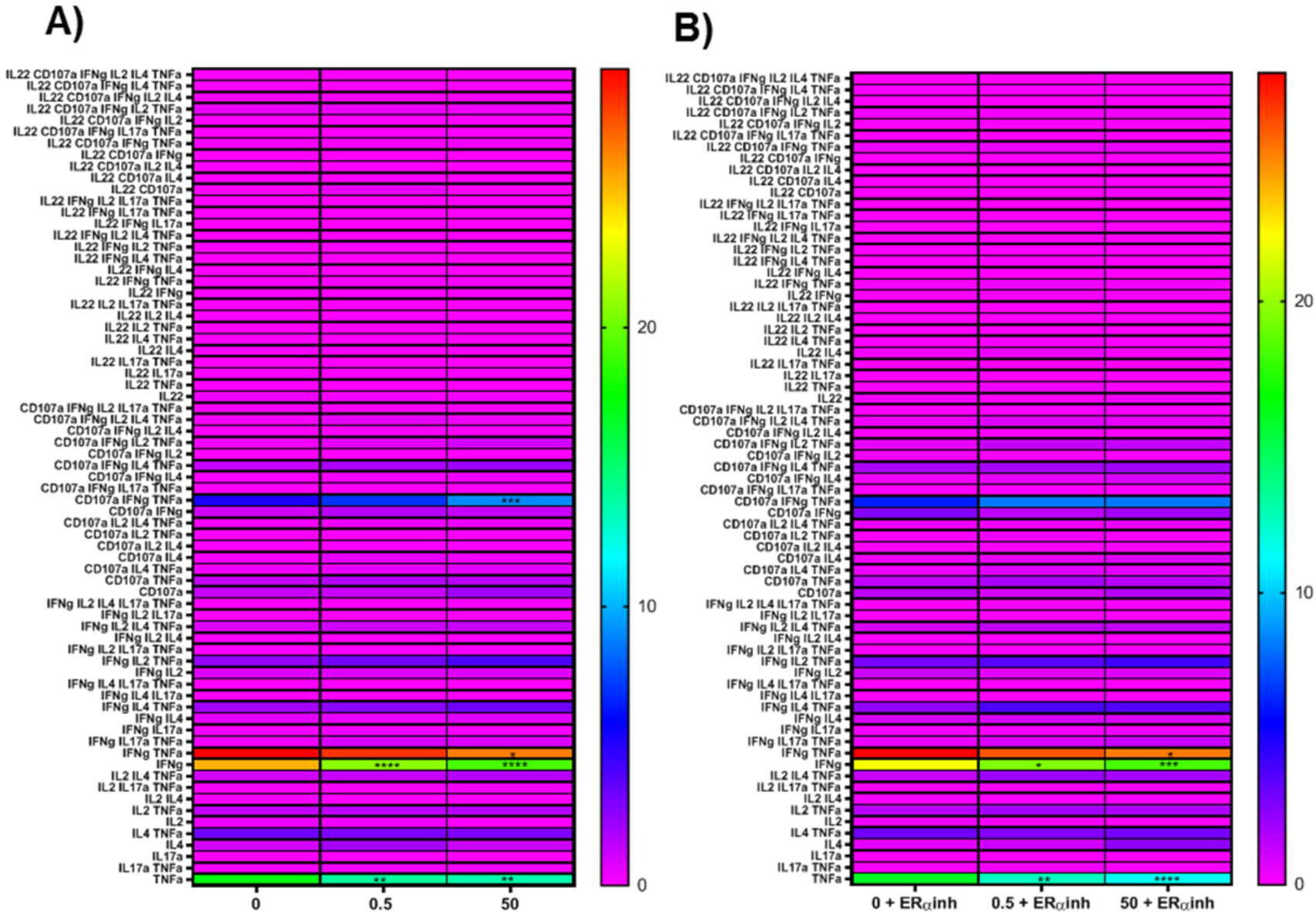


Figure 20. Estrogen Signaling through ERβ Increases the Percent Frequency of $CD8^+$ Female Ag-specific T Cells Expressing $CD107a^+IFN\gamma^+TNF\alpha^+$ and $IFN\gamma^+IL\text{-}4^+TNF\alpha^+$ and Decreases the Percent Frequency of T Cells Expressing $IFN\gamma^+TNF\alpha^+$, $IFN\gamma^+$, and $TNF\alpha^+$. Female HCV Ag-specific T cells from 15 donors were treated with 0, 0.5, or 50 nM estrogen in combination with an ERα inhibitor, and ERβ inhibitor, or both inhibitors simultaneously for 2 hours. After treatment, Ag-specific T cells were co-cultured for 5 hours with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide in the presence of protein transport inhibitors. Cells were then stained for flow cytometric analysis of CD3, CD34, CD8, and CD4 and functional markers CD107a, IFNγ, TNFα, IL-4, IL-2, IL-17a, and IL-22. Using Boolean gates in FlowJoX, and the software SPICE the frequency of $CD8^+$ T cells expressing each combination of these 7 markers was generated. The frequencies of $CD8^+$ female Ag-specific T cells expressing each of these marker combinations after treatment with **A)** estrogen, **B)** estrogen in combination with an ERα inhibitor, **C)** estrogen in combination with an ERβ inhibitor, **D)** estrogen in combination of both ERα and ERβ inhibitors were plotted. Data represents the mean of n=15 donors. Data was analyzed using MANOVA and Tukey's post-hoc. $p<0.0001$=****, $p< 0.01$=**.


A)
B)
IL22 CD107a IFNg IL2 IL4 TNFa
IL22 CD107a IFNg IL4 TNFa
IL22 CD107a IFNg IL2 IL4
IL22 CD107a IFNg IL2 TNFa
IL22 CD107a IFNg IL2
IL22 CD107a IFNg IL17a TNFa
IL22 CD107a IFNg TNFa
IL22 CD107a IFNg
IL22 CD107a IL2 IL4
IL22 CD107a IL4
IL22 CD107a
IL22 IFNg IL2 IL17a TNFa
IL22 IFNg IL17a TNFa
IL22 IFNg IL17a
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CD107a IFNg IL2 IL17a TNFa
CD107a IFNg IL2 IL4 TNFa
CD107a IFNg IL2 IL4
CD107a IFNg IL2 TNFa
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CD107a IFNg TNFa
CD107a IFNg
CD107a IL2 IL4 TNFa
CD107a IL2 TNFa
CD107a IL2 IL4
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CD107a TNFa
CD107a
IFNg IL2 IL4 IL17a TNFa
IFNg IL2 IL17a
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IL2 IL4 TNFa
IL2 IL17a TNFa
IL2 IL4
IL2 TNFa
IL2
IL4 TNFa
IL4
IL17a
IL17a TNFa
TNFa
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*
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*
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**
****
20
10
0
0
0.5
50
0 + ERαinh
0.5 + ERαinh
50 + ERαinh

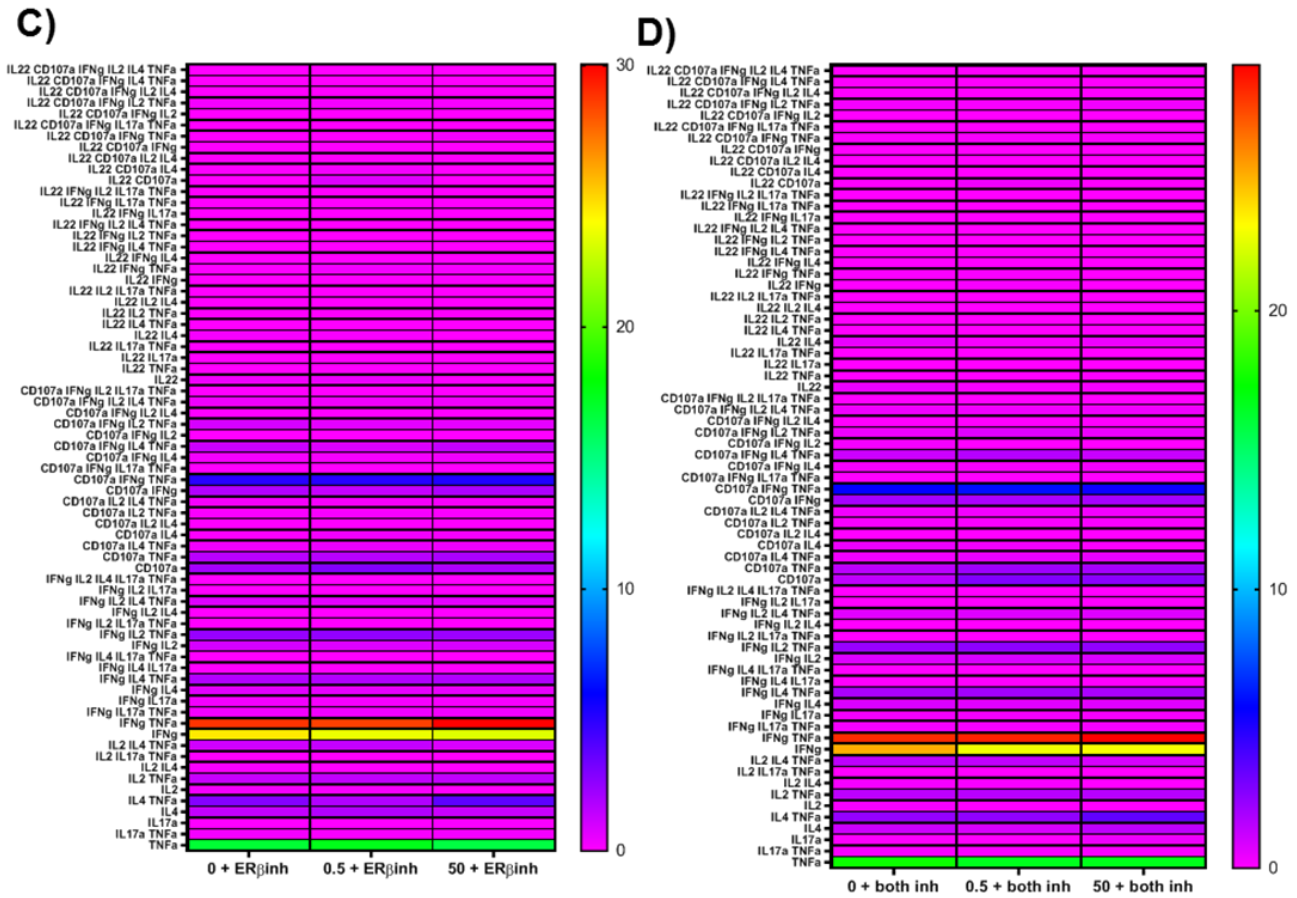


Figure 21. Estrogen Signaling Through ERβ Increases the Percent Frequency of CD8$^+$ Male Ag-Specific T Cells Expressing CD107a$^+$IFNγ$^+$TNFα$^+$ and Decreases the Percent Frequency of T Cells Expressing IFNγ$^+$TNFα$^+$, IFNγ$^+$, and TNFα$^+$. Male HCV Ag-specific T cells from 15 donors were treated with 0, 0.5, or 50 nM estrogen in combination with an ERα inhibitor, and ERβ inhibitor, or both inhibitors simultaneously for 2 hours. After treatment, Ag-specific T cells were co-cultured for 5 hours with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide in the presence of protein transport inhibitors. Cells were then stained for flow cytometric analysis of CD3, CD34, CD8, and CD4 and functional markers CD107a, IFNγ, TNFα, IL-4, IL-2, IL-17a, and IL-22. Using Boolean gates in FlowJoX, and the software SPICE the frequency of CD8$^+$ T cells expressing each combination of these 7 markers was generated. The frequencies of CD8$^+$ male Ag-specific T cells expressing each of these markers combinations after treatment with **A)** estrogen, **B)** estrogen in combination with an ERα inhibitor, **C)** estrogen in combination with an ERβ inhibitor, **D)** estrogen in combination of both ERα and ERβ inhibitors were plotted. Data represents the mean of n=15 donors. Data was analyzed using MANOVA and Tukey's post-hoc. P<0.0001=****, p< 0.001=***, p<0.01=**, p<0.05=*.

Estrogen Signaling through ERβ Enhances Female and Male CD4$^+$ Ag-specific T cell Polyfunctionality

Estrogen signaling had similar effects on CD4$^+$ T cell polyfunctionality to those observed in CD8$^+$ Ag-specific T cells. Estrogen stimulation at super-physiological concentrations significantly decreased the percent frequency of monofunctional CD4$^+$ T cells, and significantly increased the percent frequency of polyfunctional cells expressing three markers simultaneously (Figure 22 & 23 A). Super-physiological estrogen also significantly decreased the percentage of bifunctional female CD4$^+$ T cells (Figure 22 A). ERα signaling blockade had no effect of the estrogen-dependent enhancement of polyfunctionality of male CD4$^+$ T cells, but it increased CD4$^+$ T cell polyfunctionality at physiological estrogen concentration in female CD4$^+$ T cells (Figure 22 & 23 B). Indicating that ERα signaling inhibits female CD4$^+$ T cell polyfunctionality. Blockade of ERβ or ERα and ERβ simultaneously completely abrogated the estrogen-mediated increase in polyfunctionality of CD4$^+$ male and female Ag-specific T cells (Figure 22 & 23 C, D). Overall these data indicate that estrogen signaling through ERβ increases polyfunctionality of male and female CD4$^+$ T cells, and estrogen signaling through ERα inhibits female CD4$^+$ T cell polyfunctionality at physiological estrogen concentration.

The percent frequencies of male and female CD4$^+$ Ag-specific T cells expressing IFNγ$^+$TNFα$^+$, and IFNγ$^+$ or TNFα$^+$ were significantly decreased by physiological and super-physiological estrogen treatment (Figure 24 & 25 A). The percent frequency of male CD4$^+$ T cells expressing IFNγ$^+$IL-4$^+$TNFα$^+$ was also significantly increased by super-physiological estrogen treatment (Figure 25 A). A trending increase on the frequencies of CD4$^+$ T cells

expressing IFNγ$^+$ IL2$^+$IL4$^+$TNFα$^+$, and IFNγ$^+$IL-2$^+$TNFα$^+$ were observed upon estrogen treatment even if significance was not achieved. Female CD4$^+$ Ag-specific T cells also showed a trending increase on expression of IFNγ$^+$IFNγ$^+$TNFα$^+$ upon estrogen treatment (Figure 24 A). Blockade of ERα showed no effect on the estrogen-mediated changes on expression of functional phenotypes indicating that ERα signaling does not affect CD4$^+$ T cell polyfunctionality (Figure 24 & 25 B). On the other hand, ERβ blockade or ERα and ERβ simultaneous blockade during estrogen treatment abrogated the estrogen-mediated decrease of monofunctional (IFNγ or TNFα alone) and bifunctional (IFNγ$^+$TNFα$^+$) T cell phenotypes, and increase in polyfunctional phenotypes such as IFNγ$^+$IL-4$^+$TNFα$^+$ (Figure 24 & 25 C, D). Overall, taking these data together with the results obtained in Figures 22 & 23, it can be concluded that estrogen signaling through ERβ enhances CD4$^+$ Ag-specific T cell polyfunctionality by increasing expression of polyfunctional phenotypes such as IFNγ$^+$IL-4$^+$TNFα$^+$, and decreasing expression of monofunctional and bifunctional phenotypes including TNFα$^+$IFNγ$^+$, TNFα$^+$, and IFNγ$^+$.

Ideally, in order to conclude that the effects of estrogen signaling on polyfunctionality are fully mediated by ERβ signaling another set of experiments need to be performed using ERβ agonists to stimulate human Ag-specific T cells instead of estrogen. The ERβ-specific agonist diarylpropionitrile (DPN) which has a 70-fold selectivity over ERα (EC_{50} values are 0.85 nM and 66 nM for ERβ and ERα respectively) [150] can be used to determine if estrogen signaling through ERβ is directly upregulating T cell polyfunctionality upon Ag stimulation. In order to test this, male and female Ag-specific T cells need to be treated with DPN alone, DPN in

combination with PHTPP (ERβ antagonist), and DPN in combination with PHTPP and estrogen. If after DPN treatment alone the polyfunctionality of T cells is enhanced to levels similar to those observed in estrogen treated T cells, then estrogen signaling through ERβ alone enhances T cell polyfunctionality. These data can be confirmed if T cells treated with DNP and PHTPP simultaneously show no increase in polyfunctionality. If treating T cells with DPN shows no increase on T cell polyfunctionality but treating T cells with estrogen, DNP and PHTPP do show an increase, then estrogen signaling through other receptor is causing the estrogen-mediated enhancement of polyfunctionality. Inhibition of ERα during estrogen stimulation was shown to enhance polyfunctionality, even to higher extent than estrogen alone in some cases, indicating that ERα has an inhibiting effect. Estrogen signaling through its other receptor, GPER, may be enhancing T cell polyfunctionality. In order to rule out that polyfunctionality is enhanced through GPER signaling, male and female Ag-specific T cells need to be treated with estrogen in combination of a GPER antagonist. If T cell polyfunctionality is decreased in T cells treated with estrogen in combination with a GPER antagonist compared to estrogen treated T cells, then estrogen signaling through GPER enhances T cell polyfunctionality. GPER signaling was shown to rapidly activate the PI3K and the MAPK pathways which are known to contribute to T cell cytokine secretion downstream from the TCR [177] indicating that GPER signaling could have an enhancing effect on TCR signaling leading to polyfunctionality. Unfortunately, these experiments could not be performed due to lack of time and support but these results would indicate that estrogen signaling through ERβ and GPER enhance T cell polyfunctionality downstream from the TCR.

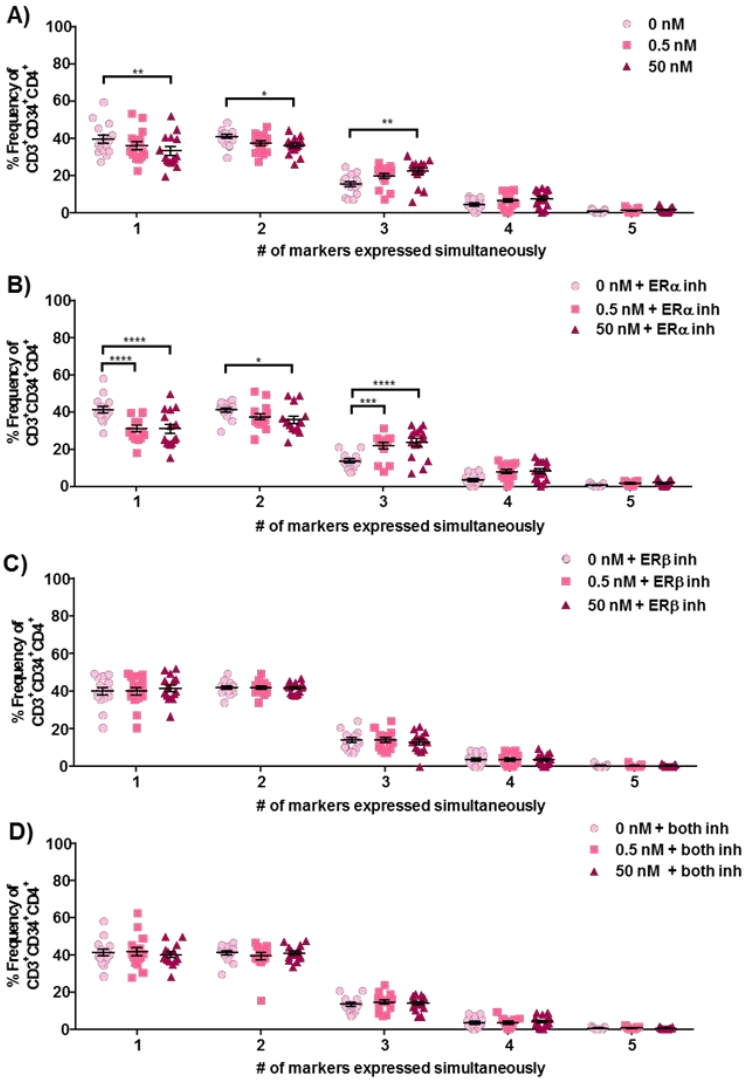


Figure 22. Estrogen Signaling Through ERβ Enhances the Percentage of Polyfunctional Female CD4$^+$ Ag-specific T Cells and Decreases the Percentage of Monofunctional and Bifunctional T Cells after Ag Stimulation. Female HCV Ag-specific T cells from 15 donors were treated with 0, 0.5, or 50 nM estrogen in combination with an ERα inhibitor, and ERβ inhibitor, or both inhibitors simultaneously for 2 hours. After treatment, Ag-specific T cells were co-cultured for 5 hours with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide in the presence of protein transport inhibitors. Cells were then stained for flow cytometric analysis of CD3, CD34, CD8, and CD4 and functional markers CD107a, IFNγ, TNFα, IL-4, IL-2, IL-17a, and IL-22. Using Boolean gates in FlowJoX, the frequency of CD4$^+$ T cells expressing each combination of these 7 markers was generated and added into 7 functional categories. The frequencies of CD4$^+$ female Ag-specific T cells expressing one through seven markers simultaneously after treatment with **A)** estrogen, **B)** estrogen in combination with an ERα inhibitor, **C)** estrogen in combination with an ERβ inhibitor, **D)** estrogen in combination of both ERα and ERβ inhibitors were plotted. Data for T cells expressing 6 or 7 markers simultaneously is not shown since the percent frequency was very low. Data represents n=15 donors, each donor plotted and SEM represented. Data was analyzed using MANOVA and Tukey's post-hoc. p<0.0001=****, p< 0.001=***, p<0.01=**, p<0.05=*.

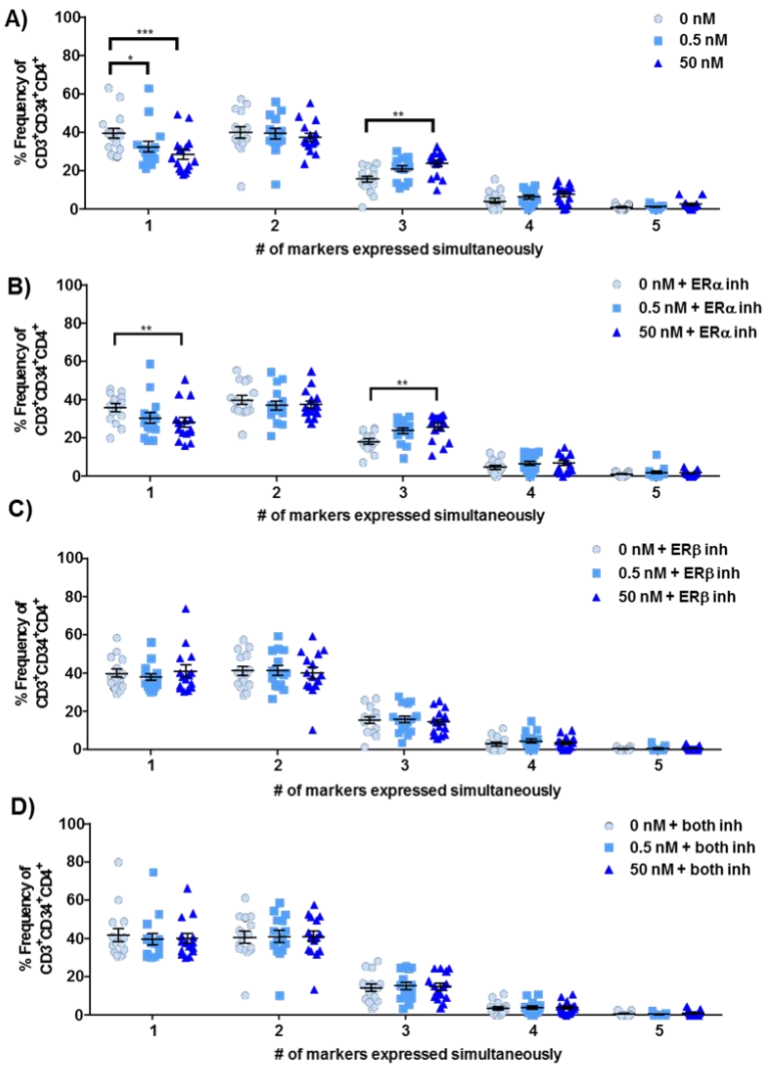
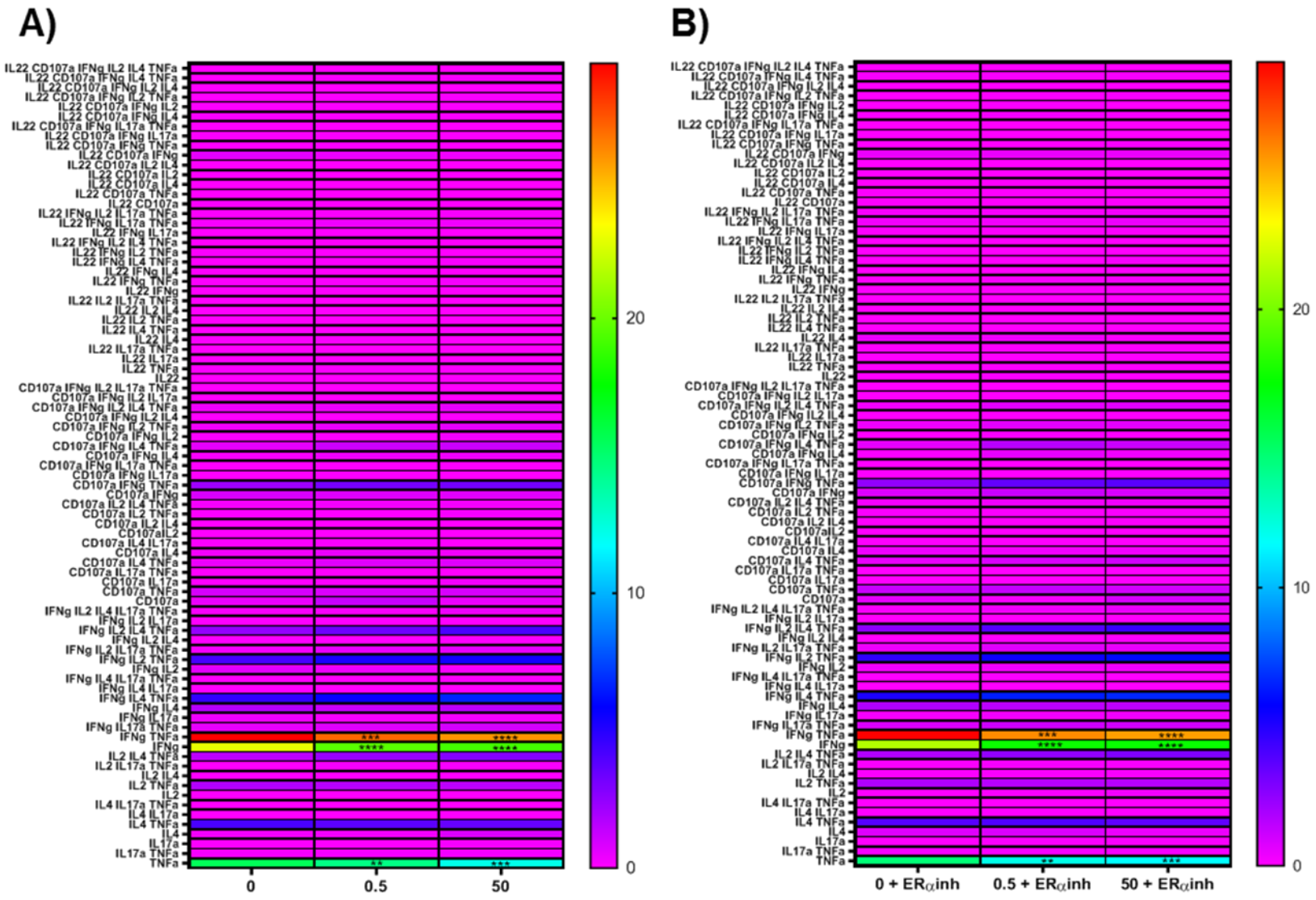


Figure 23. Estrogen Signaling Through ERβ Enhances the Percentage of Polyfunctional Male CD4$^+$ Ag-specific T Cells and Decreases the Percentage of Monofunctional T Cells after Ag Stimulation. Male HCV Ag-specific T cells from 15 donors were treated with 0, 0.5, or 50 nM estrogen in combination with an ERα inhibitor, and ERβ inhibitor, or both inhibitors simultaneously for 2 hours. After treatment, Ag-specific T cells were co-cultured for 5 hours with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide in the presence of protein transport inhibitors. Cells were then stained for flow cytometric analysis of CD3, CD34, CD8, and CD4 and functional markers CD107a, IFNγ, TNFα, IL-4, IL-2, IL-17a, and IL-22. Using Boolean gates in FlowJoX, the frequency of CD4$^+$ T cells expressing each combination of these 7 markers was generated and added into 7 functional categories. The frequencies of CD4$^+$ male Ag-specific T cells expressing one through seven markers simultaneously after treatment with **A)** estrogen, **B)** estrogen in combination with an ERα inhibitor, **C)** estrogen in combination with an ERβ inhibitor, **D)** estrogen in combination of both ERα and ERβ inhibitors were plotted. Data for T cells expressing 6 or 7 markers simultaneously is not shown since the percent frequency was very low. Data represents n=15 donors, each donor plotted and SEM represented. Data was analyzed using MANOVA and Tukey's post-hoc. $p< 0.001$=***, $p<0.01$=**, $p<0.05$=*.


A)
B)
0
0.5
50
0 + ERαinh
0.5 + ERαinh
50 + ERαinh
20
10
0

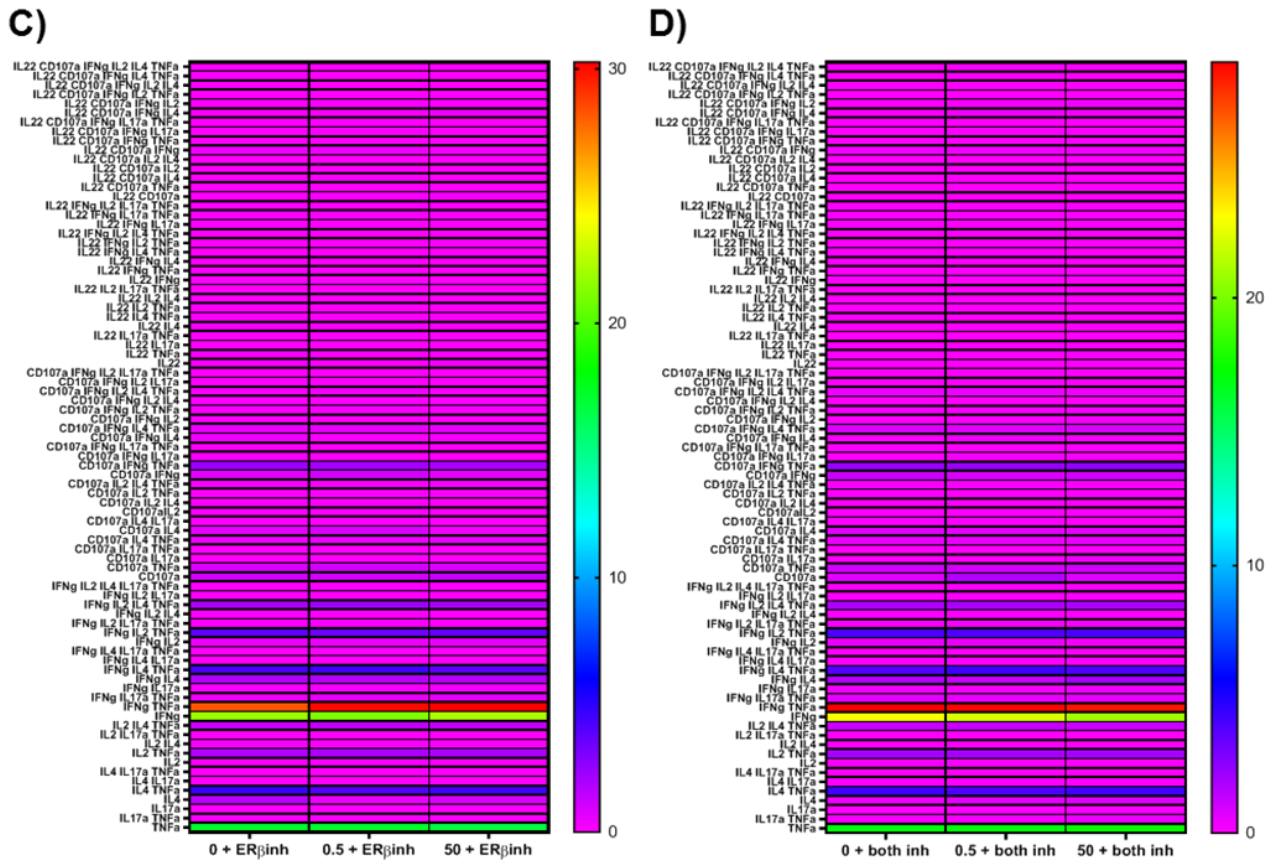
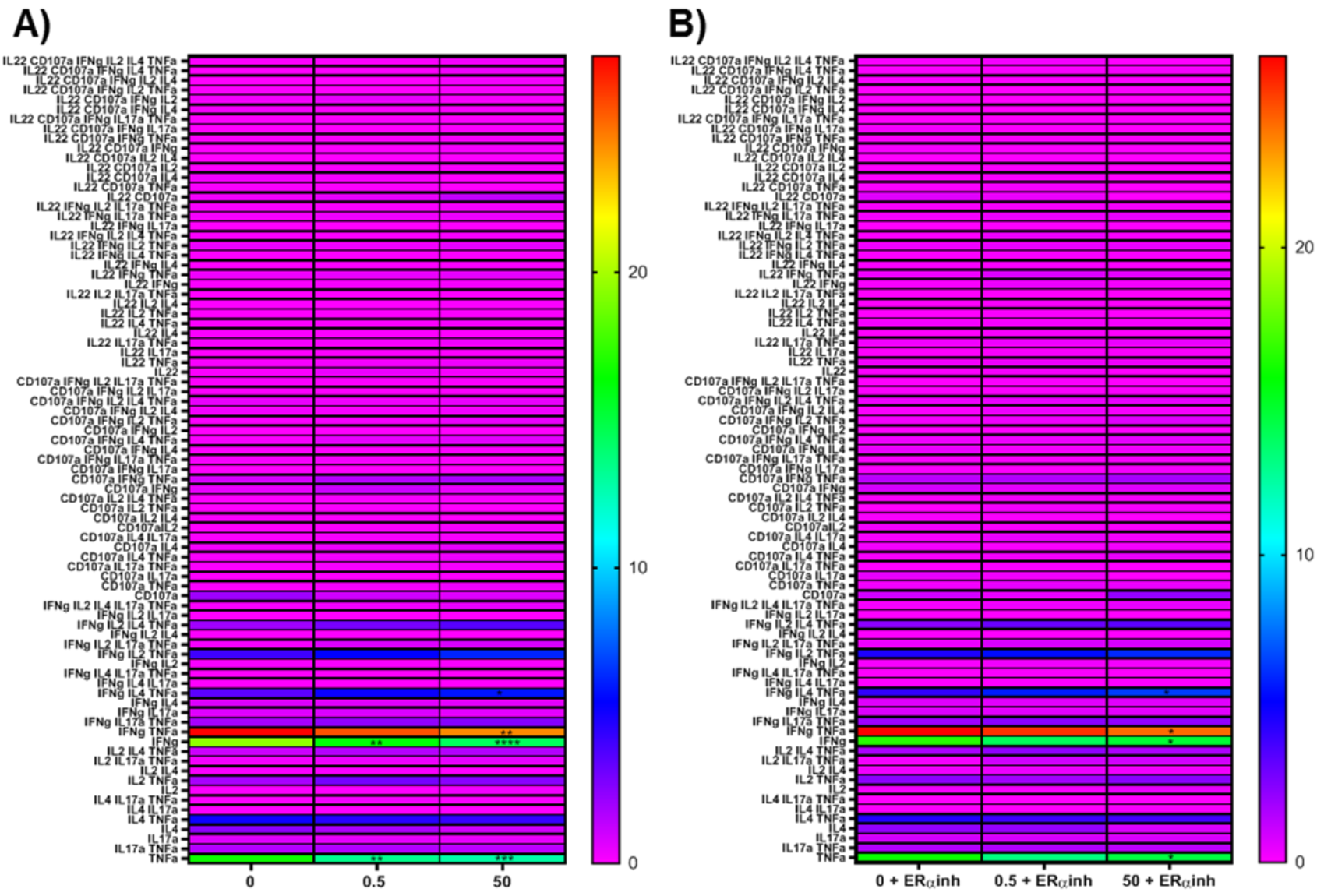


Figure 24. Estrogen Signaling Through ERβ Decreases the Percent Frequency of Female CD4$^+$ Ag-specific T Cells Expressing IFNγ$^+$TNFα$^+$, IFNγ$^+$, and TNFα$^+$. Female HCV Ag-specific T cells from 15 donors were treated with 0, 0.5, or 50 nM estrogen in combination with an ERα inhibitor, and ERβ inhibitor, or both inhibitors simultaneously for 2 hours. After treatment, Ag-specific T cells were co-cultured for 5 hours with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide in the presence of protein transport inhibitors. Cells were then stained for flow cytometric analysis of CD3, CD34, CD8, and CD4 and functional markers CD107a, IFNγ, TNFα, IL-4, IL-2, IL-17a, and IL-22. Using Boolean gates in FlowJoX and the software SPICE, the frequency of CD4$^+$ T cells expressing each combination of these 7 markers was generated. The frequencies of CD4$^+$ female Ag-specific T cells expressing one through seven markers simultaneously after treatment with **A)** estrogen, **B)** estrogen in combination with an ERα inhibitor, **C)** estrogen in combination with an ERβ inhibitor, **D)** estrogen in combination of both ERα and ERβ inhibitors were plotted. Data for T cells expressing 6 or 7 markers simultaneously is not shown since the percent frequency was very low. Data represents the mean of n=15 donors. Data was analyzed using MANOVA and Tukey's post-hoc. p<0.0001=****, p< 0.001=***, p<0.01=**, p<0.05=*.



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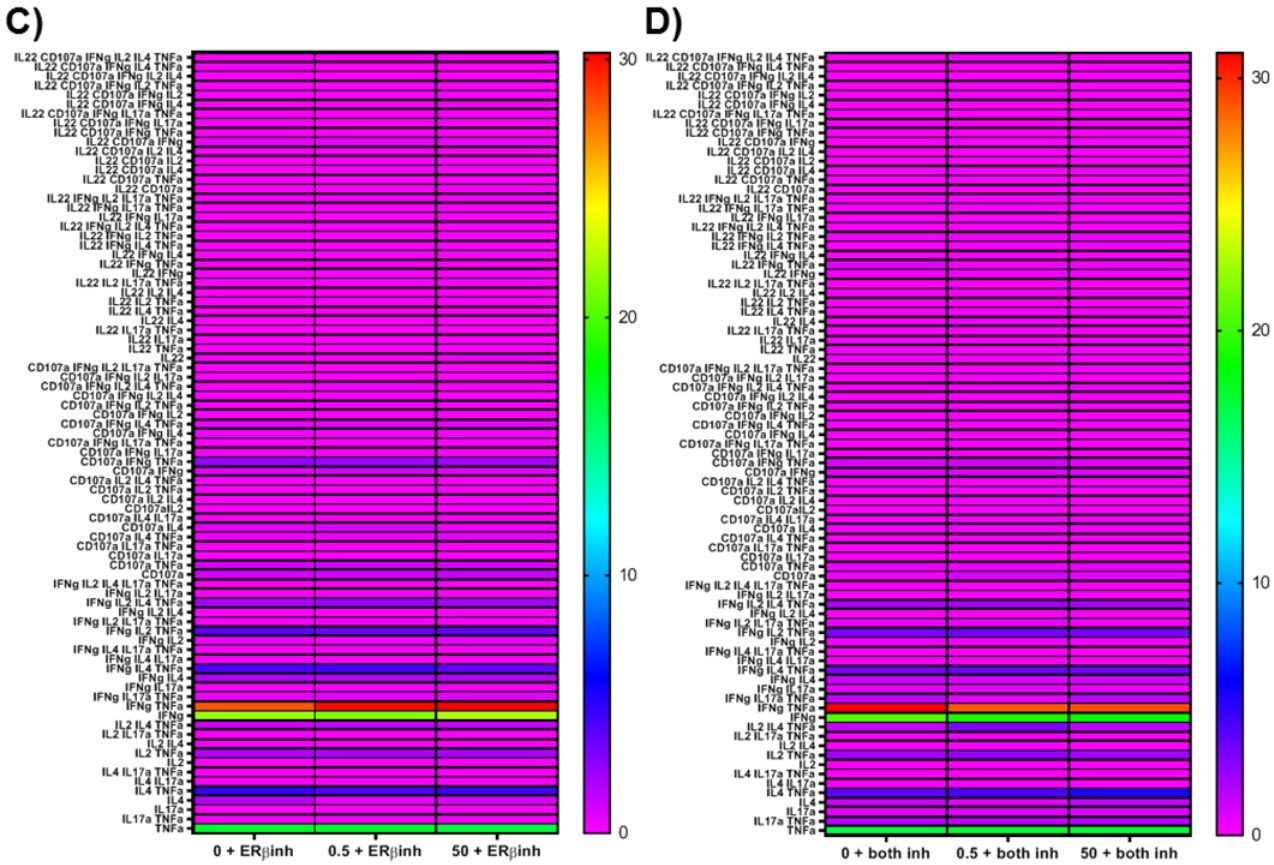

Figure 25. Estrogen Signaling Through ERβ Increases the Percent Frequency of $CD4^+$ Male Ag-specific T Cells Expressing $IFN\gamma^+IL\text{-}4^+TNF\alpha^+$ and Decreases the Percent Frequency of T Cells Expressing $IFN\gamma^+TNF\alpha^+$, $IFN\gamma^+$, and $TNF\alpha^+$. Male HCV Ag-specific T cells from 15 donors were treated with 0, 0.5, or 50 nM estrogen in combination with an ERα inhibitor, and ERβ inhibitor, or both inhibitors simultaneously for 2 hours. After treatment, Ag-specific T cells were co-cultured for 5 hours with T2 cells pulsed with HCV cognate antigen or a tyrosinase irrelevant peptide in the presence of protein transport inhibitors. Cells were then stained for flow cytometric analysis of CD3, CD34, CD8, and CD4 and functional markers CD107a, IFNγ, TNFα, IL-4, IL-2, IL-17a, and IL-22. Using Boolean gates in FlowJoX and the software SPICE, the frequency of $CD4^+$ T cells expressing each combination of these 7 markers was generated. The frequencies of $CD4^+$ male Ag-specific T cells expressing each of the markers combinations after treatment with **A)** estrogen, **B)** estrogen in combination with an ERα inhibitor, **C)** estrogen in combination with an ERβ inhibitor, **D)** estrogen in combination of both ERα and ERβ inhibitors were plotted. Data represents the mean of n=15 donors. Data was analyzed using MANOVA and Tukey's post-hoc. $p< 0.001$=***, $p<0.01$=**, $p<0.05$=*.

Estrogen Signaling through ERβ Enhances the Percent Frequency of Ag-specific T cells Expressing Polyfunctional Marker Combinations

Up to this section of the results, polyfunctionality data were statistically analyzed for $CD4^+$ and $CD8^+$ T cells within each sex separately for each of the estrogen or estrogen + inhibitor treatments individually. This indicates that statistical significance was reported by comparing the percent frequency of T cells expressing each marker combination when treated with estrogen alone (0.5 nM or 50 nM) or in combination with each ER inhibitor or both simultaneously to the percent frequency of T cells expressing each marker combination when treated with 0 nM estrogen in combination with each inhibitor or both separately. In order to statistically analyze the interaction of estrogen and estrogen receptor inhibitor treatment on the expression of each of the combination of cytokines, a mixed-effect linear regression model was used. The estrogen main effect, estrogen inhibitor main effect, and estrogen x estrogen inhibitor interaction were run for every unique marker combination. An n=10 is needed for statistically analyzing each main effect/interaction so an n=30 was achieved by analyzing Ag-specific T cells from 15 female and 15 male donors together. Analyzing the data from male and female Ag-specific T cells together has its limitations since the effect of sex in the expression of each marker combination is not taken into account but, based on previous data, it was demonstrated that estrogen signaling affects the polyfunctionality of male and female T cells similarly. Also, the bulk Ag-specific T cell population was analyzed instead of analyzing $CD8^+$ and $CD4^+$ T cell subset separately since during HCC immunotherapy T cells are infused into patients as a mixed population and superior anti-tumor immune responses are achieved when both functional subsets are found infiltrating the tumor, indicating a combined $CD8^+$ and $CD4^+$ T cell anti-tumor function [352].

$IFN\gamma^{+}IL\text{-}4^{+}TNF\alpha^{+}$ was one of the cytokine combination that demonstrated a significant interaction effect between estrogen and estrogen inhibitor treatments ($p = 0.0002$). This indicates that the effect of estrogen treatment on $IFN\gamma^{+}IL\text{-}4^{+}TNF\alpha^{+}$ expression varies significantly based on each estrogen inhibitor treatment, and vice versa. Physiological and super-physiological estrogen treatment significantly enhanced the percent frequency of cells expressing $IFN\gamma^{+}IL\text{-}4^{+}TNF\alpha^{+}$ (4.4731% and 5.1938% compared to 3.3751% in 0 nM treated T cells) (Table 3). The percent frequency of T cells expressing $IFN\gamma^{+}IL\text{-}4^{+}TNF\alpha^{+}$ was significantly decreased when T cells were treated with 0.5 or 50 nM estrogen in combination of an ERβ inhibitor was added (3.2479% and 2.9269%) or both ERα and ERβ inhibitors are added (3.182% and 2.9945%) (Table 3). On the other hand, the percent frequency of T cells expressing $IFN\gamma^{+}IL\text{-}4^{+}TNF\alpha^{+}$ significantly increased when T cells were treated with 0.5 nM or 50 nM estrogen in combination with an ERα inhibitor (5.0915% and 5.5498%) (Table 3). This indicates that physiological and super-physiological estrogen signaling through ERβ enhances the expression of $IFN\gamma^{+}IL\text{-}4^{+}TNF\alpha^{+}$, while signaling through ERα significantly inhibits expression of $IFN\gamma^{+}IL\text{-}4^{+}TNF\alpha^{+}$.

Other cytokine combinations that demonstrated a significant interaction effect between super-physiological estrogen and estrogen inhibitor treatments ($p<0.000618$). This indicates that the effect of super-physiological estrogen concentration on the expression of $IFN\gamma^{+}IL\text{-}2^{+}IL\text{-}4^{+}IL\text{-}17a^{+}TNF\alpha^{+}$, and $IL\text{-}2^{+}IL\text{-}4^{+}TNF\alpha^{+}$varies significantly based on each estrogen inhibitor treatment, and vice versa. The percent frequency of T cells expressing these marker combinations were significantly increased when treated with physiological (0.5 nM) or super-physiological (50 nM) estrogen concentrations (Table 4). A significant decrease on the frequency of cells expressing $IFN\gamma^{+}IL\text{-}2^{+}IL\text{-}4^{+}IL\text{-}17a^{+}TNF\alpha^{+}$, and $IL\text{-}2^{+}IL\text{-}4^{+}TNF\alpha^{+}$ was observed when T

cells were treated with super-physiological estrogen in combination with an ERβ inhibitor, or ERα and ERβ inhibitors simultaneously when compared to those cells treated with estrogen only (Table 4). A significant increase on the frequency of cells expressing $IFN\gamma^{+}IL\text{-}2^{+}IL\text{-}4^{+}IL\text{-}17a^{+}TNF\alpha^{+}$, and $IL\text{-}2^{+}IL\text{-}4^{+}TNF\alpha^{+}$ was observed when T cells were treated with super-physiological estrogen in combination with an ERα inhibitor (Table 4). Overall these result indicate that estrogen signaling through ERβ enhances the expression of polyfunctional combinations $IFN\gamma^{+}IL\text{-}2^{+}IL\text{-}4^{+}IL\text{-}17a^{+}TNF\alpha^{+}$, and $IL\text{-}2^{+}IL\text{-}4^{+}TNF\alpha^{+}$ in Ag-specific T cells while estrogen signaling through ERα inhibits the expression of these polyfunctional combinations.

Significant interaction effect between super-physiological estrogen and estrogen inhibitor treatments ($p<0.000618$) were also found for Ag-specific T cells expressing only $IFN\gamma^{+}$ or $TNF\alpha^{+}$ in combination with no other marker. Percent frequency of T cells expressing only $IFN\gamma^{+}$ or $TNF\alpha^{+}$ were significantly decreased when treated with physiological (0.5 nM) or super-physiological (50 nM) estrogen concentrations (Table 4). A significant increase on the frequency of cells expressing only $IFN\gamma^{+}$ or $TNF\alpha^{+}$ was observed when T cells were treated with super-physiological estrogen in combination with an ERβ inhibitor, or ERα and ERβ inhibitors simultaneously when compared to those cells treated with estrogen only. A significant decrease on the frequency of cells expressing only $IFN\gamma^{+}$ or $TNF\alpha^{+}$ was observed when T cells were treated with super-physiological estrogen in combination with an ERα inhibitor (Table 4).

Variable	Level	Mean Estimate (95% CI)	p value
Estrogen (nM)	50	-	< 0.0001*
	0.5	-	
	0 (REF)	-	
Estrogen Inhibitor	Both inh	-	< 0.0001*
	ERβ inh	-	
	ERα inh	-	
	None (REF)	-	
Estrogen x Estrogen Inhibitor Interaction	50 + Both inh	2.9945 (2.0584, 3.9306)	0.0002*
	50 + ERβ inh	2.9269 (1.9907, 3.8630)	
	50 + ERα inh	5.5498 (4.6136, 6.4859)	
	50 None	5.1938 (4.2577, 6.1300)	
	0.5 + Both inh	3.182 (2.2459, 4.1181)	
	0.5 + ERβ inh	3.2479 (2.3118, 4.1840)	
	0.5 + ERα inh	5.0915 (4.1554, 6.0276)	
	0.5 None	4.4731 (3.5370, 5.4092)	
	0 + Both inh	2.9184 (1.9823, 3.8546)	
	0 + ERβ inh	3.0636 (2.1275, 3.9997)	
	0 + ERα inh	3.862 (2.9259, 4.7981)	
	0 None	3.3751 (2.4390, 4.3112)	

Table 3. Estimated Effects of Estrogen and Estrogen Inhibitor Treatment on the Percent Frequency of Ag-specific T Cells Expressing IFNγ$^+$IL-4$^+$TNFα$^+$. The 95% confidence interval mean estimates for the percent frequency of Ag-specific T cells expressing IFNγ$^+$IL-4$^+$TNFα$^+$ is represented for each estrogen treatment (0.5, 50 nM) compared to 0 nM treatment, and for each estrogen treatment (0.5 or 50 nM) in combination with either ERα or ERβ inhibitors or both simultaneously, compared to estrogen treatment in combination with no inhibitor (none). Significant at p=0.000618.

Cytokine Combination	Variable	Level	Mean Estimate (95% CI)	p value
$INF\gamma^+IL\text{-}2^+IL\text{-}4^+$ $IL\text{-}17a^+TNF\alpha^+$	Estrogen (nM)	50	0.1721 (0.1155, 0.2287)	0.0005*
		0.5	0.0992 (0.0426, 0.1559)	
		0 (REF)	0.0521 (-0.0046, 0.1087)	
	Estrogen (50 nM) + ER Inhibitor	Both inh	0.0273 (-0.0345, 0.0891)	< 0.0001*
		ERβ inh	0.0171 (-0.0447, 0.0789)	
		ERα inh	0.2186 (0.1568, 0.2804)	
		None (REF)	0.1721 (0.1155, 0.2287)	
$INF\gamma^+$	Estrogen (nM)	50	20.0235 (17.3414, 22.7056)	0.0005*
		0.5	20.6113 (17.9292, 23.2934)	
		0 (REF)	22.7240 (20.0419, 25.4060)	
	Estrogen (50 nM) + ER Inhibitor	Both inh	22.3911 (19.6668, 25.1155)	< 0.0001*
		ERβ inh	22.4518 (19.7274, 25.1761)	
		ERα inh	19.0598 (16.3354, 21.7841)	
		None (REF)	20.0235 (17.3414, 22.7056)	
$IL\text{-}2^+IL\text{-}4^+TNF\alpha^+$	Estrogen (nM)	50	1.4389 (0.8793, 1.9985)	0.0004*
		0.5	1.5916 (1.032, 2.1513)	
		0 (REF)	1.0911 (0.5314, 1.6507)	
	Estrogen (50 nM) + ER Inhibitor	Both inh	1.2209 (0.6518, 1.7900)	< 0.0001*
		ERβ inh	1.0978 (0.5287, 1.6669)	
		ERα inh	1.7424 (1.1733, 2.3116)	
		None (REF)	1.4389 (0.8793, 1.9985)	
$TNF\alpha$	Estrogen (nM)	50	13.3153 (10.2340, 16.3965)	0.00021 *
		0.5	13.5958 (10.5145, 16.6770)	
		0 (REF)	14.9031 (11.8219, 17.9844)	
	Estrogen (50 nM) + ER Inhibitor	Both inh	15.4256 (12.3204, 18.5308)	<0.0001*
		ERβ inh	15.4904 (12.3852, 18.5956)	
		ERα inh	11.9707 (8.8655, 15.0759)	
		None (REF)	13.3153 (10.2340, 16.3965)	

Table 4. Adjusted Effects of Estrogen and Estrogen Inhibitor Treatment on Unique Cytokine Combination Expression. The 95% confidence interval mean estimates for the percent frequency of Ag-specific T cells expressing $IFN\gamma^+$ $IL\text{-}2^+IL\text{-}4^+IL\text{-}17a^+TNF\alpha^+$, $IFN\gamma^+$, $IL\text{-}2^+IL\text{-}4^+TNF\alpha^+$, and $TNF\alpha^+$ is represented for each estrogen treatment (0.5, 50 nM) compared to 0 nM treatment, and for each estrogen treatment (0.5 or 50 nM) in combination with either ERα or ERβ inhibitors or both simultaneously, compared to estrogen treatment in combination with no inhibitor (none). Significant at p=0.000618.

Overall these result indicate that estrogen signaling through ERα enhances the expression of monofunctional combinations IFNγ$^+$ or TNFα$^+$ in Ag-specific T cells while estrogen signaling through ERβ inhibits the expression of these monofunctional combinations.

Overall these statistical analysis indicates than in a human HCV Ag-specific population comprised of a mixture of CD4$^+$ and CD8$^+$ T cells that are activated with the HCV cognate antigen, estrogen signaling through ERβ significantly enhances the percentage of T cells expressing polyfunctional phenotypes such as IFNγ$^+$IL-4$^+$TNFα$^+$, IFNγ$^+$IL-2$^+$IL-4$^+$IL-17a$^+$TNFα$^+$, and IL-2$^+$IL-4$^+$TNFα$^+$ and inhibits the expression of mono-functional phenotypes such as IFNγ$^+$ or TNFα$^+$. On the other hand, estrogen signaling through ERα significantly inhibits the percentage of cells expressing polyfunctional phenotypes while enhancing the percentage of cells expressing mono-functional phenotypes. This results demonstrate that estrogen signaling through ERβ enhances the polyfunctionality of Ag-specific T cells for immunotherapy.

Chapter Discussion

This chapter of the dissertation was aimed to determine the impact of sex and estrogen signaling in regulating Ag-specific T cell polyfunctionality. Here it was found that female CD8$^+$ T cells are inherently more polyfunctional than male CD8$^+$ T cells indicating that sex intrinsically impacts CD8$^+$ T cell function upon antigen stimulation. In addition, it was shown that estrogen signaling through ERβ and not ERα enhances male and female CD8$^+$ and CD4$^+$ Ag-specific T cell polyfunctionality. Overall these results report, for the first time, the impact of

estrogen stimulation on T cell polyfunctionality, and recognize specific functional phenotypes that are directly affected by estrogen receptor signaling in Ag-specific T cells.

T cell polyfunctionality was evaluated by measuring the expression of cytokines IFNγ, TNFα, IL-2, IL-4, IL-17a, and IL-22 and the lytic marker CD107a. Analysis of these factors yielded highly complex multi-dimensional data sets that were analyzed using software packages FlowJo, Pestle, and SPICE which allowed for graphical representation of the data and allowed to make comparison between sexes and estrogen receptor inhibitor treatment groups. It was found that male and female $CD8^+$ and $CD4^+$ Ag-specific T cells are highly diverse on their phenotypic and functional complexity. Female $CD8^+$ Ag-specific T cells were more polyfunctional than male $CD8^+$ T cells upon antigen stimulation expressing higher degrees of functional phenotypes such as $CD107a^+IFN\gamma^+TNF\alpha^+$, $IFN\gamma^+IL\text{-}2^+TNF\alpha^+$, and $IFN\gamma^+TNF\alpha^+$ indicating strong type I T cell responses. On the other hand, significantly higher percentages of male $CD8^+$ Ag-specific T cells expressed $TNF\alpha^+$ compared to female T cells. This indicates that male $CD8^+$ Ag-specific T cells also produce strong type I effector responses but T cells are less likely to express several markers simultaneously.

Despite the sex differences on $CD8^+$ T cell polyfunctionality, estrogen signaling showed equal effects modulating the polyfunctionality of male and female $CD8^+$ and $CD4^+$ Ag-specific T cells. Estrogen signaling through ERβ significantly increased the frequency of T cells expressing trifunctional phenotypes like $CD107a^+IFN\gamma^+TNF\alpha^+$, and $IFN\gamma^+IL\text{-}4^+TNF\alpha^+$ and significantly decreased the frequency of T cells expressing bifunctional or monofunctional phenotypes including $IFN\gamma^+TNF\alpha^+$, $IFN\gamma^+$, and $TNF\alpha^+$. ERβ signaling enhances the expression of polyfunctional phenotypes that include effector type I cytokines that enhance the T cell anti-

tumor immune response, the lytic marker CD107a indicating T cell degranulation, and the type II cytokine IL-4 which promotes T cell survival and memory formation. Since polyfunctional $CD8^+$ and $CD4^+$ T cells were shown to generate superior anti-tumor immune responses [417, 423, 428, 433], it can be hypothesized that female $CD8^+$ T cells can modulate stronger anti-tumor immune responses compared to male $CD8^+$ T cells. Based on the findings in this chapter it can also be hypothesized that estrogen signaling through ERβ can modulate stronger $CD8^+$ and $CD4^+$ T cell anti-tumor immune responses through enhancement of their polyfunctionality.

Estrogen signaling through ERβ was also shown to enhance the polyfunctionality of the whole Ag-specific T cell population containing $CD4^+$ and $CD8^+$ T cells and independently of the sex of the 30 donors tested. Physiological and super-physiological estrogen signaling through ERβ enhanced the percent frequency of Ag-specific T cells expressing IFNγ, TNFα, and IL-4 simultaneously upon activation. Surprisingly, estrogen signaling through ERα significantly inhibited the percent of Ag-specific T cells expressing this polyfunctional phenotype. Super-physiological concentrations of estrogen significantly increased the percentage of Ag-specific T cells expressing $INF\gamma^+IL\text{-}2^+IL\text{-}4^+$ and $IL\text{-}17a^+TNF\alpha^+IL\text{-}2^+IL\text{-}4^+TNF\alpha^+$ and the super-physiological estrogen-mediated enhancement of expression was abrogated when ERβ was blocked, but significantly enhanced when ERα was blocked. Opposite results were obtained for monofunctional phenotypes $IFN\gamma^+$ and $TNF\alpha^+$ which were significantly decreased upon estrogen treatment, but significantly increased when physiological estrogen signaling through ERβ was inhibited. On the other hand, the percent of Ag-specific T cells expressing monofunctional phenotypes $IFN\gamma^+$ and $TNF\alpha^+$ was significantly decreased when ERα was blocked indicating that super-physiological estrogen signaling through ERα enhances monofunctional phenotypes.

Overall, these results indicate that estrogen signaling through ERβ enhances the polyfunctionality of the bulk Ag-specific T cell population in a dose-dependent manner, while estrogen signaling through ERα inhibits the polyfunctionality of the bulk Ag-specific T cell population. Previously, ERα signaling was shown to enhance expression of specific cytokines, IFNγ, TNFα and IL4, through its genomic pathway. This could indicate that ERβ is enhancing Ag-specific T cell polyfunctionality through modulating T cell pathways and genes that promote polyfunctionality.

T cell polyfunctionality is induced by TCR ligation of the antigen-MHC complex resulting in subsequent T cell activation, proliferation and cytokine production. Upon antigen ligation the TCR gets phosphorylated by lck and the kinase ZAP70 is recruited to phosphorylation sites. ZAP70 then activates several signaling pathways that modulate T cell function and cytokine expression. The three major pathways activated downstream from the TCR are the MAP kinase pathway, the NFκB pathway, and the NFAT pathway [6]. NFAT and NFκB are transcription factors that translocate into the nucleus when activated by TCR downstream signaling and modulate expression of immune genes and cytokines [6]. The MAP kinase pathway induces the expression of the transcription factor c-Fos which together with Jun form the dimeric transcription factor complex AP-1 [6]. AP-1 can also modulate expression of immune genes and cytokines. While the effect of ERβ on TCR downstream signaling remains unknown, there are studies confirming a relationship between estrogen signaling and activation of the MAPK, NFAT, and NFκB pathways. While the specific estrogen receptor was not reported, estrogen signaling was shown to induce rapid activation of the MAP kinase pathway in mammalian cells [434]. Estrogen activation of the MAPK pathway is preceded by a rapid

increase in cytosolic Ca^{2+} concentration indicating that estrogen promotes Ca^{2+} release from the intracellular storages [435, 436]. The estrogen-mediated increase of cytosolic Ca^{2+} can also activate NFAT [437]. NFAT is activated via dephosphorylation by the Ca^{2+}-dependent phosphatase calcineurin [438]. This indicates that estrogen can enhance intracellular Ca^{2+} levels which result on subsequent activation of the MAPK and NFAT pathways. Estrogen was also shown to rapidly activate the NFκB pathway in endothelial and breast cancer cells [439]. While treating cells with estrogen ligand enhanced NFκB signaling, ERα and ERβ were shown to inhibit NFκB binding to DNA target sites in myocardial cells impeding NFκB target gene expression, and to enhance expression of the NFκB inhibitory protein IKβ [440, 441]. The controversial effect of estrogen signaling on NFκB seems to be cell dependent and it cannot be ruled out that estrogen signaling through ERβ enhances NFκB signaling.

In addition to the evidence supporting that estrogen activates these TCR downstream signaling pathways, EREs were found in the promoters of genes coding for important TCR downstream signaling proteins like lck, ZAP70 and c-Fos. Lck contains an ERE at position 3451 bp of its promoter, and ZAP70 contains an ERE at position 4305 bp of its promoter, and c-Fos contains an ERE at location -3411 of its promoter (Table 5) [442]. This indicates that transcription of these genes could be regulated by ligand and ERE-independent ERβ canonical genomic signaling. Overall, given that ERβ enhances T cell polyfunctionality which is induced by TCR signaling, and knowing that estrogen signaling can enhance TCR signaling proteins expression and activation of downstream pathways it can be hypothesized that ERβ enhances human male and female Ag-specific T cell polyfunctionality by enhancing overall TCR signaling.

In order to exclude the possibility that ERβ mediated enhancement of $CD8^+$ and $CD4^+$ Ag-specific T cell polyfunctionality is caused by changes on monofunctional and polyfunctional T cell survival and proliferation rather than enhancement of the TCR signaling, cell viability and proliferation staining need to incorporated into these experiments. Survival of monofunctional and polyfunctional Ag-specific T cells after antigen stimulation can be assessed by a viability dye and compared between estrogen and ER inhibitor treatments. Ag-specific T cell proliferation can be accessed via Ki67 staining and equally compared between estrogen and ER inhibitor treatments.

Hs Gene Name	Hs Chr #	Hs ERE Sequence	Hs ERE position	Hs Gene Start	Hs Gene End	Hs Dist. Gene-ERE
LCK	Chr1	TGGTCAGGCTGATCT	2501902	2498451	2533350	3451
ZAP70	Chr2	AGGTCCTTCTGACCA	400360	396055	422347	4305
FOS	Chr14	AAGTCACCCTGACCT	55662161	55665572	55668973	-3411

Table 5. Estrogen Response Elements Found in the Promoters of LCK, ZAP70, and FOS. Table shows the human (Hs) gene name, the chromosome location, the ERE sequence and its position in respect to the gene starting site. EREs were found in n the EREfinder database. c-Fos ERE was previously reported by Weisz & Rosales, *Nucleic Acids Res*, 1990.

CHAPTER VI

ESTROGEN ENHANCES T CELL SURVIVAL, TUMOR INFILTRATION AND ANTI-TUMOR FUNCTION DURING IMMUNOTHERAPY FOR HCC

Introduction and Rationale

The protective role of estrogen against HCC is widely accepted and supported by the observation that the HCC female-biased survival is significantly reduced after menopause [311]. Post-menopausal females undergoing estrogen hormone-replacement therapy have a decreased risk of HCC and increased survival rates compared to non-estrogen users [311]. Previous studies demonstrated that the protective activity of estrogen against HCC is mediated through inhibition of expression and signaling of IL-6 and inactivation of the STAT3 pathway in hepatocytes and Kupffer cells, as well as inhibition of tumor-associated macrophages, which all result in reduction of chronic inflammation of the liver and subsequent transformation [293, 318, 443]. The effect of estrogen on other immune cells present in the TME that impact anti-tumor immune responses and may have critical bearings on the success of ACT immunotherapy, including T cells, remains unclear and understudied. Since higher frequencies of infiltrating lymphocytes including $CD4^+$ and $CD8^+$ T cells have been correlated with increased survival in HCC patients [333, 334], it is important to study the role of estrogen signaling regulating the function of T cells in the HCC TME. The previous chapters of this dissertation demonstrated that estrogen signaling through ERα enhances the expression of specific cytokines including IFNγ, TNFα, and IL-4, in Ag-specific T cells upon TCR ligation and estrogen signaling through ERβ significantly

enhances the polyfunctionality, or the ability to express several functional markers simultaneously, of human male and female Ag-specific T cells activated by their cognate antigen. These results indicate that estrogen signaling through both ERα and ERβ contribute to enhancing Ag-specific T cell function in response to antigen stimulation.

ACT immunotherapy using genetically modified T cells including CAR-T cells and TCR-transduced T cells showed some success for treating HCC in preclinical models, and in early stage clinical trials. However, due to high fibrosis and cirrhosis, HCC tumors are hard to penetrate physically by Ag-specific T cells [444] therefore, currently the clinical results in treating HCC remain unsatisfactory. In addition, the HCC TME comprises immunosuppressive stromal cells and molecules including checkpoint molecules that promote tumor growth by exhausting T cells [444]. These data indicate that there is a high need for ways to improve the anti-tumor function of Ag-specific T cells in order to generate more successful ACT immunotherapy for HCC. Since estrogen has a protective role against HCC through mechanisms involving inflammation, and can enhance cytokine production and polyfunctionality of T cells *in vitro*, it was hypothesized that the protective role of estrogen against HCC is partially due to the estrogen-mediated enhancement of the T cell anti-tumor immune response. Estrogen receptor signaling was shown to enhance T cell activation, proliferation, and survival in models of autoimmune disease [233], indicating that estrogen could further strengthen the efficacy of ACT immunotherapy for HCC. In this chapter of the dissertation, the impact of estrogen signaling on tumor growth and T cell function is assessed during ACT immunotherapy using human HCV Ag-specific T cells to treat a HCV^{+}HCC generated in mice.

Results

The ideal target tumor tissue to be used for studying the role of estrogen during ACT immunotherapy for HCC would be patient derived xenografts (PDX) from HCV^{+}HCC $HLA\text{-}A2^{+}$ patients. PDX are transplanted into immune-deficient mice which then receive ACT immunotherapy with human HCV Ag-specific T cells, and tumor rejection can be measured as well as T cell function. Unfortunately, HCV^{+}HCC xenografts from $HLA\text{-}A2^{+}$ patients were not available to be used for the following experiments so a genetic HCC mouse model was used instead. HCC can be generated in mice by forcing the expression of human oncogenes c-MET (MET) and β−catenin (CAT) in hepatocytes by sleeping beauty (SB) transposase-mediated somatic integration [445]. SB100 is retrovirally transduced into the liver in combination with transposon containing vectors for MET and CAT. SB recognizes and binds to inverted repeats flanking the MET and CAT sequences, excises the sequences, and inserts them into a new location containing a TA dinucleotide in the hepatocyte genome (Figure 26) [445]. This is a previously described clinically relevant HCC model that relies on the co-activation of MET and CAT which often occur in human HCC cases [371, 446, 447]. Co-delivery of both MET and CAT simultaneously on mouse livers using the sleeping beauty transposase system induces HCC within 40 to 60 days [372]. In order to include the HCV specific antigen in these HCC tumors that could be recognized by human Ag-specific T cells, a pcDNAIII expression vector containing the sequence of the full length HCV NS3 (Figure 7) [51, 358] was also delivered in combination with MET and CAT. Using this model, HCC tumors expressing the HCV NS3 antigen were generated. Ideally these tumors generated in mice would be treated syngeneically using mouse HCV Ag-specific T cells. On the other hand, considering that the effects of estrogen cytokine production and polyfunctionality were observed specifically in human T cells, it was decided to

use human male and female HCV Ag-specific T cells to perform ACT immunotherapy for HCC in these mice. In order to test human T cells in the mouse system, the strain of mice used were the NSG-A2^{+} mice which are completely immune-deficient HLA-A2 expressing mice. NSG-A2^{+} mice lack functional mature B cells, T cells, NK cells and have defective DCs and macrophages, which prevents them from targeting and depleting or "rejecting" adoptively transferred human T cells. Furthermore, human T cells were shown to survive and be fully functional in NSG-A2^{+} mice [448-450], indicating this is a valid mouse model for the study of human T cell based immunotherapy.

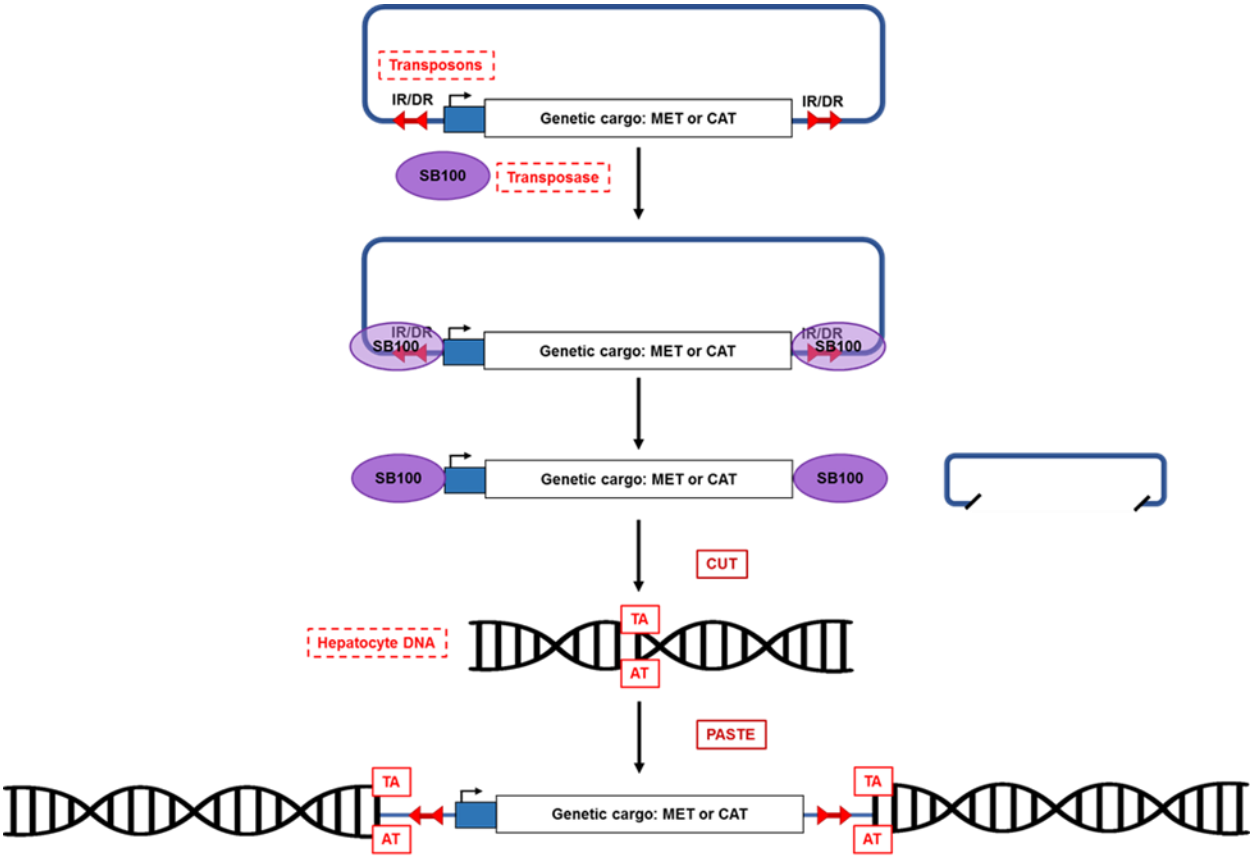

Figure 26. Mechanisms of Sleeping Beauty 100 (SB100)-mediated Transposition. Diagram of SB100 transposase-mediated cut-and-paste transposition of the sequences of the oncogenes MET and CAT into hepatocyte's genome. A transposon, defined by the mirrored sets of red double arrows (IR/DRs) is shown on each end of the plasmid containing the sequence of MET and CAT oncogenes. The transposon harbors the expression cassette consisting of a promoter (blue square) that can direct the transcription of MET and CAT. SB100 binds to the IR/DRs and cuts the transposons out of the plasmid. A DNA molecule, in this case genomic DNA in mouse hepatocytes, containing the TA sequence becomes the recipient of the transposed transposon. In this process the TA sequence at the insertion site is duplicated.

While the MET-CAT HCC tumor model was previously used to study HCC oncogenesis and pharmacological drug design and delivery, it has never been used before to study ACT immunotherapy. To ensure that human HCC Ag-specific T cells could recognize the HCV antigen presented in the context of HLA-A2 in mouse HCC cells, HCV^{+}HCC tumors generated in NSG-$A2^{+}$ mice were digested into a single cell suspension and incubated with male and female human Ag-specific T cells. T cell activation in response to target tumor antigen was measured via IFNγ release by ELISPOT. It was observed that female and male Ag-specific T cells secreted IFNγ in response to HCV^{+}HCC cells but not in response to HCV^{-}HCC cells generated in mice challenged with MET/CAT (Figure 27). These resulting data demonstrate that human Ag-specific T cells can recognize HCV antigen expressing HCC tumors generated in mice but do not respond to hepatocytes or tumor cells in the absence of the cognate Ag. The lack of response to tumors missing HCV Ag expression rules out the possible off-antigen reactivity to mouse tissue. Overall these results indicate that HCC Ag-specific T cells can be safely used to treat HCC bearing mice with ACT immunotherapy.

In order to study the role of estrogen signaling in the T cell anti-tumor immune response generated during ACT immunotherapy *in vivo*, physiological estrogen was removed via ovariectomy (OVX) which consists of surgically ablating the ovaries of female mice. The ovaries are the main source of estrogen in the body of female mice and removing them was shown to significantly reduce the serum estrogen concentration to levels similar to those observed in male mice [451]. Physiological estrogen serum concentration in female mice is around 30-60 pg/mL and ovariectomy was shown to reduce this concentration to around 1.5-3 pg/mL [451, 452].

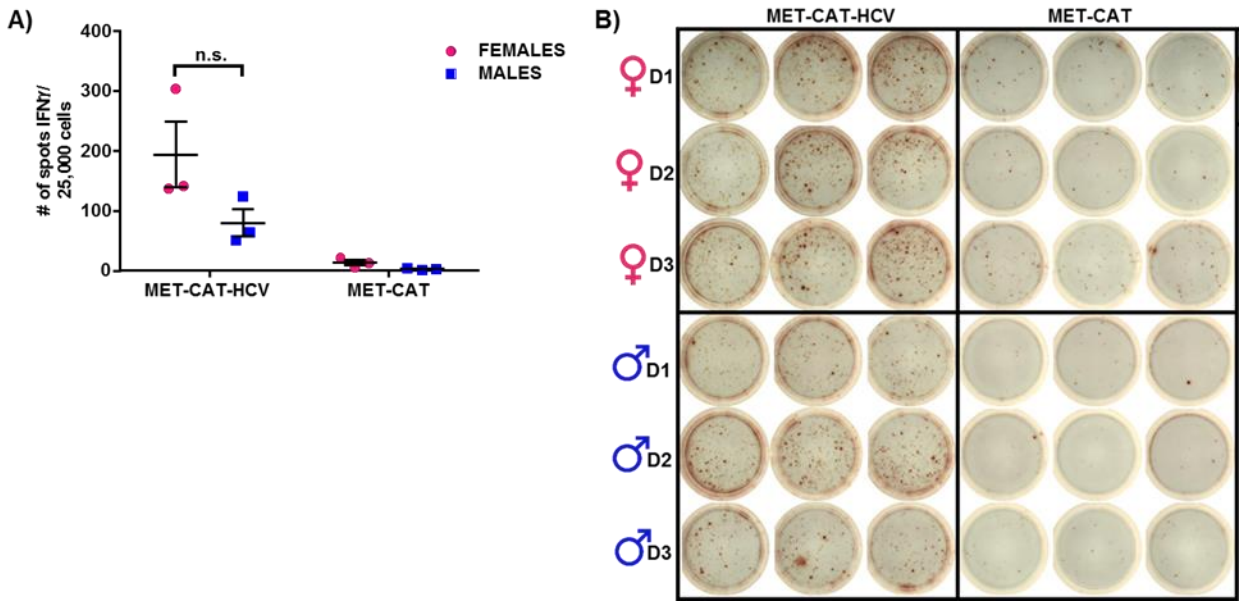

Figure 27. Human HCV Ag-specific T Cells Secrete IFNγ in Response to HCV$^+$ HCC Cells Generated in NSG-A2$^+$ Mice. NSG-A2$^+$ mice were challenged with c-MET and β-catenin human oncogenes in combination with an expression vector for the HCV NS3 peptide to generate HCV-expressing HCC. HCC were left to develop for 60 days and then were collected and digested into a single cell suspension. Tumor cells were co-cultured with human male or female HCV Ag-specific T cells at a 1:1 ratio for 18 hours, and IFNγ secretion was measured via ELISPOT. **A)** Female and male Ag-specific T cells secreted IFNγ in response to HCV expressing-HCV but not to HCV$^-$HCC. **B)** ELISPOT plate demonstrating IFNγ secretion from three different female and three different male donors (D1, D2, D3). Data represents n=3 donors, each donor plotted and the standard error of the mean indicated. Data was analyzed using 2-way ANOVA with Tukey's post-hoc. No significance was observed.

These serum estrogen concentrations can be correlated to the ones found in human females before (30-800 pg/mL) and after menopause (less than 20 pg/mL). Ovariectomy of female mice was considered a clinically relevant model to study the effect of physiological estrogen presence or absence during ACT for HCC. Sham surgeries omitting the ablation of the ovaries were performed in control female and male mice to take into account possible effects of surgery-mediated inflammation on all experimental groups. Before tumor challenge, mice were left to stabilize for 3 weeks which is the minimum time reported for accumulated serum estrogen concentration to significantly decrease after ovariectomy [453, 454].

Estrogen Inhibits Tumor Growth during Adoptive T cell Transfer Immunotherapy

Using mice at 6-8 weeks old, treatment groups consisted of ovariectomized females, sham females and sham males were challenged with MET and CAT oncogenes in combination with the HCV NS3 expression vector. After tumor initiation, at day 20 or day 40 after MET/CAT/HCV injection, sham/OVX females and sham males received ACT immunotherapy of 10^7 sex-matched human HCV Ag-specific T cells via tail vein. 20 days after ACT immunotherapy, livers were collected and analyzed for tumor presence, and T cells were isolated from livers and spleens for analysis. All mice in these experiments received HCV Ag-specific T cells generated from one male and one female healthy donor. Phenotypical analysis of the Ag-specific T cells before adoptive transfer demonstrated similar percentage and MFI of $CD3^+CD34^+$ in male and female T cells indicating equal anti-HCV TCR expression in both sexes (Figure 28 A). While female Ag-specific T cells showed a 1:1 $CD8^+$:$CD4^+$ ratio, the male T cells showed a 2:1 $CD8^+$:$CD4^+$ (Figure 28 B). The percentage of $CD8^+$ T cells expressing intracellular Granzyme B was approximately 70% in female T cells compared to 85% in male T cells indicating a higher percentage of CTLs in transferred male Ag-specific T cells (Figure 28 C). Both male and female $CD4^+$ T cells expressed low percentages of T-BET and high percentages of GATA3 indicating low Th1 and high Th2 $CD4^+$ populations (Figure 28 D, E). Approximately 18% of female T cells expressed RORγt compared to only 2% of male T cells indicating a higher Th17 population in female $CD4^+$ Ag-specific compared to male T cells (Figure 28 F). Surprisingly a high percentage of both male and female $CD4^+$ T cells expressed FoxP3 (50-55%)

indicating a high T_{reg} population (Figure 28 G). These data indicate that the Ag-specific T cells transferred into female mice consisted of a more heavily $CD4^+$ helper T cell population including

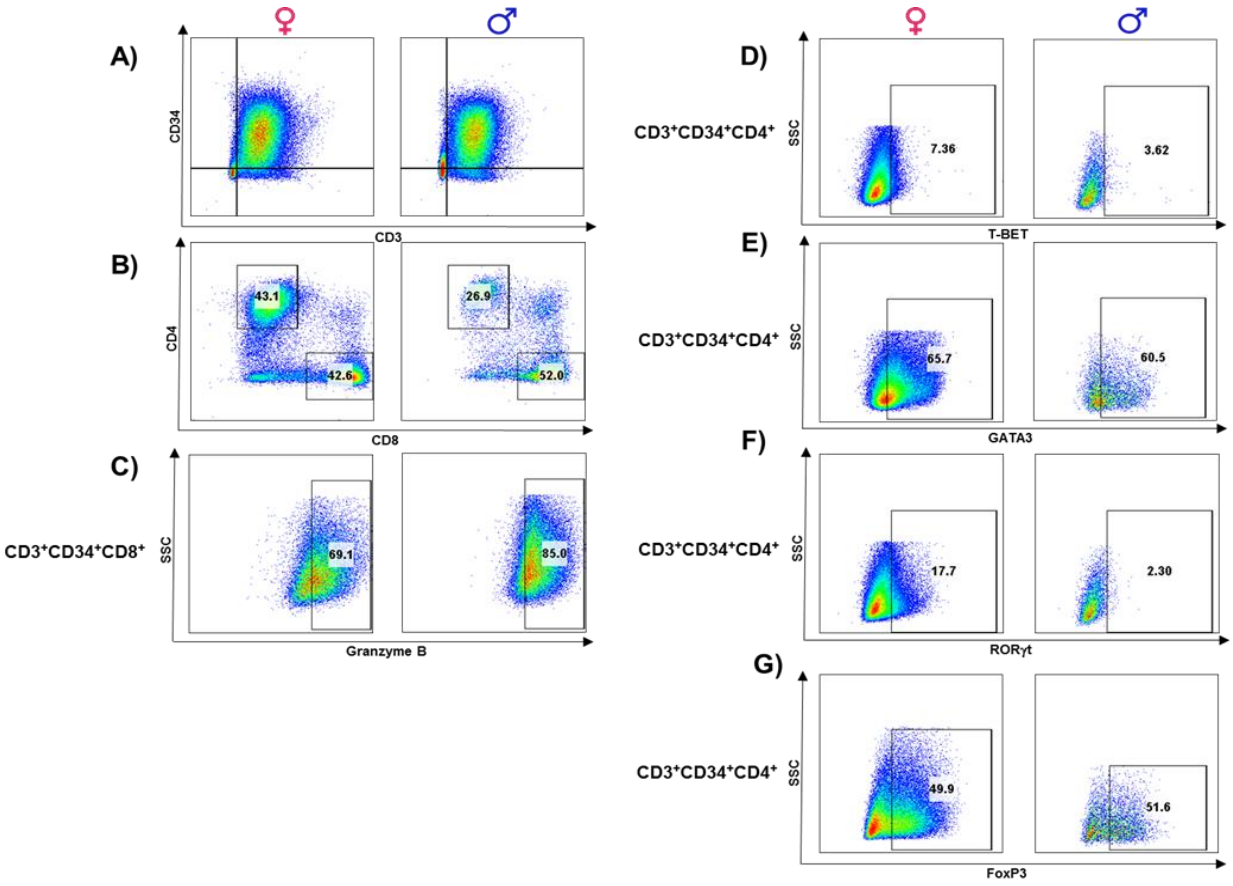

Figure 28. Phenotype of Human Female and Male Ag-specific T Cells Used for ACT Immunotherapy. Flow cytometry dot plots representing the percent frequency of human female and male T cells that were **A)** $CD3^+CD34^+$. **B)** Percent frequency of female and male $CD3^+CD34^+$ that were either in the $CD8^+$ or $CD4^+$ subset. **C)** Percent frequency of $CD3^+CD34^+CD8^+$ female and male T cells expressing Granzyme B. Percent frequency of $CD3^+CD34^+CD4^+$ T cells expressing **D)** T-BET, **E)** GATA3, **F)** RORγt, and **G)** FoxP3.

high percentage of Th2 and Th17 cells, while Ag-specific T cells transferred into male mice consisted of a more $CD8^+$ CTL population.

HCC tumors in mice are commonly quantified by measuring the ratio of tumor over body weight [455]. Since mice used for these experiments were a range of ages, and male mice were bigger than female mice, no significant differences in tumor over body weight ratios were found between sexes or treatment groups. It is important to note that the transformed livers were three times heavier than livers of non-challenged mice, healthy livers weighting approximately 1.5 grams and fully transformed livers weighting approximately 4 grams. Alternatively, to using the ratio of liver tumor:body weight, the BCLC staging classification was used to compare tumor burden between experimental groups. According to this staging method, HCC tumors are classified into four different categories according to the number of tumors, the size of the tumors, the presence of vascular invasion, and the spread of nodules or presence of extrahepatic metastases [320]. According to these parameters, HCC tumors are considered stage 0 (very early stage) when only one small nodule <2 mm is found, stage A (early stage) when between 1 and 3 small nodules are found, stage B (intermediate stage) when several nodules >3 mm are found, stage C (advanced stage) when several large nodules are found and there is portal invasion, or stage D (terminal stage) when several large nodules are present and there is extrahepatic spread [319-321]. Extrahepatic spread in mice HCC models is described as presence of metastases in the lungs, the spleen, the kidneys, and the heart [456]. HCC tumors that were left to develop for 20 days before ACT were on the very early stage (stage 0) since tumor nodes were not detected. Only OVX females and sham males, which have minimal estrogen levels, that did not receive ACT showed transformation of the liver and tumor node presence (Figure 29 B lower panel). This indicates that ACT immunotherapy prevented tumor development since none of the mice that received ACT had tumor nodules present compared to untreated mice (Figure 29 B upper panel).

Tumors that were left to develop for 40 days prior to ACT were in very advanced HCC stage and resulted in an almost complete transformation of the liver (Figure 29 C). HCC tumors were multinodular, nodules were very large, and some mice even presented tumor nodules on the spleen which were characterized as metastases (represented on Figure 29 C), indicating that these tumors were in the intermediate (stage B), advanced or very advanced stages (stage C and D). In mice that did not receive ACT immunotherapy, tumor burden was greater in OVX females and sham males compared to sham females (Figure 29 C lower panel). Tumor burden was overall reduced in mice that received ACT indicating T cell tumor recognition and killing. Sham females treated with ACT immunotherapy showed less tumor burden than OVX females and sham males counterparts (Figure 29 C upper panel). These data overall support the hypothesis that estrogen has a protective roll against HCC. The HCC tumor burden in ACT treated mice was quantified and showed that even if tumor burden was improved with ACT immunotherapy, OVX females had the overall worst diagnosis when compared to sham females or sham males. A significantly greater percentage of ACT treated OVX female mice developed HCC tumors in the very advance stage D (multinodular and spleen metastasis presence) compared to sham females (Figure 29 D). HCC tumor burden on OVX females treated with ACT was also worse compared to sham males showing higher percentage of mice in the very advanced stage even if significance was not achieved. Overall, sham females that received immunotherapy showed significantly less advanced tumor burden compared to OVX females, and trending less advanced tumor burden compared to sham males (Figure 29 D). These results indicate that removal of endogenous estrogen enhances HCC tumor burden and decreases ACT immunotherapy efficacy.

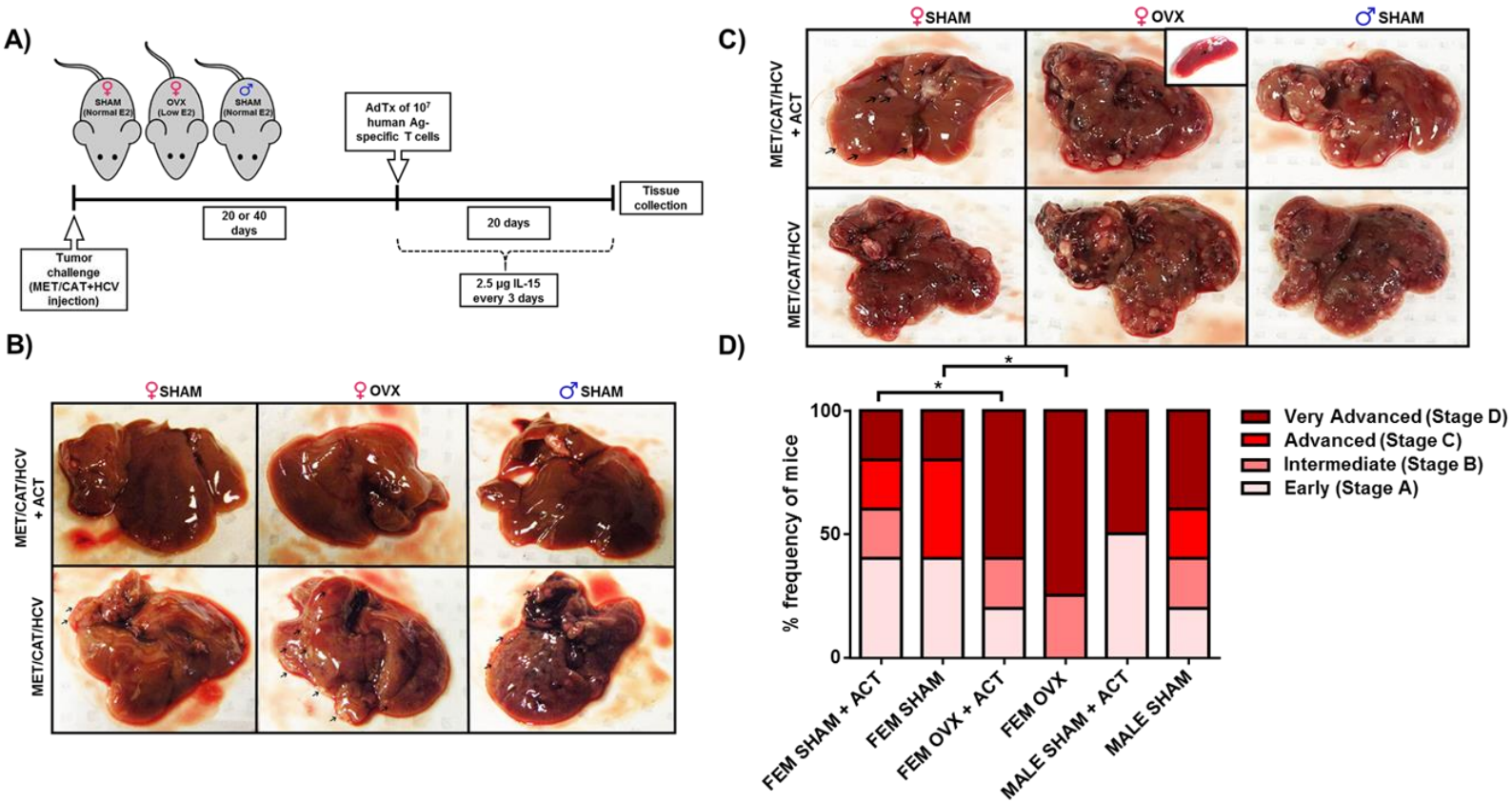

Figure 29. Removal of Endogenous Estrogen via Ovariectomy Enhances HCC Tumor Burden and Reduces ACT Immunotherapy Efficacy. A) Experimental set up. **B)** Representative pictures of livers of sham females, OVX females, and sham males that were challenged with MET and CAT oncogenes in combination with an HCV antigen expression vector. Upper panels show livers of mice that received ACT immunotherapy with sex-matched human HCV Ag-specific T cells 20 days after tumor challenge and that were sacrificed 20 days after therapy administration. Lower panels show mice counterparts that did not receive ACT immunotherapy. **C)** Representative pictures of mice that received ACT immunotherapy with sex-matched human HCV Ag-specific T cells 40 days after tumor challenge and were sacrificed 20 days after therapy administration. Arrows point at tumor nodules. Small panel shows a representative picture of a spleen metastasis (MET). **D)** HCC tumor burden was determined using the BCLC staging based on the number of nodules, the percent transformation of the liver, and presence of spleen metastases in mice that received ACT 40 days after tumor challenge compared to untreated mice. ACT day 20: n=6 sham females, n=7 OVX females, n=10 sham males. ACT day 40: n=5 sham females, n=5 OVX females, n=6 sham males. Data was analyzed with 2-way ANOVA with Tukey's post-hoc. $p<0.05$.

Estrogen Presence during Adoptive T cell Transfer Immunotherapy Enhances Human CD4$^+$ Ag-specific T cell Tumor Infiltration

After assessing tumor burden, HCC tumors were digested and T cells were isolated from whole tumors and analyzed via flow cytometry in order to determine Ag-specific T cell tumor infiltration. T cells obtained from the tumors of each mouse were analyzed separately and no tumors were pooled together. Even if the Ag-specific T cell numbers recovered were low they were sufficient for analysis and for making meaningful comparisons. Significantly greater numbers of human Ag-specific T cells were found infiltrating the livers of sham females, an average of approximately 2000-4000 cells, compared to OVX females, an average of 1500-2000 cells, at both time points of ACT (Figure 30 B). A trend was observed indicating increased numbers of infiltrating T cells in sham females compared to sham males but significance was not achieved (Figure 30 B). The number of infiltrating CD8$^+$ Ag-specific T cells was not significantly different in early stage tumors treated with ACT immunotherapy at day 20 after tumor challenge. On the other hand, significantly higher numbers of infiltrating CD8$^+$ T cells were found in advanced tumors of sham females, an average of approximately 1700 cells, compared to OVX females and males that received ACT immunotherapy at day 40 after tumor challenge, >500 cells (Figure 30 C). The numbers of infiltrating CD4$^+$ Ag-specific T cells were significantly higher in sham females compared to OVX females and males at both time points of ACT (Figure 30 D). Overall, these data indicates that endogenous estrogen enhances tumor infiltration of CD4$^+$ Ag-specific T cells no matter on tumor burden, and enhances the infiltration of CD8$^+$ Ag-specific T cells in advanced tumors.

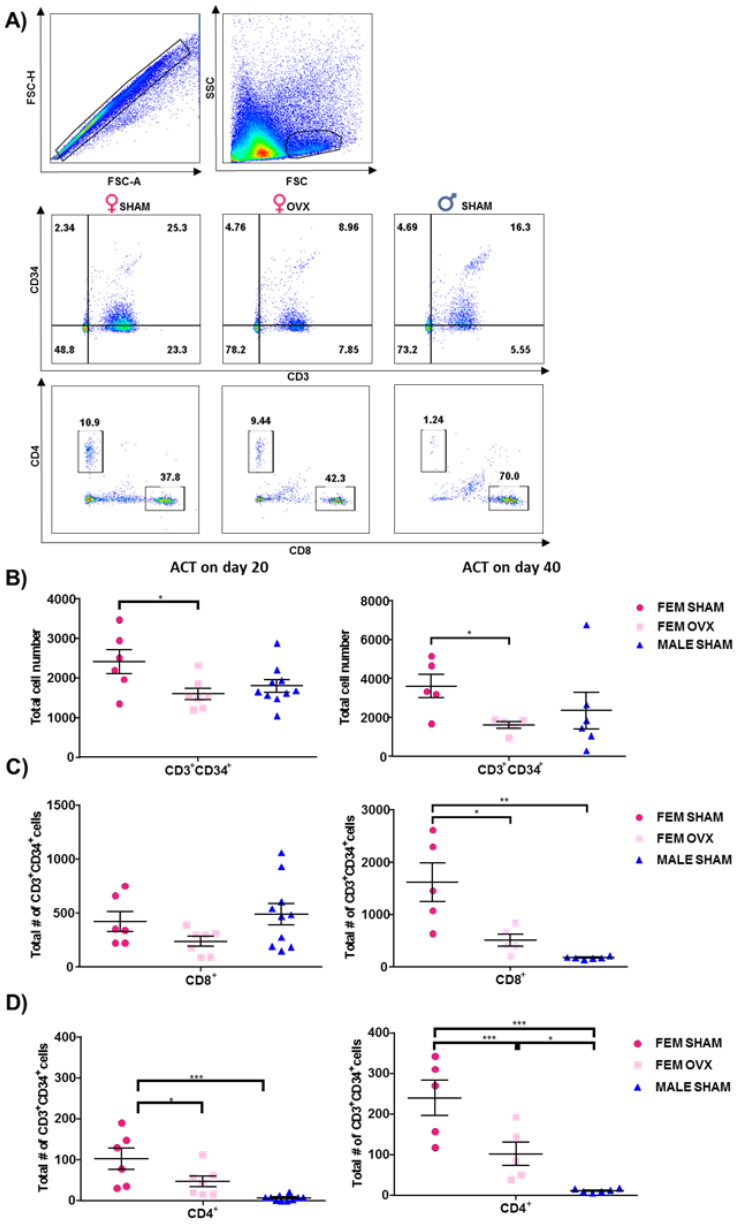

Figure 30. **Endogenous Estrogen Enhances Human Ag-specific T Cell Tumor Infiltration.** Human Ag-specific T cells were adoptively transferred into OVX/sham female or sham male mice 20 or 40 days after HCC tumor challenge, and then isolated from tumors 20 days after transfer for flow cytometric analysis. **A)** Representative flow cytometry dot plots containing the gating strategy performed to analyze HCV Ag-specific T cells isolated from tumors of one female sham, one female OVX, and one male sham mouse. Total number of **B)** $CD3^+CD34^+$, **C)** $CD3^+CD34^+CD8^+$, or **D)** $CD3^+CD34^+CD4^+$ HCV Ag-specific T cells found infiltrating the liver of sham females, OVX females, and sham males that were treated with ACT immunotherapy either 20 or 40 days after tumor challenge. ACT day 20: n=6 sham females, n=7 OVX females, n=10 sham males. ACT day 40: n=5 sham females, n=5 OVX females, n=6 sham males. Each mouse plotted and the standard error of the mean indicated. Data were analyzed by 1way-ANOVA and Tukey's post-hoc. $p<0.001$=***, $p<0.01$**, $p<0.05$=*.

The subset distribution of $CD4^+$ Ag-specific T cells infiltrating the tumors of OVX and sham females was analyzed via flow cytometry staining. No significant differences were found on expression of any of these transcription factors between sham and OVX females indicating that endogenous estrogen does not affect $CD4^+$ T cell subset differentiation during ACT immunotherapy. High percent frequency of $CD4^+$ T cells infiltrating the livers of female sham and OVX mice expressed GATA3 and RORγt compared to other transcription factors, which are the prototypic transcription factors for the Th2 and Th17 $CD4^+$ T cell subsets (Figure 31).

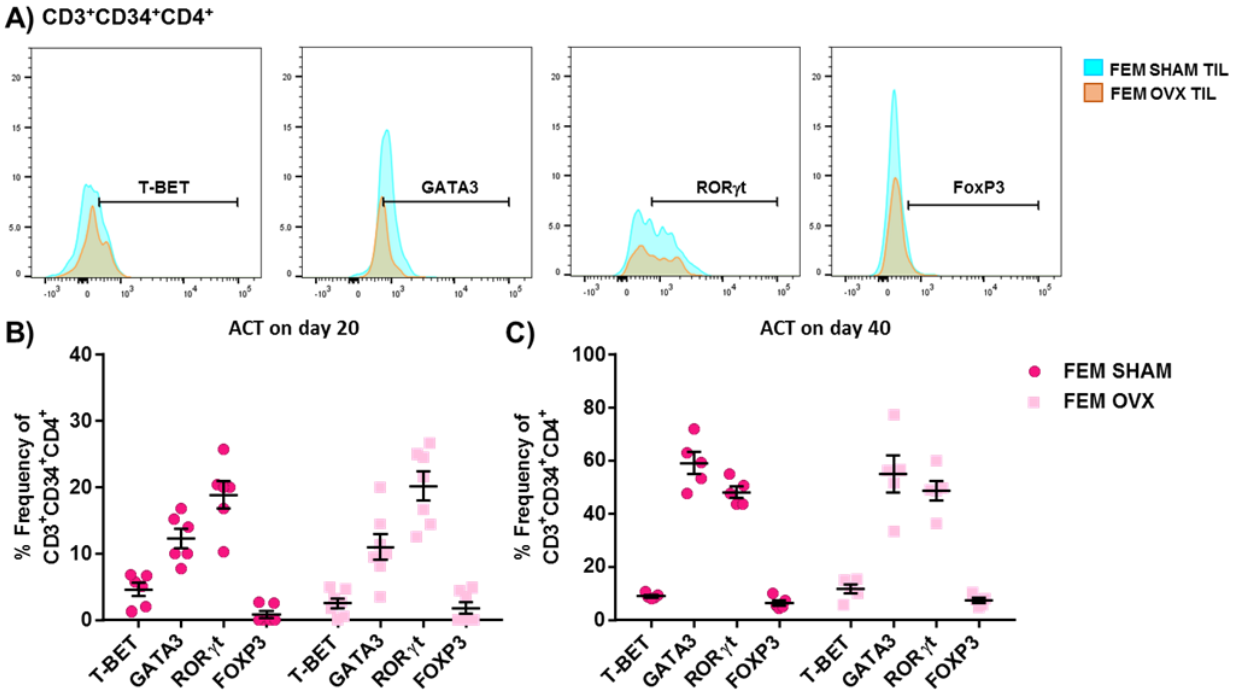

Figure 31. The $CD4^+$ Ag-specific T Cell Subset Distribution was not Affected by Removal of Endogenous Estrogen. Human $CD4^+$ Ag-specific T cells isolated from livers of sham and OVX female mice were intracellularly stained for the transcription factors T-BET, GATA3, RORγt, and FOXP3 which correspond to the Th1, Th2, Th17, and T_{reg} subsets respectively. **A)** Representative histograms showing the expression of T-BET, GATA3, RORγt, and FOXP3 in tumor infiltrating $CD3^+CD34^+CD4^+$ T cells from one sham female and one OVX female mouse. Percent frequencies of tumor infiltrating $CD3^+CD34^+CD4^+$ T cells from sham females and OVX females that received ACT immunotherapy **B)** 20 days or **C)** 40 days after HCC tumor challenge. ACT day 20: n=6 sham females, n=7 OVX females. ACT day 40: n=5 sham females, n=5 OV females. Each mouse plotted and the standard error of the mean indicated. Male group is not included since not enough $CD4^+$ T cell numbers were present infiltrating the liver to perform this analysis.

Surprisingly, low frequency of $CD4^+$ T cells, less than 10%, expressed the transcription factor T-BET indicating low percentage of Th1 $CD4^+$ Ag-specific T cells infiltrating the tumors of sham and OVX mice. Low frequencies of $CD4^+$ T cells, less than 10%, expressed FoxP3, indicative of lack of immunosuppressive regulatory $T_{regs,}$ infiltrating the tumors of sham and OVX females (Figure 31). These results indicate that $CD4^+$ T cells infiltrating the tumors of female mice belonged mostly to the Th2 and Th17 subsets.

In order to demonstrate that the significant differences in $CD4^+$ Ag-specific T cell infiltration between sham females and sham males were not due to the differences in $CD8^+$:$CD4^+$ ratios observed in Ag-specific T cells populations before transfer (Figure 28), opposite sex ACT immunotherapy was performed. Female mice received 10^7 male human Ag-specific T cells, and male mice received 10^7 female human Ag-specific T cells on day 20 after tumor challenge. On day 20 after ACT immunotherapy, livers were collected, and Ag-specific T cells were isolated. It was observed that female and male mice that received opposite sex ACT had similar numbers of $CD3^+CD34^+$ Ag-specific T cells infiltrating the tumor, approximately 2500 to 4000 TILs (Figure 32 B). The numbers of infiltrating $CD8^+$ Ag-specific T cells were comparable in male and females that received opposite sex ACT, m approximately 1200 TILs (Figure 32 C). On the other hand, female mice that received male human Ag-specific T cells had significantly greater number, around 500, of infiltrating $CD4^+$ Ag-specific T cells compared to males, around 200, that received human female T cells (Figure 32 C). These results indicate that females have increased $CD4^+$ Ag-specific T cell tumor infiltration independently of the sex of the T cell donor. These results in combination with the ones presented in Figure 30 indicate that endogenous estrogen enhances tumor infiltration of $CD4^+$ T cells from male and female source.

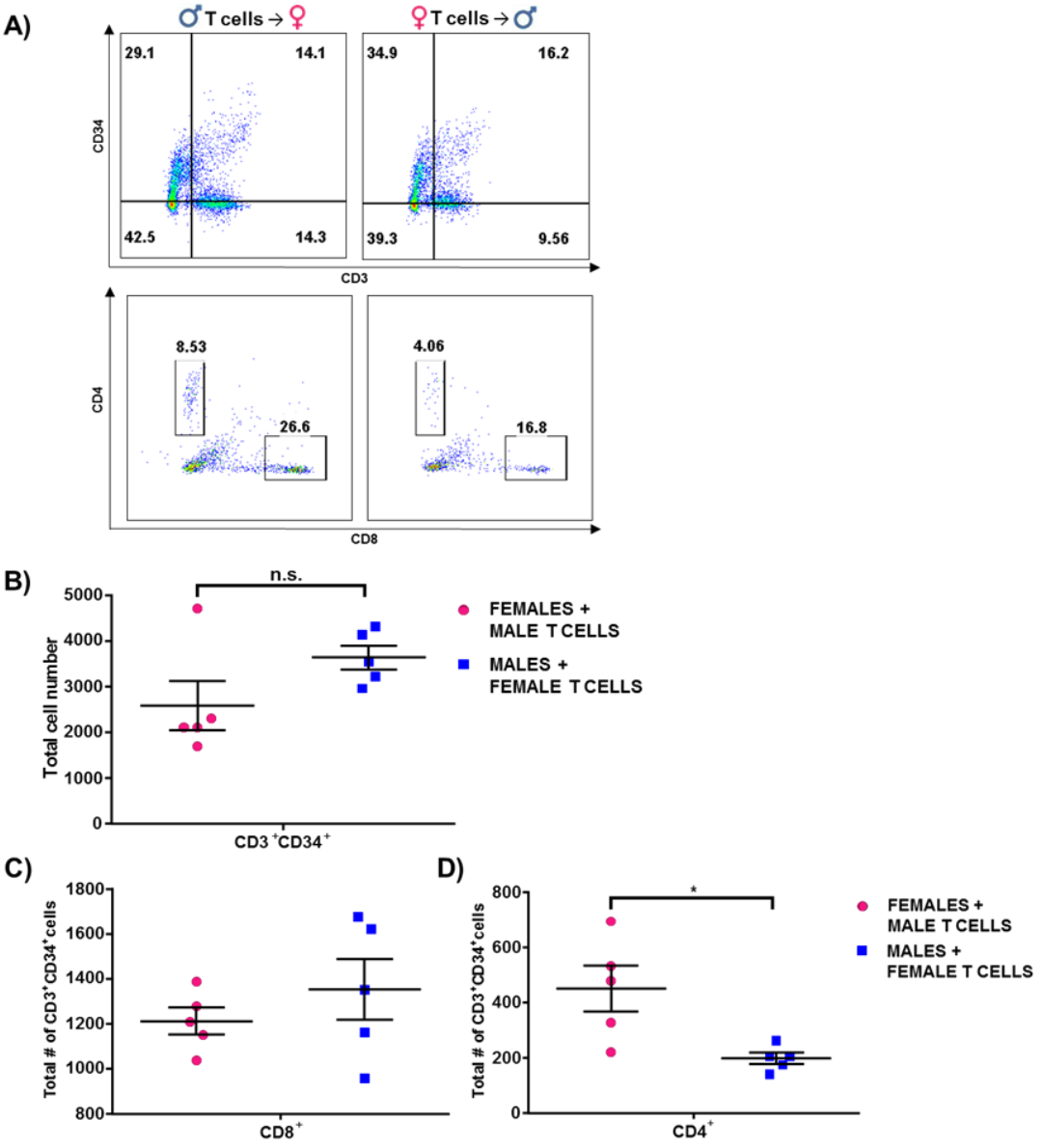

Figure 32. Endogenous Estrogen Enhances Tumor Infiltration of CD4$^+$ Ag-specific T Cells Independently of the Donor's Sex. Female and male NSG-A2$^+$ mice were challenged with MET-CAT-HCV oncogenes and 20 days after they received ACT immunotherapy with T cells of the opposite sex. 20 days after ACT immunotherapy, livers were collected and T cells were analyzed via flow cytometry. **A)** Representative dot plots of infiltrating Ag-specific T cells from one female (received male T cells) and one male (received female T cells) mouse. **B)** Total number of CD3$^+$CD34$^+$ Ag-specific T cells found in the liver of female and male mice. Total number of **C)** CD3$^+$CD34$^+$CD8$^+$ or **D)** CD3$^+$CD34$^+$CD4$^+$ T cells found infiltrating the liver of female or male mice. Data represents n=5 sham females, n=5 sham males. Each mouse plotted and the standard error of the mean indicated. Data analyzed with Student's t test. $p<0.05$=*.

Estrogen Presence during Adoptive T cell Transfer Immunotherapy Enhances Human $CD4^+$ Ag-specific T cell Survival

In order to determine if the overall survival of adoptively transferred cells outside of the tumor was affected by estrogen removal, the number of circulating human T cells present in the spleen was quantified. While no differences were observed in mice that received ACT 20 days after tumor challenge and were bearing early stage tumors, there were significantly higher numbers of $CD3^+CD34^+$ and $CD3^+CD34^+CD8^+$ T cells in the spleens of sham female mice with advanced stage tumors compared to OVX females and males which received ACT 40 days after tumor challenge (Figure 33 B, C). $CD3^+CD34^+CD4^+$ T cell numbers were significantly higher in the spleens of sham female mice compared to OVX females and males at both time points of ACT immunotherapy and independently of tumor stage (Figure 33 D). These data demonstrate that estrogen enhances the persistence of adoptively transferred Ag-specific T cells. Estrogen removal is deleterious for T cell survival with an even more significant impact on the survival of the $CD4^+$ T cell subset.

Ideally, conclusions of these experiments would be supported by data measuring the effect of endogenous estrogen on the viability and proliferation state of tumor infiltrating and circulating T cells in order to determine if decreased $CD4^+$ numbers found in the tumors and spleens of OVX females and sham males are due to increased T cell apoptosis or decreased proliferation. To test this, HCC tumors and spleens from sham/OVX females and sham males can be fixed and sectioned, and then stained for human $CD4^+$ to detect the T cells in combination with staining for fragmented DNA (a feature of apoptotic cells) by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) [457].

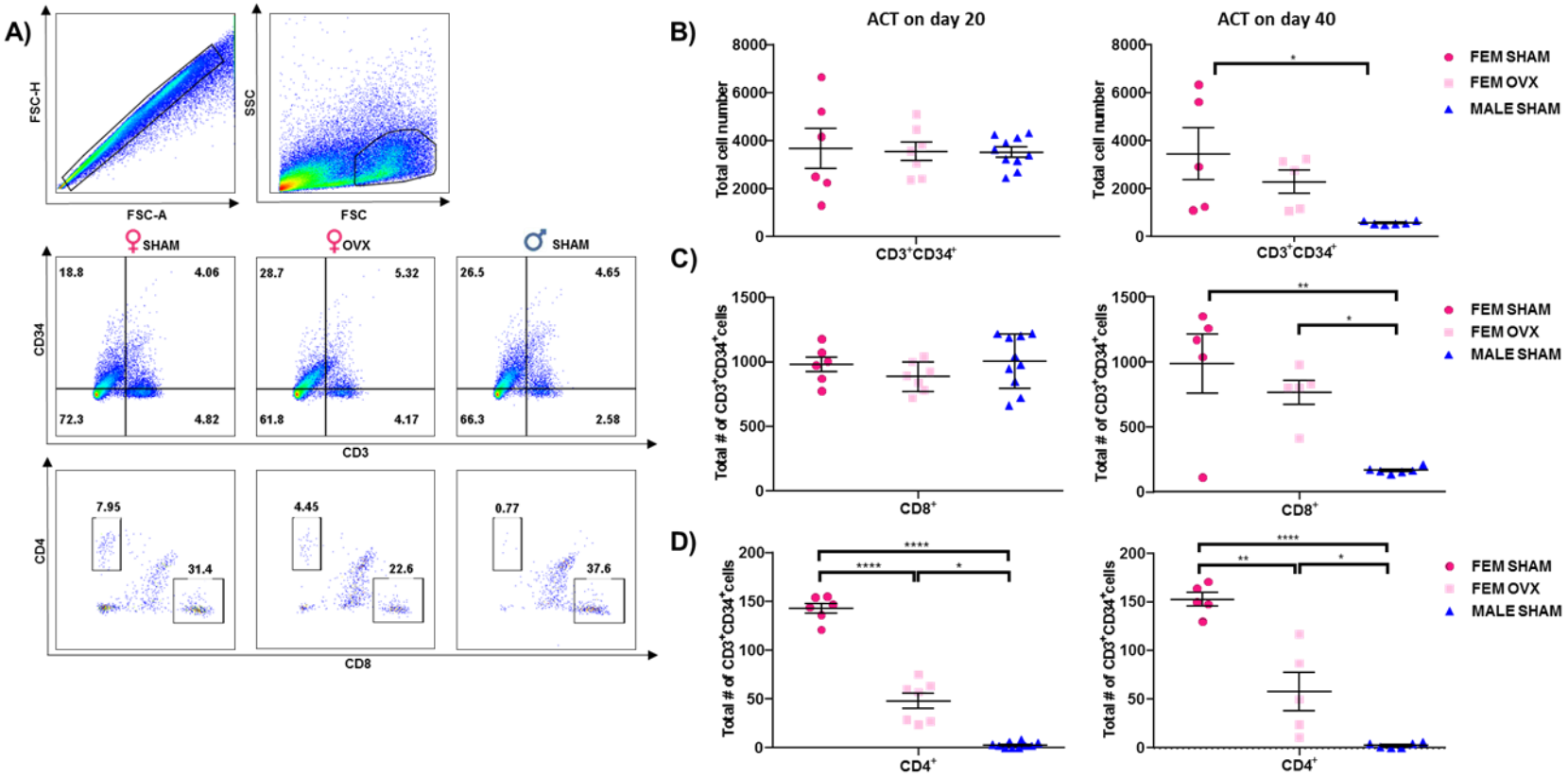

Figure 33. **Endogenous Estrogen Enhances Human Ag-specific T Cell Survival.** Human Ag-specific T cells were adoptively transferred into OVX/sham female or sham male mice 20 or 40 days after HCC tumor challenge, and then isolated from spleens 20 days after transfer for flow cytometric analysis. **A)** Representative flow cytometry dot plots containing the gating strategy performed to analyze HCV Ag-specific T cells isolated from spleens of one female sham, one female OVX, and one male sham mouse. Total number of **B)** $CD3^+CD34^+$, **C)** $CD3^+CD34^+CD8^+$, or **D)** $CD3^+CD34^+CD4^+$ HCV Ag-specific T cells found in the spleens of sham females, OVX females, and sham males that were treated with ACT immunotherapy either 20 or 40 days after tumor challenge. ACT day 20: n=6 sham females, n=7 OVX females, n=10 sham males. ACT day 40: n=5 sham females, n=5 OVX females, n=6 sham males. Each mouse plotted and the standard error of the mean indicated. Data were analyzed by 1way-ANOVA and Tukey's post-hoc. $p<0.0001$=****, $p<0.001$=***, $p<0.01$**, $p<0.05$=*.

If $CD4^+$ T cells in OVX mice show increased TUNEL staining compared to sham females, then estrogen inhibits $CD4^+$ T cell apoptosis. This could happen through enhancing of expression of anti-apoptotic genes such as Bcl-2. Lack of estrogen during ACT, removes the protective effect of the hormone in enhancing expression of anti-apoptotic genes. If similar TUNEL staining is found in $CD4^+$ T cells from sham and OVX mice, then it would be concluded that removal of endogenous estrogen would have no effect on the apoptosis of $CD4^+$ T cells. This could be due to $CD4^+$ T cells dying through other mechanisms such as necrosis, or could simply be that estrogen does not modulate $CD4^+$ T cell survival. In this case, enhanced proliferation would be the reason for increased $CD4^+$ T cell numbers observed in the presence of estrogen which should be confirmed in conjunction with proliferation staining using Ki67. If $CD4^+$ T cells in female sham mice show increased Ki67 staining compared to OVX mice, then it can be resolved that estrogen enhances the proliferation of $CD4^+$ T cells. T cell proliferation is induced through Ag recognition and TCR activation and downstream signaling thus indicating the estrogen could be regulating TCR downstream signaling. Based on data presented in previous chapters, estrogen enhanced $CD4^+$ T cell cytokine production and polyfunctionality which are downstream of the TCR indicating that estrogen enhancing proliferation through TCR-mediated signaling is the most likely scenario. In addition, estrogen could be enhancing signaling of pathways such as MAP kinase which enhance T cell proliferation independently from the TCR. On the contrary, if Ki67 staining is equally found in $CD4^+$ T cells from both sham and OVX mice, then it would have to be considered that estrogen may not modulate $CD4^+$ T cell proliferation. In this case reduced number of $CD4^+$ T cells found in OVX mice could be due to reduced survival which would rule out an effect of estrogen on proliferation/TCR signaling enhancement. However, it should also remain a consideration that Ki67 staining is a single snap shot of time in the overall

process and may need to be repeated at multiple time points after adoptive transfer in order to truly determine whether proliferation occurs in the TME. If enhanced proliferation occurs in the lymph nodes but T cells do not survive infiltration to the TME, the result of the stain may not be able to be observed through the current measurement. If $CD4^+$ T cells are positive for both Ki67 and TUNEL staining in the absence of estrogen, then this could be due to cells proliferating but not surviving due to apoptosis-induced proliferation. This could indicate T cell exhaustion due to constant Ag stimulation, and estrogen being protective against T cell exhaustion which would be beneficial for immunotherapy.

Estrogen Enhances Tumor Infiltrating Lymphocyte Activation during Immunotherapy

Next, estrogen's ability to modulate T cell activation of infiltrating adoptively transferred T cells was tested. In order to measure T cell activation, Ag-specific T cells were stained for cell surface expression of CD69 and CD25. CD69 and CD25 are activation markers which expression is upregulated after antigen stimulation and TCR signaling activation [458]. A significantly greater percentage of Ag-specific T cells expressing CD25 was found infiltrating the livers of sham females compared to the estrogen depleted, OVX females at both time points of ACT (Figure 34 B). Similarly, significantly greater frequency of Ag-specific T cells infiltrating sham female tumors expressed CD69 than the ones infiltrating the tumors of OVX female at both time points of ACT (Figure 34 C). Significantly greater percentage of TILs from sham females expressed CD69 compared to TILs found in sham males (Figure 34 C). These data demonstrate removing endogenous estrogen diminishes the activation state of TILs.

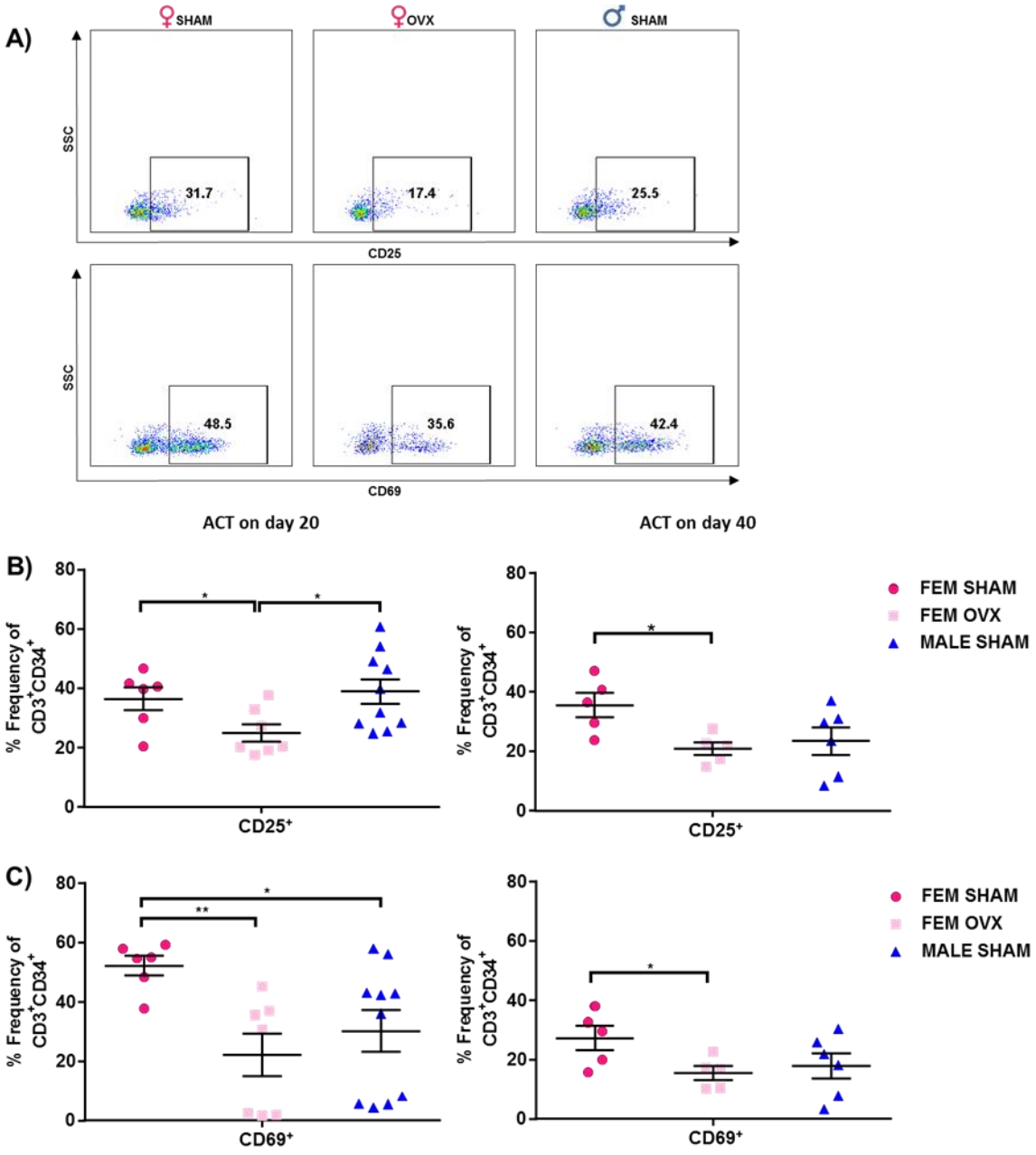

Figure 34. Removal of Endogenous Estrogen Diminished Ag-specific T Cell Activation State. Human Ag-specific T cells infiltrating the livers of sham females, OVX females, and sham males were isolated and stained for CD25 and CD69 extracellular markers. **A)** Representative flow cytometry dot plots of $CD3^{+}CD34^{+}$ TILs expressing CD25 or CD69 from one sham female, one OVX female, and one shame male mouse. **B)** Expression of CD25 in tumor infiltrating Ag-specific T cells from sham/OVX females and sham males that received ACT immunotherapy 20 or 40 days after tumor challenge. **C)** CD69 expression CD25 in tumor infiltrating Ag-specific T cells from sham/OVX females and sham males that received ACT immunotherapy 20 or 40 days after tumor challenge. ACT day 20: n=6 sham females, n=7 OVX females. AdTx day 20: n=6 sham females, n=7 OVX females, n=10 sham males. ACT day 40: n=5 sham females, n=5 OVX females, n=6 sham males. Each mouse plotted and the standard error of the mean indicated. Data were analyzed by 1way-ANOVA and Tukey's post-hoc. $p<0.01$=**, $p<0.05$=*.

Endogenous Estrogen Enhances TIL Cytotoxic and Helper Cytokine Production during Immunotherapy

Ag-specific T cells found infiltrating the livers of HCC challenged sham/OVX females and sham males expressed diverse levels of CD25 and CD69 indicating differential T cell activation state and TCR signaling in response to antigen. Differences in T cell activation and TCR signaling can result in altered T cell cytokine expression and T cell polyfunctionality in response to antigen. It was previously demonstrated that estrogen signaling enhances the secretion of certain cytokines including Granzyme B, IFNγ, TNFα, and IL-4, upon T cell activation. In order to investigate if estrogen presence during immunotherapy affects TIL cytokine production, equal numbers of human Ag-specific TILs from sham/OVX females and sham males were co-cultured *in vitro* with target T2 cells pulsed with HCV cognate Ag or tyrosinase irrelevant peptide. Activated TILs were then stained extracellularly for CD107a (a lytic marker indicative of Granzyme B degranulation), and intracellularly for IFNγ, TNFα, IL-4. As predicted, minimal numbers of Ag-specific TILs expressed any of these cytokines when co-cultured with T2 cells pulsed with tyrosinase (Figure 35 A). No significant differences in the percentage of HCV activated $CD8^+$ Ag-specific TILs expressing IFNγ, TNFα, D107a were found between sham females, OVX females, and sham males (Figure 35 B). Significantly greater numbers of female sham $CD8^+$ Ag-specific TILs expressed IL-4 in comparison to OVX female TILs (Figure 35 B). This indicates that endogenous estrogen enhances the expression of IL-4 in $CD8^+$ Ag-specific TILs upon antigen stimulation. Significantly higher numbers of $CD4^+$ TILs from sham females expressed IFNγ, TNFα, IL-4, and CD107a compared to OVX female TILs (Figure 35 C).

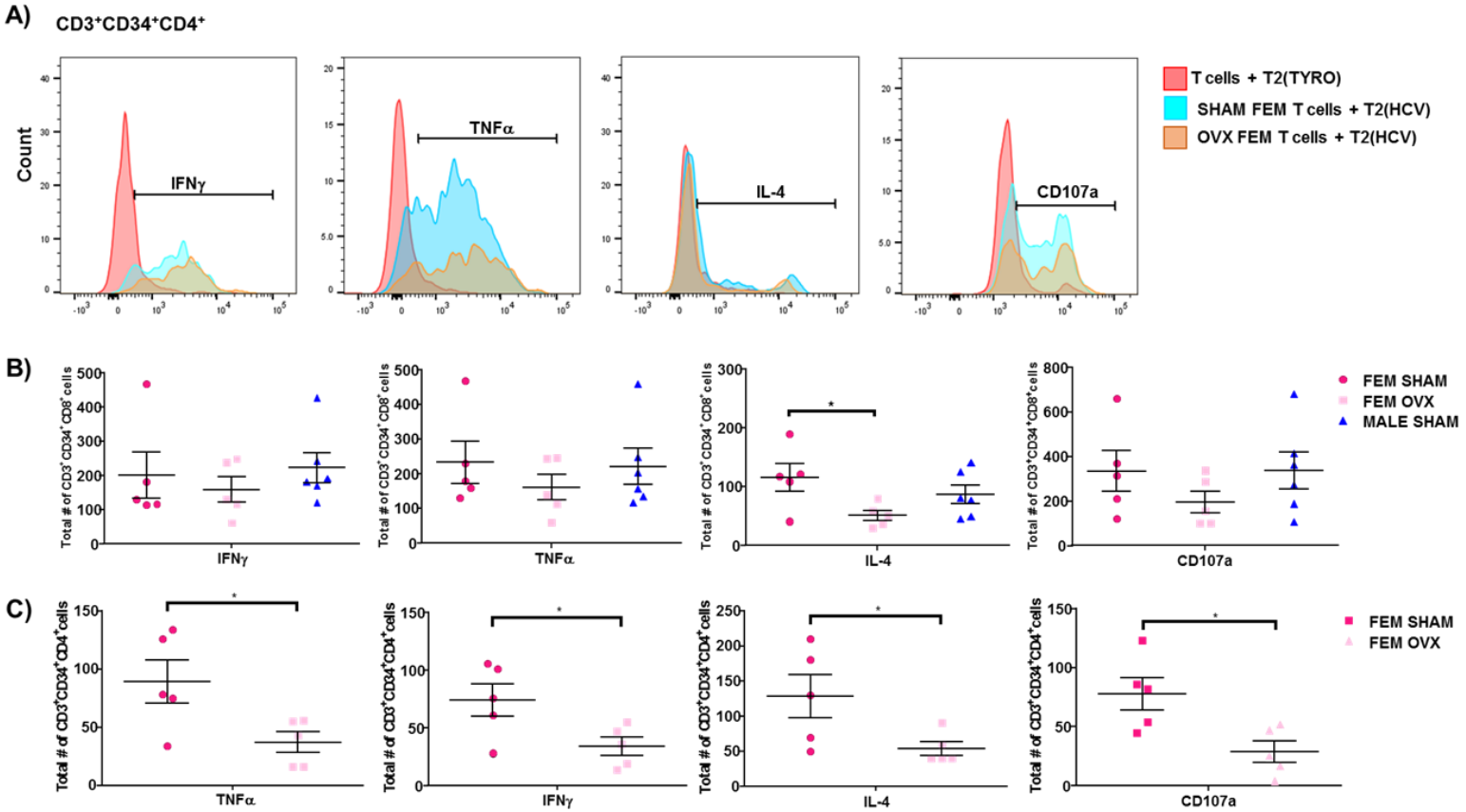

Figure 35. Removal of Endogenous Estrogen Enhances Expression of Th1 and Th2 Cytokines in $CD4^+$ TILs. Isolated TILs from livers of sham/OVX females and sham males were co-cultured with T2 cells expressing the HCV antigen for 5 hours in the presence of protein-transport inhibitors, cytokine expression was then measured by flow cytometry staining. **A)** Representative histograms of $CD3^+CD34^+CD4^+$ Ag-specific T cells from one sham and one OVX female expressing each of the cytokines after stimulation with HCV cognate antigen or tyrosinase irrelevant peptide. **B)** Total number of $CD3^+CD34^+CD8^+$ human Ag-specific TILs from sham/OVX females and sham males expressing IFNγ, TNFα, IL-4 or CD107a that were transferred 40 days after tumor challenge. **C)** Total number of $CD3^+CD34^+CD4^+$ human Ag-specific TILs from sham and OVX females expressing IFNγ, TNFα, IL-4 or Cd107a that were transferred either 40 days after tumor challenge. ACT day 40: n=5 sham females, n=5 OVX females, n=6 sham males. Each mouse plotted and the standard error of the mean indicated. Data were analyzed by 1way-ANOVA and Tukey's post-hoc. $p<0.05$=*.

This demonstrates that removing endogenous estrogen significantly reduces the expression of cytotoxic and helper cytokines in $CD4^+$ Ag-specific TILs activated with their cognate Ag.

Removal of Endogenous Estrogen Decreases $CD4^+$ TIL Polyfunctionality during Immunotherapy

To assess if polyfunctionality of adoptively transferred T cells is enhanced and/or regulated by the presence and availability of endogenous estrogen, TILs from sham and OVX females and sham males were activated using T2 cells as described before. Activated T cells were then stained using fluorescent antibodies against six different intracellular cytokines (TNFα, IFNγ, IL-2, IL-4, IL-17a, and IL-22), and the cell surface lytic marker CD107a as described in Chapter V. All the different 128 combinations of these markers that were expressed simultaneously by activated TILs were calculated using tandem analysis with FlowJoX Boolean gates, Pestle, and SPICE. Then, the percent frequencies of TILs expressing each marker combination were added into 7 categories depending on the number of markers expressed simultaneously, from one through seven markers. T cells were considered polyfunctional if they expressed more than two markers simultaneously. The frequencies of cells expressing 6 and 7 markers simultaneously were under 1% so they were not depicted on the graphs in Figure 36. Significantly higher frequency of $CD8^+$ female TILs, regardless of the estrogen level, expressed 3 or 4 markers simultaneously compared to males (Figure 36 A). A significantly higher frequency of male $CD8^+$ TILs expressed two markers simultaneously compared to sham and OVX female TILs (Figure 36 A). These data demonstrate that adoptively transferred $CD8^+$ female T cells are inherently more polyfunctional than male T cells regardless of physiological estrogen presence. A significantly higher frequency of $CD4^+$ TILs from sham females expressed

three markers simultaneously compared to OVX female TILs (Figure 36 B), demonstrating that removal of endogenous estrogen reduces $CD4^+$ T cell polyfunctionality.

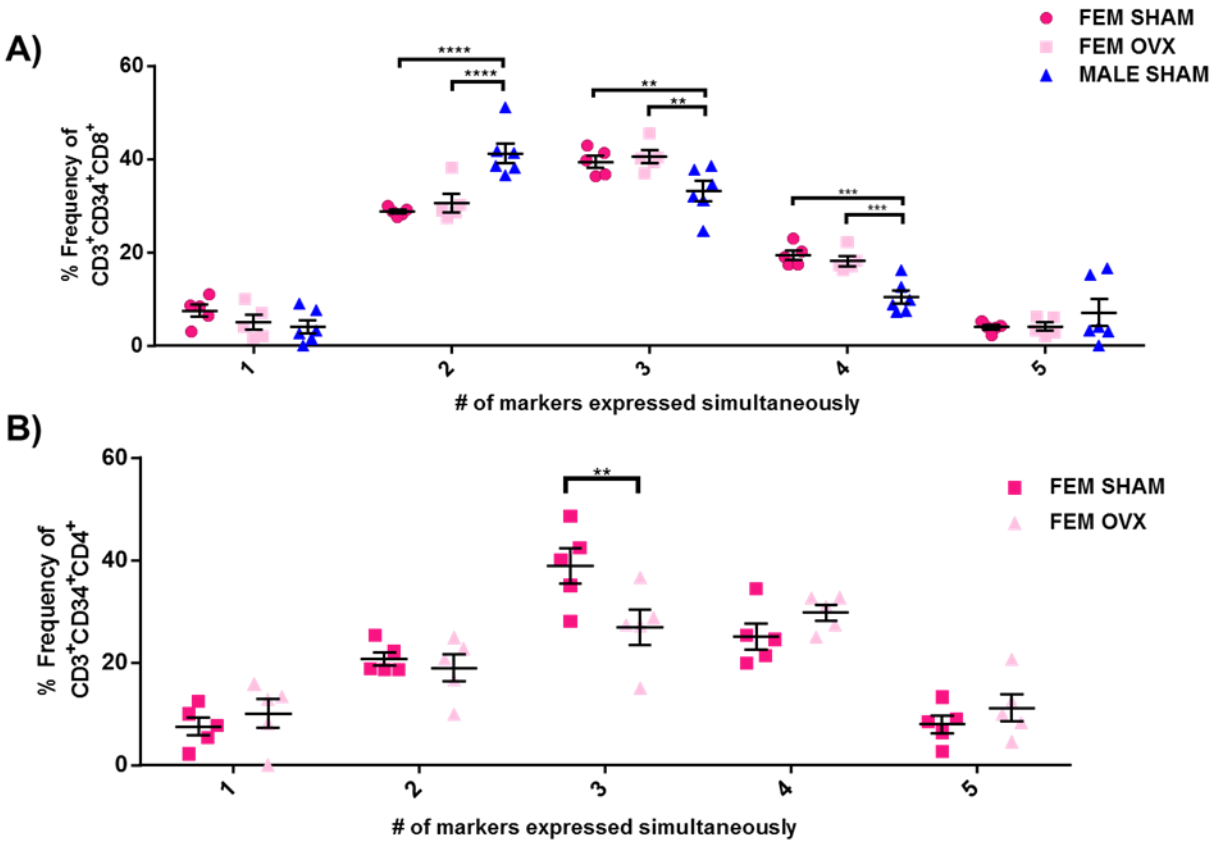

Figure 36. Female $CD8^+$ Ag-specific TILs are More Polyfunctional than Male $CD8^+$ TILs Independently of Estrogen, but Removal of Endogenous Estrogen Diminishes Female $CD4^+$ TIL Polyfunctionality. Human $CD8^+$ and $CD4^+$ Ag-specific T cells were isolated from HCC livers of sham females, OVX females, and sham male mice and co-cultured with T2 target cells at a 1:1 ratio for 5 hours in the presence of protein transport inhibitors. Then, cells were intracellularly stained for cytokines TNFα, IFNγ, IL-2, IL-4, IL-17a, and IL-22 and the lytic marker CD107a. Using FlowJoX Boolean combinatorial gates the % frequency of T cells expressing every combination of these 7 markers simultaneously was generated and added into 7 categories. **A)** The percent frequency of $CD3^+CD34^+CD8^+$ TILs that expressed one through five markers simultaneously when activated with their cognate antigen. **B)** The percent frequency of $CD3^+CD34^+CD4^+$ TILs that expressed one through five markers simultaneously when activated with their cognate antigen. ACT day 40: n=5 sham females, n=5 OVX females, n=6 sham males. Each mouse plotted and the standard error of the mean indicated. Data were analyzed by MANOVA and Tukey's post-hoc. $p<0.0001$=****, $p<0.001$=***, $p<0.01$=**, $p<0.05$=*.

Analysis of all the possible marker combinations simultaneously expressed showed that significantly higher frequencies of sham female $CD4^+$ T cells expressed combinations including $CD107a^+IFN\gamma^+TNF\alpha^+$, $IFN\gamma^+IL\text{-}2^+TNF\alpha^+$ and $IFN\gamma^+IL\text{-}17^+TNF\alpha^+$ than OVX female T cells (Figure 37). On the other hand, even if significance was not achieved, higher frequencies of OVX female $CD4^+$ TILs expressed combinations including IFNγ alone, TNFα alone, or $IFN\gamma^+TNF\alpha^+$ (Figure 37). Overall, these data demonstrate that endogenous estrogen enhances the expression of polyfunctional phenotypes of three markers in $CD4^+$ TILs used for immunotherapy. In addition, removal of endogenous estrogen enhances the expression of mono- and bifunctional phenotypes in $CD4^+$ TILs found after ACT immunotherapy.

Overall, the data presented in this chapter demonstrate that estrogen removal during ACT immunotherapy for HCC reduces Ag-specific T cell survival and tumor infiltration, impairs T cell activation and decreases $CD4^+$ T cell cytokine production and polyfunctionality. These results were obtained by comparing sham female to OVX female mice. Surgically removing the ovaries not only eliminates the main source of estrogen on the body but was also shown to decrease progesterone and increase testosterone serum levels [459]. In order to conclude that estrogen mediates T cell function during ACT immunotherapy, the effects of progesterone and testosterone on T cell function need to be ruled out. In order to test this, a second set of experiments in which estrogen is added back to OVX females during ACT immunotherapy needs to be performed. Estrogen can be given back orally or subcutaneously via estradiol capsules to obtain physiological estrogen concentrations in the serum of OVX females [460]. If adding back estrogen to OVX mice via estradiol capsules enhances Ag-specific T cell tumor infiltration, activation state, cytokine production and polyfunctionality to levels similar to those found in

sham females, then estrogen signaling alone mediates all these aspects of T cell function. This would indicate that estrogen can directly enhance the efficacy of ACT immunotherapy. Based on the results obtained in the experiments testing the effects of estrogen signaling *in vitro* that indicated estrogen enhances expression of T cell cytokines and polyfunctionality in response to Ag-stimulation this is the most likely scenario. If rescuing estrogen in OVX mice does not enhance Ag-specific T cell function to levels similar to those seen in sham female mice, then estrogen alone does not mediate T cell function. This could indicate that the reduction in progesterone or the increase in testosterone serum concentrations induced by ovariectomy are detrimental for T cell tumor infiltration, activation, cytokine production, and polyfunctionality. Progesterone was previously shown to exert immunosuppressive effects on human T cells during pregnancy by inhibiting IFNγ expression [461], promoting naïve T cell differentiation into T_{regs} [462], and by inhibiting Th1 differentiation [463] so it is not expected that the removal of ovaries and subsequent decrease in serum progesterone impairs T cell function. Testosterone was also shown to have immunosuppressive effects on T cells with humans with high testosterone levels exhibiting lower activation-induced pro-inflammatory cytokine production [464], decreased Th1 differentiation [465], and increased IL-10 secretion by $CD4^+$ T cells [466]. This indicates that the increase of serum testosterone after ovariectomy could mediate the reduced T cell proliferation, survival, cytokine production and polyfunctionality observed in OVX mice. Antagonistic effect of testosterone and estrogen on T cell function is also a possibility that could be tested by giving sham mice testosterone capsules with levels similar to those observed in males or OVX females, or alternatively treating OVX mice with a testosterone blocker and comparing T cell function during immunotherapy with sham females.

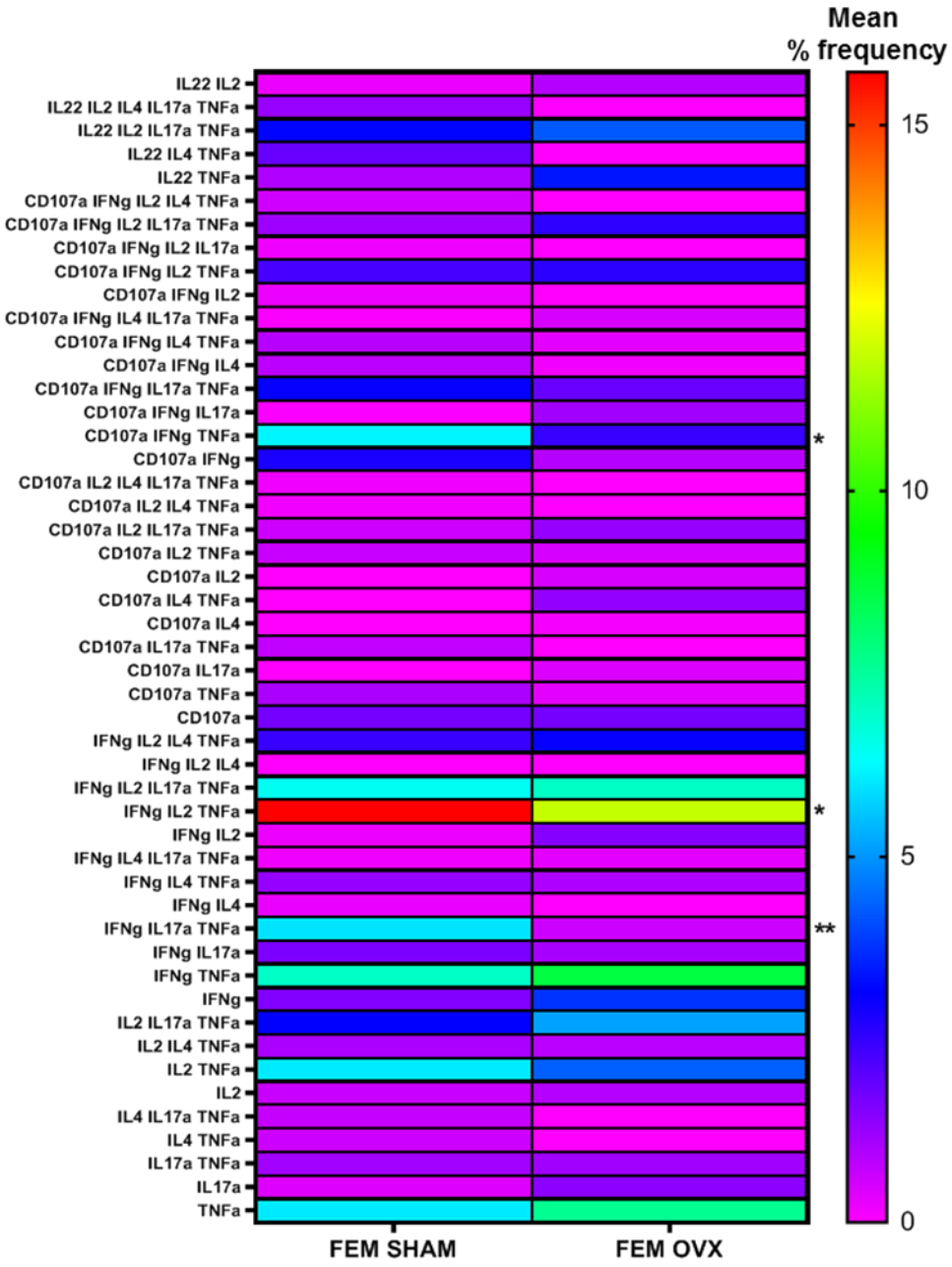

Figure 37. Removal of Endogenous Estrogen Diminishes the Frequency of $CD4^+$ Ag-specific TILs Expressing $CD107a^+IFN\gamma^+TNF\alpha^+$, $IFN\gamma^+IL\text{-}2^+TNF\alpha^+$ and $IFN\gamma^+IL\text{-}17a^+TNF\alpha^+$. Human $CD8^+$ and $CD4^+$ Ag-specific T cells were isolated from HCC livers of sham and OVX females and co-cultured with T2 target cells at a 1:1 ratio for 5 hours in the presence of protein transport inhibitors. Then, cells were intracellularly stained for cytokines TNFα, IFNγ, IL-2, IL-4, IL-17a, and IL-22 and the lytic marker CD107a. Using FlowJoX Boolean gating and the software SPICE all the possible combinations of these 7 markers expressed simultaneously by T cells were generated. The heat map shows the mean % frequency of $CD3^+CD34^+CD4^+$ Ag-specific TILs obtained from 5 mice that expressed each of the marker combinations depicted on the left. ACT day 40: n=5 sham females, n=5 OVX females. Data were analyzed by MANOVA and Tukey's post-hoc. $p<0.01$=**, $p<0.05$=*.

Chapter Discussion

This chapter of the dissertation was aimed to characterize the effects of sex and estrogen on the T cell anti-tumor immune response generated during ACT immunotherapy. A forced oncogene expression HCC model in combination with a HCV NS3 antigen expression vector was used to generate HCV-expressing liver tumors in mice. Using this novel model, it was demonstrated that estrogen enhances the anti-tumor function of HCV Ag-specific T cells by increasing T cell tumor infiltration, survival, cytokine expression, and polyfunctionality. While some of these effects were observed in both $CD8^+$ and $CD4^+$ T cell populations when advanced stage tumors were present, enhanced T cell anti-tumor function was specially observed in the $CD4^+$ T cell subset. Removal of estrogen during ACT immunotherapy decreased $CD4^+$ Ag-specific T cells infiltration and survival, and decreased overall $CD4^+$ T cell functions including cytokine production and polyfunctionality. These results indicate that estrogen signaling during ACT immunotherapy could improve the anti-tumor immune response of $CD4^+$ male and female Ag-specific T cells.

Using this HCV^+HCC tumor model, it was first demonstrated that estrogen has a protective role against HCC where sham female mice, which have circulating physiological estrogen levels, showed reduced tumor burden compared to ovariectomized female and males, which had minimal circulating estrogen. While these results do support the findings of previous studies that estrogen has a protective effect on hepatocytes directly, more importantly, it was found that ACT immunotherapy using HCV Ag-specific T cells reduced HCC tumor burden. Furthermore, the differences were maintained based on the presence of physiological estrogen, in sham females, or very low estrogen, in OVX females and males. Thus suggesting that ACT

immunotherapy for HCC is enhanced by estrogen, and estrogen signaling should be considered in the development and optimization of T cell based immunotherapies. ACT immunotherapy using Ag-specific T cells was tested in mice 20 days after tumor challenge which developed early stage tumors when not treated, and no visible tumors when treated with ACT. On the other hand, mice that were treated with ACT immunotherapy 40 days after tumor challenge developed very advanced HCC tumors. The effect of ACT immunotherapy on tumor burden was partial with some of the ACT treated mice still presenting very advanced tumors. This could be due to the highly the fibrotic and cirrhotic HCC TME which was previously shown to inhibit T cell tumor infiltration and subsequent T cell anti-tumor responses [467]. Another possibility being $CD8^+$ T cells getting exhausted due to continuous antigen stimulation by HCV expression in these large tumors.

Tumor burden in ovariectomized females was far greater than in control females and surprisingly also greater than in males. This indicates that female adoptively transferred T cells for immunotherapy require physiological estrogen stimulation to generate a superior anti-tumor immune response. It was previously reported that HCC incidence and mortality significantly increase in post-menopausal women compared to pre-menopausal women and post-menopausal estrogen users. The results obtained in this study indicate that the increase in cancer incidence after menopause may be partially mediated by the dampening of T cell anti-tumor immune responses. Therefore, when designing of immunotherapies for post-menopausal women, their lack of physiological estrogen, and consequent reduced T cell anti-tumor responses need to be taken into account.

Part of the mechanism of enhanced therapeutic outcome can be demonstrated by the finding that estrogen enhanced T cell survival and T cell infiltration of HCC tumors. Specifically, estrogen enhanced the persistence of $CD4^+$ T cells which were found in greater number in the spleen and infiltrating the tumors of sham females which have physiological estrogen compared to OVX females and males. It was previously reported that $CD4^+$ helper T cells are critical for maintenance of CTL responses during cancer and infection [78-80]. $CD4^+$ T cells are not only necessary for supporting $CD8^+$ T cell survival but it has been shown that $CD4^+$ T cell subsets Th1 Th2 and th17 are required for successful anti-tumor immune responses [468-470]. It was found that the majority of the $CD4^+$ T cells that persisted in females were from the Th2 and Th17 subsets ruling out immunosuppression by T_{regs}. The effect of estrogen signaling on $CD8^+$ T cells was found to be controversial since it mainly affected their survival and infiltration when advanced tumors were present. This could indicate that estrogen signaling is especially important for $CD8^+$ T cells survival and function in environments where strong immune tolerance and T cell exhaustion is being mediated by the tumor. Also, HCC tumors where shown to produce high local estrogen concentrations indicating that super-physiological estrogen could affect $CD8^+$ T cells greater than physiological estrogen.

In addition to infiltration, significantly higher frequencies of TILs from estrogen competent females expressed activation markers CD25 and CD69 than those of estrogen depleted females or males. Expression of CD69 and CD25 is upregulated upon T cell activation via TCR signaling, and is maintained during antigenic stimulation [471]. Estrogen signaling through ERα and ERβ was shown to enhance some important signaling molecules downstream from the TCR like NFκb, AP-1, and the NFAT pathways [211, 472, 473]. Up-regulation of these

activation markers indicate that estrogen enhances TCR signaling in response to Ag stimulation and subsequently increases the T cell activation state. Estrogen regulation of TCR signaling components and T cell activation was previously shown in a colitis model for autoimmunity [233], indicating that these findings not only apply to T cell function in cancer but also during autoimmunity.

Functionally, expression of cytotoxic cytokines including IFNγ and TNFα and the lytic marker CD107a was increased in antigen stimulated $CD4^+$ TILs from sham females compared to OVX females. Expression of the Th2 cytokine IL-4 was also increased in antigen stimulated $CD4^+$ and $CD8^+$ TILs from sham females compared to OVX females. While IFNγ, TNFα are cytokines expressed by cytotoxic and Th1 effector T cells, IL-4 is expressed mainly by T2 helper T cells. Estrogen can enhance the expression of these cytokines which are in the two ends of the inflammatory spectrum indicating that estrogen may be regulating the balance between cytotoxic/effector and Th2 helper T cell responses agreeing with the results obtained in the previous chapters in which estrogen signaling effect on Ag-specific function was investigated *in vitro*.

In combination with the results from previous chapters indicating that estrogen enhances $CD4^+$ and $CD8^+$ cytokine production directly as well as increases T cell polyfunctionality, results of this chapter demonstrate similar effects directly on T cells *in vivo*. Specifically, estrogen enhanced the polyfunctionality of $CD4^+$ TILs. The frequency of $CD4^+$ Ag-specific T cells expressing three markers simultaneously in a polyfunctional manner was significantly higher in estrogen intact females compared to OVX, estrogen depleted females. Three marker combinations that were greatly expressed in $CD4^+$ TILs in the presence of physiological estrogen

included $CD107a^{+}IFN\gamma^{+}TNF\alpha^{+}$, $IFN\gamma^{+}IL\text{-}17a^{+}TNF\alpha^{+}$, and $IFN\gamma^{+}IL\text{-}2^{+}TNF\alpha^{+}$. These combinations include markers that are required for superior T cell anti-tumor immune responses, indicating that estrogen enhances anti-tumor immunity during ACT immunotherapy.

Overall, the studies in this chapter demonstrate that physiological estrogen removal during ACT immunotherapy for HCC has a detrimental effect on T cell survival and anti-tumor function. These effects are especially observed on the $CD4^{+}$ T cell subset indicating that estrogen signaling enhances the survival, tumor infiltration and polyfunctionality of $CD4^{+}$ T cells. $CD4^{+}$ T cells were previously shown to be critical for generating superior T cell anti-tumor responses, and for enhancing the efficacy of ACT immunotherapy by supporting CTL survival, function and by destroying tumor cells directly. The results presented demonstrate a previously unappreciated role of estrogen signaling on $CD4^{+}$ T cell function that is an avenue to enhancing infiltration, effectiveness, and survival of adoptive transferred T cells for immunotherapy.

CHAPTER VII

OVERALL DISCUSSION AND FUTURE DIRECTIONS

Discussion

Adoptive cell transfer immunotherapy using TCR gene-modified or Ag-specific T cells is rapidly evolving field. Several pre-clinical and clinical studies have had various levels of efficacy using TCR transduced T cells to treat different solid tumors and hematopoietic malignancies. Although evidence suggests the use of genetically modified Ag-specific T cells can be effective, several challenges remain in order to improve these therapeutics. While many studies have focused on optimizing Ag-specific T cells to overcome the immunosuppressive TME and to enhance tumor infiltration and Ag recognition, other host factors that can affect Ag-specific T cells during ACT immunotherapy remain understudied.

Evidence of this lack in research includes characterizing and an in depth understanding of the effects of sex and sex hormone receptor signaling on Ag-specific T cell function. Sex influences multiple aspects of adaptive immunity in humans. T cell populations and T cell subset phenotypes vary significantly depending on sex. Females have greater $CD4^+$ T cell counts and higher $CD4^+$:$CD8^+$ ratios whereas age-matched males have higher $CD8^+$ T cell frequencies [181-184]. Females have higher numbers of activated and proliferating T cells in peripheral blood compared to males, and transcriptional analyses demonstrates that activated female T cells upregulate more antiviral and pro-inflammatory genes compared to T cells from males [185].

While some of these sex-differences in T cell phenotype are genetically and environmentally mediated, a lot of these differences heighten or decrease with puberty and reproductive senescence indicating a sex hormone receptor involvement. Estrogen is considered the female sex hormone and it is present at physiological concentration (0.5 nM) in females and at low concentrations in males and post-menopausal females (<0.1 nM). Estrogen signaling was shown to affect the differentiation, maturation, and function of T cells as described in the introduction of this dissertation. For example, estrogen signaling was shown to enhance T cell differentiation [203, 474], to increase the secretion of cytokines such as IL-10 and IFNγ [194], and to induce the expression of the T_{reg} transcription factor FoxP3 [196]. Throughout available scientific literature, most of the important aspects concerning the role of estrogen on T cells were investigated in autoimmunity models and at hormone concentrations limited to physiological and pregnancy estrogen levels. Other ranges of estrogen dosages were not investigated. Knowing that sex and estrogen have direct effects on T cell differentiation and function, it is important to carefully characterize their effects on Ag-specific T cells for immunotherapy in order to identify possible mechanisms that can be targeted to enhance T cell anti-tumor immune responses and immunotherapy efficacy.

The effects of sex and estrogen signaling on T cells and other immune cells result in differences on pathogenesis of diseases that arise from immune cell function dysregulation, like autoimmunity, or diseases that are heavily controlled by immune responses, like infections and cancer. One malignancy that shows a pronounced sex-bias and is heavily affected by estrogen is HCC. HCC is significantly more prevalent in males compared to pre-menopausal females [360, 361]. Post-menopausal females that are not undergoing estrogen HRT are also more prone to develop HCC than pre-menopausal females and estrogen users [311]. The protective role of

estrogen against HCC was shown to be partially mediated by estrogen receptor signaling inhibition of IL-6-mediated chronic inflammation and tumor associated macrophage polarization [293, 318]. While this research beautifully demonstrated the anti-HCC role of estrogen, the impact of estrogen signaling on other HCC tumor infiltrating immune like T cells remains unclear and needs further investigation. ACT immunotherapy using genetically modified T cells showed some success on treating HCC in pre-clinical models and clinical trials but the efficacy of ACT fighting HCC remains unsatisfactory. These data indicate that there is a high need for ways to improve the anti-tumor function of Ag-specific T cells in order to generate more successful ACT immunotherapy for HCC. The work performed in this dissertation was aimed to characterize the role of estrogen signaling on T cell function *in vitro*, and during ACT immunotherapy against HCC *in vivo*. In the following sections, the results obtained and their implications for T cell biology and tumor immunology are discussed.

First, the effect of estrogen signaling was studied on the function of Ag-specific T cells *in vitro*. It was observed that estrogen stimulation, especially at super-physiological concentrations, was able to modulate Ag-specific T cell cytokine expression and secretion in response to cognate Ag stimulation. Estrogen signaling through ERα and not ERβ enhanced the expression and/or secretion of TNFα, IFNγ, and Granzyme B in Ag-specific T cells from males and females in a dose dependent manner in response to Ag stimulation. IFNγ, TNFα, and Granzyme B are cytokines produced by cytotoxic $CD8^+$ T cells and Th1 $CD4^+$ T cells and can directly destroy tumor cells. IFNγ is cytotoxic to certain malignant cells, and it enhances MHC class I expression [406]. Granzyme B directly lyses malignant cells, and TNFα promotes T cell activation, co-stimulation, and promotes certain cancer cell death [406]. Estrogen signaling through ERα and

not ERβ completely controlled the expression of IL-4 in response to Ag stimulation and, in addition, it was shown to enhance IL-4 expression and secretion in Ag-specific T cells from males and females in a dose dependent manner. IL-4 promotes T cell and B cell survival, induces Ig class switch to IgE and IgG in B cells, drives long-term development of $CD8^+$ T cell memory, and in combination with TGFβ it drives Th9 T cell subset differentiation which are T cells that augment anti-tumor responses in ACT models [387-389, 407, 408]. Overall these data demonstrate for the first time that estrogen signaling through ERα enhances the expression and secretion of cytokines that promote stronger $CD4^+$ and $CD8^+$ T cell anti-tumor responses hence estrogen signaling through ERα can enhance the efficacy of ACT immunotherapy. Most importantly, the largest effects of ERα signaling enhancing cytokine expression were observed at super-physiological estrogen concentrations which mimic the elevated estrogen concentrations in the HCC TME. This indicates that infiltrating T cells from male and females will could be generating stronger anti-tumor responses in response to estrogen stimulation in the TME.

EREs were found in the promoters of TNFα and IFNγ indicating that ERα ligand-bound ERE-independent signaling could be enhancing transcription of these genes in response to estrogen stimulation. In addition, an ERE on the promoter of T-BET, a transcription factor that induces IFNγ expression, was found indicating that ERα signaling could be also upregulating transcription of T-BET. Apart from inducing IFNγ expression, T-BET is well known to promote Th1 differentiation while suppressing Th2 differentiation. No ERE was found on the promoter of IL-4 which was surprising due to the strong effects of ERα signaling on IL-4 expression observed in T cells in response to Ag stimulation. Considering IL-4 related genes, an ERE was found on the promoter of GATA3, a transcription factor that induces IL-4 expression. This

indicates that ERα could enhance IL-4 expression by enhancing GATA3 transcription through a ligand-bound ERE-dependent signaling. GATA3 is well known to promote Th2 differentiation. Based on these results, estrogen signaling through ERα enhances expression of prototypic Th1 and Th2 cytokines indicating that estrogen signaling does not favor differentiation towards one subset over the other, but it most likely plays a balancing role between Th1 and Th2 T cell responses. Estrogen signaling not only enhances expression of cytokines that generate stronger anti-tumor immune responses and facilitate T cell survival, but it balances cytotoxic type I and survival type II T cell responses. Type I and type II T cell responses were both shown to enhance the anti-tumor immune response during ACT immunotherapy, especially when both $CD4^+$ helper Th1 and Th2 cells and cytotoxic $CD8^+$ T cells were transferred as a mixture into patients indicating a synergistic effect between them [82, 475]. Studies in the past have reported contradicting evidence on the effect of estrogen on T cell cytokine production. While some groups demonstrated an inhibitory effect of estrogen on the expression of TNFα and IL-4, other groups simultaneously reported enhancing effects of estrogen signaling on the expression of these cytokines. The effects observed were concentration dependent, and some studies reported that pregnancy estrogen levels (higher than physiological) induce IL-4 expression and inhibit TNFα expression, while physiological estrogen levels induce TNFα and IFNγ expression. Based on this evidence, it was believed that physiological estrogen signaling promotes type I T cell responses and Th1 $CD4^+$ T cell function while pregnancy estrogen promotes type II T cell responses and Th2 $CD4^+$ T cell function. The results reported in this dissertation help clarify some of the contradicting evidence in the field and demonstrate for the first time that in the case of human Ag-specific T cells from both sexes, the effect of estrogen is concentration and

receptor independent and that at both physiological and super-physiological concentrations induce Th1 and Th2 T cells responses in a dose dependent manner (Figure 38).

An attribute of genetically modified T cells that was previously correlated with enhanced clinical outcome is their ability to express multiple functional markers simultaneously in response to Ag stimulation, or their polyfunctionality. One clinical study in which patients with aggressive refractory non-Hodgkin's lymphoma were treated with CD19 CAR-T cells demonstrated that highly polyfunctional T cells mediated stronger anti-tumor immune responses and tumor rejection compared to those with lower polyfunctionality. Polyfunctionality of CAR-T cells was determined immediately before CAR-T cell infusion by measuring the expression of 32 different effector, cytotoxic, helper, and chemotactic cytokines upon Ag stimulation. Cells that expressed >2 of these markers simultaneously were considered polyfunctional. Polyfunctional $CD8^+$ and $CD4^+$ CAR-T cells expressed combinations of markers including but not limited to IFNγ, Granzyme B, IL-8, IL-17a, and IL-2 [428]. While T cell populations only showed approximately 20% of polyfunctional cells, they correlated with clinical outcome significantly more than those patients that received monofunctional CAR-T cells [428]. Polyfunctionality of Ag-specific T cells also correlated with better disease outcome when patients were treated with immunotherapy in combination with other cancer therapies such as chemotherapy. B cell lymphoma challenged mice were treated with chemotherapy and then given ACT immunotherapy with polyfunctional or monofunctional $CD4^+$ Ag-specific T cells. Mice showed reduced tumor burden when they were given adoptive transfer of polyfunctional Ag-specific $CD4^+$ T cells compared to monofunctional $CD4^+$ T cells and compared to mice treated with chemotherapy alone [417]. T cell polyfunctionality was also shown to correlate to clinical

prognosis and response to immunotherapeutic interventions in solid tumors including melanoma and breast cancer. Advanced melanoma patients that were treated with Ipilumab (anti-CTLA-4 Ab) showed better disease prognosis when polyfunctional tumor Ag-specific $CD4^+$ and $CD8^+$ T cells were present infiltrating the tumor compared those patients with infiltrating monofunctional T cells. $CD4^+$ and $CD8^+$ polyfunctional cells expressed combinations of markers including but not limited to IFNγ, TNFα, IL-2, and Granzyme B [426]. In addition, polyfunctional but not monofunctional tumor-reactive $CD8^+$ T cells accumulated in the peripheral lymphocyte pool of those patients with advanced melanoma that were treated with autologous TIL immunotherapy and showed significant tumor regression compared to non-responders [476]. These polyfunctional $CD8^+$ T cells persisted up to 1 year after infusion and even if they expressed PD-1, they proved to be cytotoxic when re-stimulated with tumor Ag [476]. Polyfunctional $CD8^+$ Ag-specific T cells were found infiltrating the tumors of breast cancer patients, and when isolated, polyfunctional $CD8^+$ TILs were shown to retain cytotoxic ability against tumor target cells despite high PD-1 expression compared to monofunctional $CD8^+$ TILs [477]. Overall, these studies demonstrated that $CD8^+$ and $CD4^+$ Ag-specific T cell polyfunctionality not only correlates with stronger T cell anti-tumor responses and increased tumor rejection, but also with augmented persistence of cytotoxic T cells that can exert strong responses in response to Ag stimulation long time after infusion. Since estrogen signaling was shown to directly enhance the expression of several cytokines in Ag-specific T cells, it was hypothesized that estrogen signaling increases Ag-specific T cell polyfunctionality which can result in better immunotherapy efficacy.

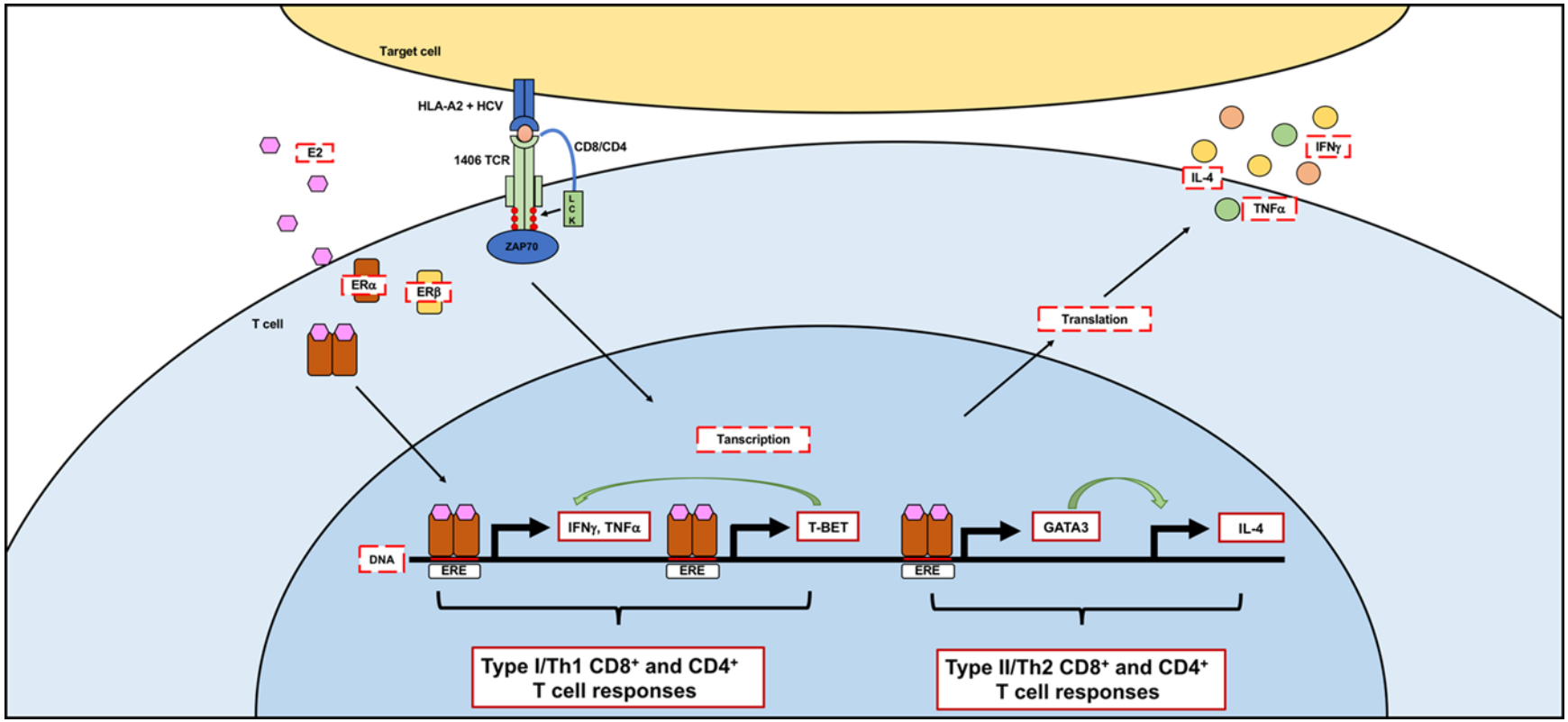

Figure 38. Proposed Mechanism I: Estrogen Signaling Through ERα Enhances Expression of TNFα, IFNγ, and IL-4 and Modulates the Balance Between Th1/Type I and Th2/Type II T Cell Responses and Differentiation. Estrogen signaling through ERα gnomically enhances the expression and secretion of Type I/Th1 cytokines IFNγ and TNFα and the Type II/Th2 cytokine IL-4. ERα is also able to genomically enhance the expression of Th1 transcription factor T-BET and Th2 transcription factor GATA3 through the EREs on their promoters. Overall this leads to the hypothesis that estrogen signaling through ERα mediates balance between Type I and Type II, and Th1 and Th2 T cell differentiation.

Considering these facts, the effect of estrogen signaling regulating Ag-specific T cell polyfunctionality in response to antigen stimulation was determined by activating human male and female HCV Ag-specific T cells with target cells expressing the HCV Ag. One of the most striking results found in this dissertation was that estrogen signaling through ERβ and nor ERα enhances male and female $CD8^+$ and $CD4^+$ Ag-specific T cell polyfunctionality. Estrogen signaling through ERβ increased the percentage of Ag-specific T cells that expressed three markers simultaneously in response to Ag stimulation and decreased the percentage of T cells that expressed only one marker in response to Ag stimulation. The marker combinations that were shown to be enhanced by ERβ signaling when $CD4^+$ and $CD8^+$ T cells were analyzed separately included $CD107a^+IFN\gamma^+TNF\alpha^+$ and $IFN\gamma^+IL\text{-}4^+TNFa^+$ which are combinations including cytotoxic/effector and Th2 T cell cytokines, again known to mediate stronger anti-tumor immune responses and T cell survival. ERβ signaling reduced the percentage of T cells expressing $IFN\gamma^+TNF\alpha^+$, and $IFN\gamma^+$ or $TNF\alpha^+$ in combination with no other marker. This does not mean that expression of these markers is overall downregulated by ERβ, but it means that expression of these markers in combination with other markers such as IL-4 and CD107a is enhanced. Interestingly when the data was analyzed for the bulk Ag-specific T cell population without dividing it into $CD8^+$ and $CD4^+$ subsets, additional polyfunctional marker combinations were shown to be affected by estrogen signaling through ERα and ERβ. Estrogen signaling through ERβ was shown to increase the percentage of Ag-specific T cells expressing polyfunctional marker combinations such as $IFN\gamma^+IL\text{-}4^+TNFa^+$, $IFN\gamma^+IL\text{-}2^+IL\text{-}4^+IL\text{-}17a^+TNF\alpha^+$, and $IL\text{-}2^+IL\text{-}4^+TNF\alpha^+$. Strikingly, inhibition of ERα during estrogen stimulation enhanced the percentage of T cells expressing these polyfunctional combinations even more than estrogen

alone, indicating that ERα signaling actually inhibits Ag-specific T cell polyfunctionality. While these polyfunctional phenotypes were expressed by low percentage of cells, other studies confirmed that even if there is a small percentage of polyfunctional cells in a T cell population, stronger anti-tumor responses are still achieved compared to highly monofunctional T cell populations [428, 433]. Estrogen signaling through ERβ was shown to decrease the percentage of Ag-specific T cells expressing monofunctional marker combinations such as IFNγ^+ and TNFα^+. On the other hand, inhibition of ERα during estrogen stimulation decreased the percentage of T cells expressing these monofunctional combinations even more than estrogen alone indicating that ERα signaling actually promotes expression of monofunctional phenotypes over polyfunctional ones. The effect of estrogen signaling on Ag-specific T cell polyfunctionality had been never studied before and these are completely novel findings that could help in the design of stronger immunotherapies.

T cell polyfunctionality was reported to be induced by TCR signaling and antigen sensitivity. T cells that are stimulated with low sensitivity antigens display reduced TCR downstream signaling activation and are less polyfunctional than those activated with high sensitivity antigens [421, 478]. These data leaded to the hypothesis that estrogen signaling through ERβ enhances TCR downstream signaling and subsequent polyfunctionality. Some TCR downstream signaling molecules were shown to be estrogen sensitive. For example, lck, ZAP70, and c-Fos contain EREs on their promoters indicating possible ERβ genomic regulation of their expression. Also, estrogen was shown to enhance other TCR downstream pathways such as the MAP kinase, the NFAT, and the NFκB pathways [436, 437, 439]. This indicates that ERβ

signaling could be enhancing TCR downstream signaling pathways which subsequently result in polyfunctionality (Figure 39).

Another important implication of T cell polyfunctionality is that it promotes T cell memory formation. In vaccination studies against viruses, bacteria, and parasites the polyfunctionality of pathogen Ag-specific T cells after vaccination was not only associated with favorable disease outcome but also with higher effector function and effector memory formation [479-482]. Long-lasting polyfunctional memory cells are found at low frequencies in the peripheral circulation but they can rapidly proliferate and elicit strong effector response upon Ag-stimulation [483]. ERβ signaling could be promoting the development of more polyfunctional memory Ag-specific T cells during ACT immunotherapy, indicating an enhancement of long-lasting anti-tumor T cell responses. This is not only important to destroy the primary tumor and possible metastatic sites but also in order to prevent relapse. The ERβ-mediated enhancement of polyfunctional T cells could mean the generation of more long-lasting memory Ag-specific T cells that are reactive to a specific tumor Ag and circulate the circulation of patients surveilling for new tumor sites expressing that Ag and eliminating them. Overall, this indicates that ERβ signaling could not only enhance the short-term efficacy of immunotherapy but also enhance immunotherapy over time through the generation of highly polyfunctional memory cells (Figure 39).

Interestingly the effect of estrogen signaling on cytokine production and polyfunctionality was observed equally in Ag-specific T cells from male and female donors. This indicates that estrogen signaling through ERα and ERβ exerts the same effects on T cells no matter on the sex of the donor. Estrogen signaling can be used as a way of enhancing ACT

immunotherapy in both males and female patients. This is especially important for males and post-menopausal females that have enhanced incidence rates of cancer such as HCC.

During ACT immunotherapy both cytotoxic $CD8^+$ and $CD4^+$ Ag-specific T cells target tumor cells. Cytotoxic $CD8^+$ directly lyse the tumor cells by secretion of perforin and Granzyme B upon Ag stimulation. With the exception of T_{regs} which are immunosuppressive, $CD4^+$ T cells (Th1, Th2, and Th17 subsets) can either directly eliminate tumor cells through cytotoxic mechanisms or indirectly by modulating other immune infiltrating cells found in the TME. For example, $CD4^+$ Th cells can express CD40L which interacts with CD40 on DCs and promotes the expression of MHC and other co-stimulatory molecules [484]. Furthermore, $CD4^+$ Th cells can directly enhance CTL anti-tumor responses. $CD4^+$ Th cells secrete IL-2 which is a growth factor that enhances survival and helps recruit CTLs to the tumor site [484]. IFNγ production by $CD4^+$ Th1 cells results in the upregulation of MHC molecules on tumor cells leading to enhanced T cell (CTL and Th) recognition [484]. In addition to being required for the generation of optimal primary CTL responses, a number of studies have shown a critical need for $CD4^+$ T cells in the generation and maintenance of memory $CD8^+$ T cells [484]. Overall these data indicate that functional $CD8^+$ and $CD4^+$ T cells are required for the generation of superior anti-tumor responses during immunotherapy. Since estrogen signaling was showed to enhance the function of both female and male $CD8^+$ and $CD4^+$ T cells, it was hypothesized that it also enhances the $CD8^+$ and $CD4^+$ anti-tumor responses during immunotherapy for HCC *in vivo.*

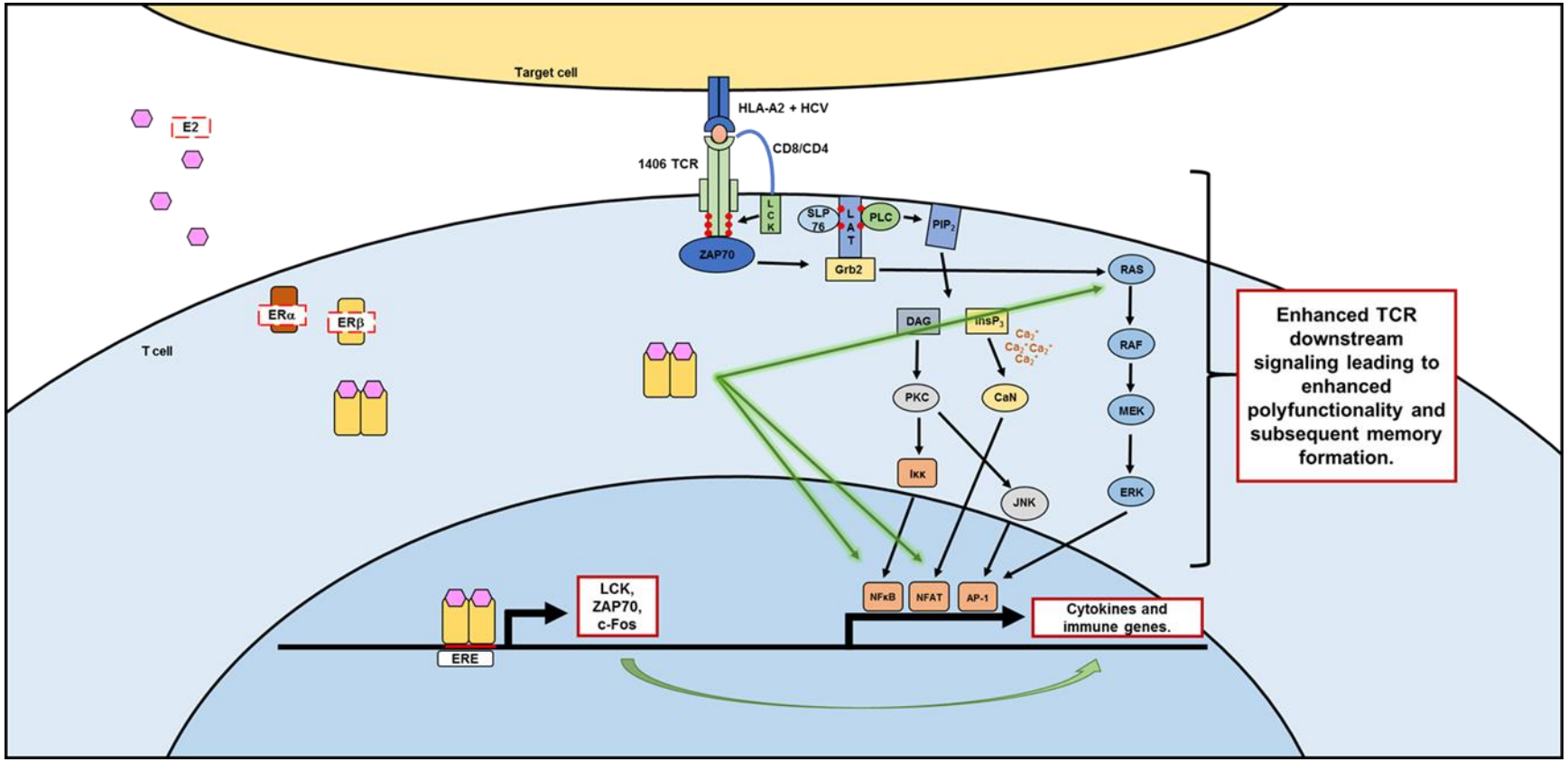

Figure 39. Proposed Mechanism II: Estrogen Signaling through ERβ Enhances Ag-specific T Cell Polyfunctionality through Enhancement of TCR Downstream Signaling Pathways. Estrogen signaling through ERβ enhances male and female Ag-specific T cell polyfunctionality by genomically inducing the expression of TCR downstream signaling molecules like lck, ZAP70, and c-Fos through the ERE on their promoters, and by enhancing signaling through the MAP kinase, the NFAT and the NFκB pathways. Enhancement of these pathways through ERβ results of the expression of polyfunctional combination of several markers simultaneously by one T cell, which can subsequently lead to memory T cell formation.

The role of estrogen signaling during ACT immunotherapy was then studied *in vivo*. Female mice, with physiological circulating estrogen concentrations, and ovariectomized female mice and male mice, with minimal circulating estrogen concentrations, were challenged with HCV^{+}HCC and given ACT with sex-matched HCV Ag-specific human T cells. First, HCC tumor burden was assessed and, as expected, male mice showed increased tumor burden compared to sham females. OVX females showed the most advanced tumor burden compared to sham males and females due to loss of the protective role of estrogen against HCC. ACT immunotherapy reduced tumor burden on sham and OVX females and males indicating therapeutic potential. OVX females still showed very advanced tumors after ACT treatment, indicating that loss of estrogen not only enhanced tumor burden but also decreased the T cell anti-tumor responses generated during immunotherapy. Analysis of the phenotype of infiltrating T cells revealed reduced tumor infiltration of Ag-specific T cells, especially $CD4^{+}$ T cells, in OVX females and males. Lack of physiological estrogen during ACT immunotherapy not only reduced tumor infiltration but also overall $CD4^{+}$ T cell survival. In addition, lack of physiological estrogen during ACT immunotherapy hindered Ag-specific T cell activation state. TILs isolated from sham and OVX females and sham males were then activated with target cells expressing their cognate Ag and cytokine expression and polyfunctionality were measured. While $CD8^{+}$ T cells did not show significant differences in polyfunctionality between sham and OVX female TILs, they showed an overall reduction on IL-4 expression in OVX females further indicating that estrogen directly regulates IL-4 expression in Ag-specific T cells. $CD4^{+}$ TILs from OVX females showed decreased expression of IL-4, TNFα, IFNγ, and the lytic marker CD107a compared to sham females. Furthermore, polyfunctionality of $CD4^{+}$ TILs was also decreased in the absence of physiological estrogen with significantly lower percentages of OVX

female TILs expressing 3 markers simultaneously compared to sham female $CD4^+$ TILs. Overall, these results indicated that physiological estrogen signaling during ACT immunotherapy enhances the survival, tumor infiltration, and function of $CD4^+$ Ag-specific T cells. Considering the important role of $CD4^+$ T cells both directly targeting tumor cells and enhancing $CD8^+$ T cell function and survival, estrogen signaling was found to be an important factor to consider into enhancing the efficacy of ACT immunotherapy.

In summary, the results presented in this dissertation are encouraging that estrogen signaling on Ag-specific T cells could serve as a way to enhance the efficacy of immunotherapy for HCC. Estrogen signaling through ERα was found to enhance the expression of key T cell effector cytokines including IFNγ, TNFα, and Granzyme B on Ag-specific T cells from male and female donors when activated with their cognate Ag *in vitro* and *in vivo*. IFNγ, TNFα, and Granzyme B are required for tumor cell destruction. Estrogen signaling through ERα was also shown to completely regulate the expression of the Th2 cytokine IL-4 on male and female Ag-specific T cells activated with their cognate Ag *in vivo* and *in vitro* which is not only necessary for Th2 differentiation but promotes T cell survival and memory formation. Enhancement of these cytokines is believed to represent the ability of ERα signaling to regulate the balance between Th1 and Th2 differentiation or type I and type II T cell responses. Estrogen signaling through ERβ was shown to enhance T cell polyfunctionality on male and female Ag-specific T cells activated with their cognate Ag. ERβ signaling is believed to enhance TCR downstream signaling pathways which result on increased T cell polyfunctionality (Figure 40). Notably estrogen effects on T cell cytokine production were observed at physiological and super-physiological concentrations in a dose dependent manner. Using an *in vivo* model for HCC, it

was found that physiological estrogen not only has a protective role against HCC, but it also enhances ACT immunotherapy. Estrogen presence during ACT immunotherapy for HCC was found to increase the tumor infiltration, survival, activation state, cytokine production and polyfunctionality of Ag-specific T cells. These effects were especially observed on the $CD4^+$ T cell subset. Reduction of $CD4^+$ T cell survival, cytokine expression, and polyfunctionality in the absence of physiological estrogen reduces ACT immunotherapy efficacy by not only lacking proper $CD4^+$ anti-tumor responses but also by the lack of support to the $CD8^+$ subset anti-tumor responses. Overall these results showed that estrogen signaling at physiological and super-physiological concentrations can enhance the function of male and female $CD8^+$ and $CD4^+$ Ag-specific T cells. This regulation of T cell function is especially important during immunotherapy *in vivo*, where physiological estrogen enhanced survival, tumor infiltration, and function of $CD4^+$ Ag-specific T cells which result on enhanced immunotherapy efficacy through the support of $CD8^+$ anti-tumor responses. In conclusion, estrogen signaling on Ag-specific T cells could enhance the efficacy of ACT immunotherapy to treat cancer in male and female patients.

Future Directions

The questions posed, results observed, and findings drawn from the data generated in this dissertation are novel and unique and provide critical information that will open the doors to new advancements in clinical outcomes as well as new research endeavors in the field of immunotherapy. The focus of this study was to characterize and mechanistically understand the biology of sex and estrogen signaling on Ag-specific T cell activation, function and survival in the context of cancer treatment.

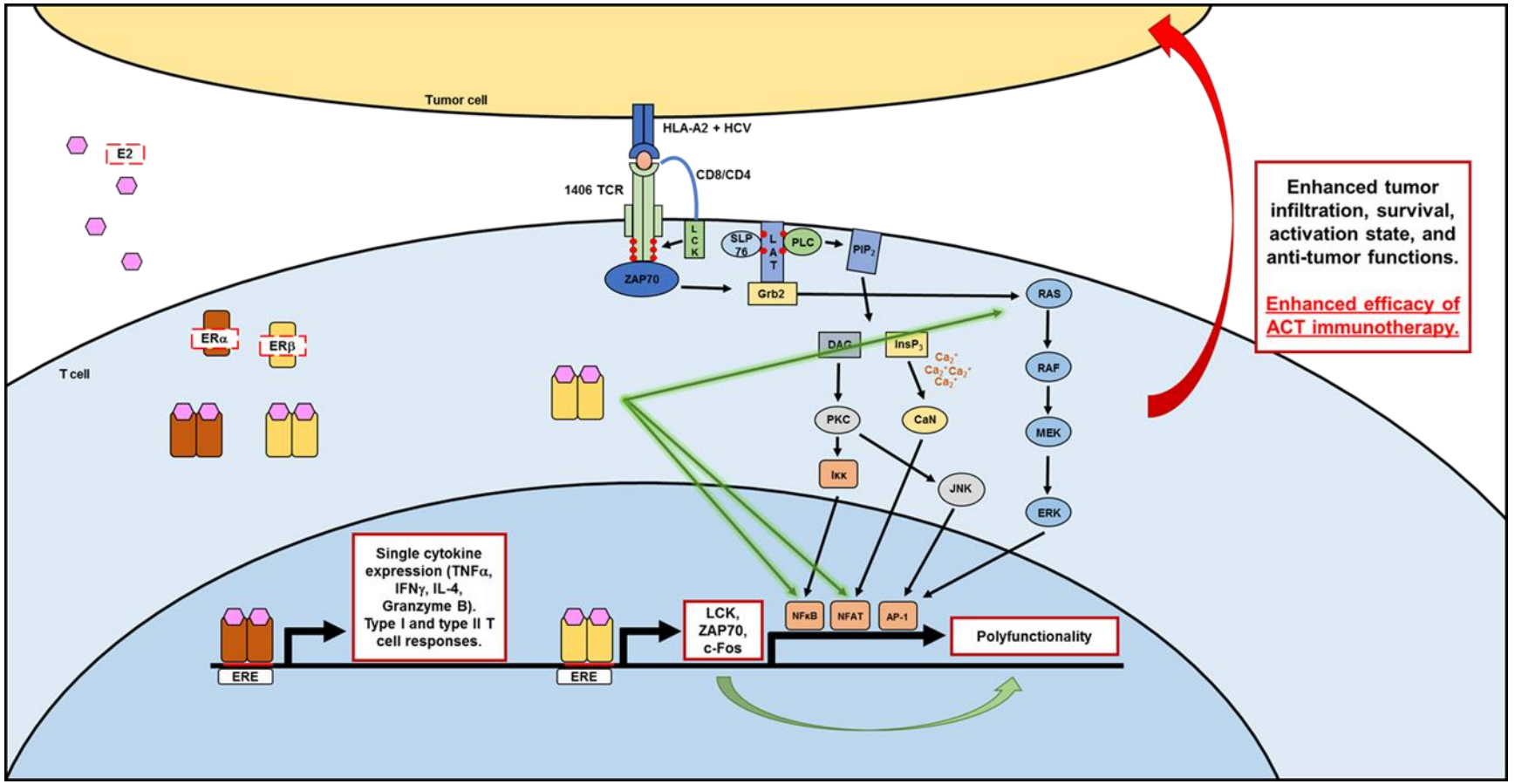

Figure 40. Overall Proposed Mechanism: Estrogen Signaling through ERα and ERβ Enhances Ag-specific T Cell Function Resulting in the Enhancement of ACT Immunotherapy Efficacy. Estrogen signaling though ERα results in the enhancement of expression of key Type I/Th1 and Type II/Th2 cytokines and estrogen signaling through ERβ results in the enhancement of male and female Ag-specific T cell polyfunctionality. Physiological estrogen enhances the survival, tumor infiltration, activation, and function of $CD4^+$ Ag-specific T cells *in vivo* which results in stronger anti-tumor immune responses during ACT immunotherapy.

My data, as presented, clearly demonstrates that although T cells are intrinsically different due to sex, meaning T cells from male donors are intrinsically different from T cells isolated from female donors, estrogen signaling has a profound effect on the function of T cells from both males and female donors. Therefore, it can be concluded that estrogen signaling mediates similar effects on the function of T cells independently of sex. Estrogen signaling occurs upon ligation of its nuclear receptors, ERα and ERβ, and results in enhanced function of Ag-specific T cells that can be used for immunotherapy *in vitro* and *in vivo*. These are very encouraging findings that indicate estrogen signaling can be used as a mean of enhancing ACT immunotherapy for both male and female cancer patients. In this dissertation, human Ag-specific T cells were acutely treated with estrogen (2-hour treatment) which elicited an enhancement of T cell cytokine production and polyfunctionality, especially at super-physiological concentrations. According to these findings, genetically modified T cells from cancer patients used for ACT immunotherapy can be assessed for function in response to Ag stimulation, and if patients' T cells do not express and produce important cytokines for anti-tumor function including but not limited to, IFNγ, TNFα, or Granzyme B, then they can be acutely treated with super-physiological estrogen concentrations prior to infusion. This estrogen treatment will enhance the effector functions through ERα ligation, and the polyfunctionality, specifically the combined simultaneous production of anti-tumor effector and helper molecules, of T cells through ERβ ligation which will result in generating stronger and more durable anti-tumor immune responses once transferred into the patient.

Since adoptively transferred T cells will be circulating in the patients for long periods of time, it is possible that an acute estrogen treatment will not be enough to maintain enhanced T cell function in the long run. Because T cells benefit from estrogen presence for longer time

periods, estrogen could be added to patient's T cells cultures during the *in vitro* Ag-specific T cell expansion performed prior to adoptive cell transfer. This approach could be first tested in mice, where HCV Ag-specific T cells are given physiological or super-physiological estrogen during the 3 to 5-day expansion performed prior to ACT immunotherapy. The effect of these prolonged treatments would need to be tested on T cell subset distribution and function. Based on the results obtained in this dissertation, prolonged estrogen treatment could affect the $CD4^+$ T cell subset distribution skewing it towards the Th1 and Th2 subsets. On the other hand, profound differences in T cell function are expected after prolonged estrogen stimulation including enhanced cytokine production and polyfunctionality. Long-term high concentration estrogen exposure was shown to downregulate the expression of ERα and ERβ both mRNA and protein levels in breast cancer cells and non-reproductive estrogen target tissues [485-487]. This estrogen-regulated feedback loop directly inhibits the transcription of the ERs through ligand-bound ER genomic signaling. The suppressive effects of estrogen on ER expression were observed after 6 hour estrogen stimulation and remained for 24 to 48 hours [485]. Decreased expression of ERs on Ag-specific T cells would mean loss of the beneficial ER signaling mediated enhancement of T cell cytokine production and polyfunctionality. Overall, indicating that acute estrogen treatment of Ag-specific T cells would be a better approach than long-term estrogen exposure.

The work of this dissertation also determined the different roles of ERα compared to ERβ signaling on T cell function. While ERα enhanced the expression and secretion of specific cytokines, one of the most intriguing discoveries of this study indicated that ERβ increases the overall T cell polyfunctionality through mechanisms believed to enhance TCR downstream

signaling. Knowing that ERβ signaling results in enhanced T cell function and polyfunctionality which can result in enhanced T cell memory formation, ERβ signaling can be used to generate polyfunctional and long-lasting Ag-specific T cell responses. In order to enhance ERβ signaling, Ag-specific T cells could be treated with an ERβ-specific agonist prior to adoptive transfer. In addition, the ERβ-specific agonist could be delivered intra-tumorally during ACT immunotherapy to enhance the function of infiltrating Ag-specific T cells. Delivering the ERβ agonist into the TME would not only affect T cells but also tumor cells and other infiltrating immune cells. In the context of HCC, estrogen signaling was shown to inhibit tumor growth and TAM polarization which means that delivering an ERβ agonist may exert anti-tumor functions as well as inducing stronger T cell anti-tumor immune responses. While this would work when tested in NSG-$A2^{+}$ mice deficient of other infiltrating immune cells, the role of ERβ signaling on the function of other immune cells in the TME including B cells, MDCSs, and DCs is unknown and would need to be further investigated. In order to limit the beneficial effect of ERβ signaling to T cells, human Ag-specific T cells can be genetically modified to overexpress ERβ. ERβ overexpression would ensure estrogen ligand binding to and activation of ERβ signaling over other estrogen receptors, including ERα and GPER, present in the T cells. Enhanced ERβ signaling in T cells would increase polyfunctionality, production of several anti-tumor cytokines simultaneously by T cells, and increased T cell memory formation hence enhancing the efficacy of ACT immunotherapy. This would be especially important for malignancies such as HCC which can produce high concentrations of local estrogen, or malignancies located on sexual tissues like the ovaries or the breasts.

In addition to providing ways to enhancing immunotherapy to treat cancer, the results obtained in this study can enhance the treatment of other diseases such as infections and auto immune diseases. Treatment of infections using ACT immunotherapy showed some success in pre-clinical and clinical studies treating viral infections to which vaccines do not exist, or that exhibit drug resistance including HIV, CMV, and Epstein-Barr virus (EBV) infections [488-490]. In spite of some success, ACT immunotherapy for viral infections was sometimes rendered unsuccessful due to large viral loads which were not able to be completely eliminated by transferred T cells due to T cell exhaustion under to continuous Ag stimulation, and by reduced transgenic T cell proliferation leading to reduced T cell numbers [491]. Interestingly, ACT immunotherapy for HIV infected patients using cytotoxic $CD8^+$ T cells was shown to require production of high amount of Granzyme B [492], and the presence of functional helper $CD4^+$ T cells [493, 494]. The results of this dissertation showed that both the secretion of Granzyme B by $CD8^+$ Ag-specific T cells, and the persistence and anti-tumor function of $CD4^+$ T cells were enhanced by estrogen signaling. This indicates that ACT immunotherapy for HIV could be enhanced by estrogen signaling enhancing the function of $CD4^+$ and $CD8^+$ T cells.

$CD4^+$ T cell subsets were shown to be able to mediate several aspects of autoimmune inflammation. While Th1 and Th17 cells drive the chronic autoimmune response, T_{regs} were shown to exert immunosuppressive functions in other T cell functions during autoimmunity [495]. In some autoimmune diseases such as RA it was shown that T_{regs} function is severely impaired [495]. The results presented in this dissertation indicated that estrogen enhances T cell function including pro-inflammatory cytokine production and polyfunctionality. Based on these findings, removal of estrogen or inhibition of ERα and ERβ signaling on T cells may relieve the

pathogenesis of autoimmunity. The use of ERα and ERβ antagonists separately and simultaneously during autoimmunity in mouse models may reduce the secretion and expression on pro-inflammatory cytokines and reduce the differentiation of activated T cells towards the Th1 and Th2 subsets.

In summary, the findings of this dissertation demonstrate that estrogen signaling is an important factor to consider in the design and optimization of T cell-based immunotherapies for cancer and infections. In addition, characterizing the role of estrogen signaling on T cell function may have far reaching importance in terms of regulating T cells during autoimmune disease or even as a result of chronic inflammation due to Ag stimulation. The results in this dissertation have provided a new foundation in which to augment T cell-based therapy efficacy for several cancers and other further diseases.

Clinical Trial Design

The studies performed in this dissertation revealed that physiological (0.5 nM) and super-physiological (50 nM) estrogen signaling through ERα enhances the expression and secretion of specific Type 1/Th1 cytotoxic effector T cell cytokines such as IFNγ and Granzyme B, as well as Type 2/Th2 helper T cell, cytokines including TNFα and IL-4, on HCV Ag-specific T cells from male and female donors. It was also shown that estrogen signaling through ERβ at both physiological and super-physiological concentrations enhanced overall T cell polyfunctionality, or the ability of HCV Ag-specific T cells from male and female donors to express more than two functional markers upon Ag stimulation. *In vivo*, it was shown that physiological estrogen enhances the infiltration, survival, activations state, cytokine expression, and polyfunctionality of Ag-specific TILs, especially of the $CD4^+$ subset, after ACT immunotherapy against HCC.

Overall, these data indicate that estrogen signaling could improve the efficacy of ACT immunotherapy against HCC by enhancing the anti-tumor function, tumor infiltration, and survival of adoptively transferred Ag-specific T cells. Since the *in vivo* studies performed in humanized mice were proven to be successful, and tumor reduction was greatly achieved in females containing physiological estrogen (SHAM females) compare to estrogen-depleted females (OVX females) and males in a safe manner, a clinical trial can be designed to evaluate if estrogen could enhance Ag-specific T cell function and reduce tumor burden during ACT immunotherapy for patients suffering from HCC.

For this clinical trial, HLA-$A2^+$ pre-menopausal (containing physiological estrogen) and post-menopausal (estrogen-depleted) women and age-matched men suffering from chronic HCV infection-derived HCC will be recruited. Circulating T cells from these patients will be collected from blood samples and transduced with the 1406 HCV TCR as described before and sorted to obtain a > 99% pure transduced T cell population. Then, Ag-specific T cells will be expanded *in vitro* using irradiated Ag-presenting cells expressing the HCV cognate Ag. Before transfer, Ag-specific T cells from pre- and post-menopausal women and men will be treated with either no estrogen, physiological (0.5 nM), or super-physiological (50 nM) estrogen overnight. According to the data obtained in the pre-clinical studies, short-term estrogen treatment was enough to enhance Ag-specific T cell function including cytokine expression and polyfunctionality upon Ag stimulation. Acute estrogen treatment will also prevent the estrogen-mediated self-downregulation of expression of ERα and ERβ observed after long-term estrogen exposure [485-487]. Patients from each respective group will be receive local liver stereotactic radiation therapy to a total dose of 50 Gy in four 12.5 Gy fractions to control tumor burden before immunotherapy

and to enhance tumor Ag presentation [496], then they will receive one dose of $10x10^6$ Ag-specific T cells/kg treated with either no estrogen, physiological, or super-physiological estrogen. After adoptive transfer, patients will receive low-dose rhIL-2 (30 $\mu g/m^2$) every five days to ensure Ag-specific T cell survival [497]. Tumor burden will be monitored via liver ultrasound and serum concentration of α-fetoprotein (AFP) [498]. Tumor burden will be compared on patients from each respective group that received Ag-specific T cells treated with estrogen compared to untreated T cells. Blood samples will be collected periodically to observe the phenotype and counts of Ag-specific T cells remaining on the patients' circulation. Number of remaining $CD4^+$ and $CD8^+$ Ag-specific T cells and their phenotype (Type I/II, T_{regs}, Th1/2/17/22) will be measured, their activation state will be assessed via flow cytometry by staining for activation markers (CD25, CD69, CD44, and CD62L), and their function will be measured upon Ag re-stimulation *ex vivo* to observe if Ag-specific T cells are under exhaustion conditions. Also, over 3 months after ACT, survivor patients will be tested for Ag-specific T cell memory formation by collecting blood samples and surveilling for T cell central memory markers ($CD45RO^+CCR7^-CD28^-CD27^-$) as well as their ability to elicit effector responses upon HCV Ag re-stimulation. Since estrogen signaling through ERβ enhances T cell polyfunctionality, which is correlated with T cell memory formation [476], greater number of central memory T cells are expected to be found in pre-menopausal women and post-menopausal women and men whose T cells were estrogen treated.

According to the pre-clinical data previously observed, men and post-menopausal women that receive untreated Ag-specific T cells will have the worse tumor burden compared to pre-menopausal women. Tumor burden will decrease in men and post- and pre-menopausal women

that receive estrogen treated Ag-specific T cells in a dose dependent manner comparing physiological to super-physiological estrogen treated T cells. Ag-specific T cells from pre-menopausal women will show enhanced survival, especially those in the Th1 and Th2 $CD4^+$ subsets, as well as enhanced cytokine expression and polyfunctionality compared to those from post-menopausal women and men. Survival and infiltration of Ag-specific T cells should increase in a dose dependent manner with estrogen treatment in all three patient groups. If possible, a liver biopsy will be collected after ACT immunotherapy in order to analyze the phenotype and function of TILs (including activation state, subset differentiation, and infiltration level), which it is predicted to be better in the presence of physiological estrogen (pre-menopausal women), and in patients that received estrogen-treated T cells.

If estrogen treated T cells elicit enhance anti-tumor responses and significantly reduce tumor burden more than untreated T cells, a second clinical trial will be performed in order to determine if signaling through ERα, ERβ, or both receptors simultaneously is necessary for the estrogen-mediated enhancement of the Ag-specific T cell anti-tumor immune response. In that case, HLA-$A2^+$ pre- and post-menopausal women and age-matched males suffering from HCV-derived HCC will receive ACT immunotherapy of Ag-specific T cells that were treated with an ERα-specific agonist (PPT), and ERβ-specific agonist (DNP), or both simultaneously overnight at a concentration of 100 nM. Then, treated Ag-specific T cells will be infused into the patients of each group as described before, and tumor burden, T cell survival, phenotype, and function will be assessed. Since it was shown that estrogen signaling through ERβ enhances overall Ag-specific T cell polyfunctionality, it can be predicted that patients that receive Ag-specific T cells

treated with an ERβ agonist will show enhanced anti-tumor immune responses and reduced tumor burden compared to those patients that received ERα agonist-treated T cells.

These clinical studies will help determine if estrogen signaling can be used to enhance the efficacy of ACT immunotherapy. This is especially important because it will show that short-term estrogen or ER-specific agonist treatment of Ag-specific T cells and not the whole patient, which is safe and easy to achieve, can alone increase the T cell anti-tumor immune response. In the case of men and post-menopausal women which show the highest incidence rates for HCC, estrogen treatment of the Ag-specific T cells will offer a simple yet effective way to enhance a cancer treatment that while has shown promising results, it is still inefficient in removing large solid tumors.

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VITA

Flor Navarro Negredo was born in Zaragoza, Spain to Fernando Navarro and Adela Negredo on November 22nd 1992. She attended Northern Illinois University on an academic scholarship as a Spanish Native Speaker for the Foreign Language Residence Program. She earned a Bachelor of Science degree, *cum laude*, in Biochemistry in August 2014. During her time as an undergraduate, Flor worked in the laboratory of Dr. Timothy Hagen, Ph.D., characterizing small molecule inhibitors for *Plasmodium falciparum* methionine aminopeptidase 2 (MetAP2).

In August 2014, Flor matriculated into the Integrated Program in Biomedical Sciences at Loyola University Chicago and joined the Biochemistry and Molecular Biology department. After rotating the laboratory of Dr. Caroline Le Poole, Ph.D., studying the role of C-C motif chemokine ligand 22 (Ccl22) during regulatory T cells trafficking in melanoma, she joined the laboratory of Dr. Stephanie K. Watkins, Ph.D., where she completed her graduate studies. In her dissertation work, she characterized the effect of sex, and the impact of estrogen receptor signaling on the function of antigen-specific T cells for adoptive T cell transfer immunotherapy against hepatocellular carcinoma. Flor's research generated a cutting edge publication that was awarded the Robert S. Birch Award for the best original scientific paper published in *Gender and the Genome* by The foundation for Gender-Specific Medicine. Flor's dissertation research was supported by the American Cancer Society Research Scholar Grant RSG-16-242-01 awarded to Stephanie K. Watkins, Ph.D.

After completion of her graduate studies, Flor will continue her research endeavors as a Postdoctoral Research fellow. She is considering an offer to work in the laboratory of Dr. Asis Palazon, Ph.D., at the Center for Cooperative Research in Biosciences BioGUNE in Bilbao, Spain. There Flor will study the role of hypoxia in the tumor microenvironment during adoptive T cell transfer immunotherapy with the aim of developing hypoxia-responsive immunotherapeutic approaches.