Estrogen regulates the liver immune microenvironment and the immunotherapy response to colorectal carcinoma liver metastases

Yasmine Benslimane

Experimental Medicine

McGill University, Montreal

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of *Master of Science*

April 2023

Table of Contents

Abstract

Liver metastases (LM) represent a major cause of cancer-related death and remain a clinical challenge. Up to 70% of colorectal cancer (CRC) patients develop LM, and CRC incidence is increasing in the younger population. LM and the female sex were identified as two factors predicting a poorer response to immunotherapy (IT), but the reasons remain unclear. We previously reported on a sexual dimorphism in the control of the tumor microenvironment (TME) of CRC liver metastases and identified estrogen as a key regulator of the immunosuppressive tumor microenvironment in the liver. Here we aimed to assess the effect of estrogen deprivation on the cytokine/chemokine profile induced by metastatic CRC cells and their effect on the innate and adaptive immune responses in the liver. Moreover, we analyzed the benefit of combining a selective estrogen-receptor degrader (SERD) with immune checkpoint blockade in the treatment of CRC liver metastases. We show here that estrogen depletion by ovariectomy altered the cytokine/chemokine repertoire in the TME of CRCLM, decreased macrophage polarization to the anti-inflammatory M2 phenotype and increased the accumulation of $CCL5^+CCR5^+$ $CD8^+$ T cells, $CCL5^+CCR5^+$ NKT cells, and $CXCR3^+$ macrophages in the liver TME. Analysis of liver resections from patients with CRCLM revealed a higher proportion $CD56^+$ NK cells and a lower proportion of $CD68^+CD163^+$ M2-like macrophages in older female compared to younger female patients. Importantly, treatment with the SERD Fulvestrant markedly enhanced the therapeutic benefit of anti-PD-1 immunotherapy by increasing the accumulation of $CD8^+CCL5^+$, $CD8^+CCR5^+$ T cells and NK cells in the mouse liver TME and resulted in a significant reduction in the outgrowth of liver metastases. Taken together, our results suggest that inhibition of estrogen signaling reprograms the liver TME in LM and could potentiate the therapeutic effect of immunotherapy in metastatic IT-resistant colon cancer.

Résumé

Les métastases hépatiques (MH) représentent une cause majeure de décès associés au cancer et restent un défi clinique. Jusqu'à 70 % des patients atteints d'un cancer colorectal (CCR) développent des métastases au foie, et l'incidence de CCR est en augmentation dans la jeune population. Les MH et le sexe féminin ont été identifiés comme facteurs de prédiction d'une faible réponse à l'immunothérapie (IT), mais les raisons restent incertaines. Nous avons précédemment démontré un dimorphisme sexuel dans le contrôle du microenvironnement tumoral (MET) des MH du CCR et identifié l'œstrogène comme un régulateur clé du MET immunosuppresseur dans le foie. Ici, nous avons cherché à évaluer l'effet de la réduction de taux d'œstrogènes sur le profil des cytokines/chimiokines induit par les MH du CCR et leur effet sur les réponses immunitaires innées et adaptatives dans le foie. De plus, nous avons analysé l'avantage de combiner un inhibiteur de récepteur à œstrogènes SERD (*Selective Estrogen Receptor Degrader*) à un inhibiteur de point de contrôle immunitaire (*immune checkpoint blockade*) dans le traitement des MH du CCR. Nous démontrons ici que la réduction du taux œstrogènes induit par l'ovariectomie a modifié le répertoire des cytokines/chimiokines dans le MET des MH, a réduit la polarisation des macrophages vers le phénotype anti-inflammatoires M2 et a augmenté l'accumulation de cellules T $CD8^+$ $CCL5^+/CCR5^+$, de cellules NKT $CCL5^+/CCR5^+$ et de macrophages $CXCR3^+$ dans le MET hépatique. L'analyse des résections hépatiques de patients atteints de CRCLM a révélé une proportion plus élevée de cellules NK $CD56^+$ et une proportion plus faible de macrophages de type-M2 $CD68^+CD163^+$ chez les femmes plus âgées par rapport aux patientes plus jeunes. Il est important de noter que le traitement avec le SERD Fulvestrant a nettement amélioré l'efficacité thérapeutique de l'immunothérapie anti-PD-1 en augmentant l'accumulation de lymphocytes T $CD8^+$ $CCL5^+/CCR5^+$ et de cellules NK dans le MET hépatique, ce qui a entraîné une réduction significative de la croissance de métastases hépatiques. Ainsi, nos résultats suggèrent que l'inhibition de la signalisation des œstrogènes reprogramme le MET du MH et pourrait améliorer l'effet thérapeutique de l'immunothérapie dans le cancer du côlon métastatique au foie résistant à l'immunothérapie.

Foreword

This thesis is presented in manuscript form, in partial agreement with the terms listed by the Faculty of Medicine, division of Experimental Medicine at McGill University. This thesis includes the following publication:

Chapter 2:

Benslimane Y, Amalfi K, Lapin S, Perrino S, Leibovitch M, Chambon J, Brodt P.

Estrogen receptor blockade potentiates immunotherapy of colorectal carcinoma liver metastases by altering the immunosuppressive microenvironment

[Manuscript prepared for submission, Abstract submitted and recommended for Nature Cancer]

Contributions of Authors

Yasmine Benslimane, B.Sc.

Wrote this thesis, first author of the drafted manuscript, and performed all the experimental work.

Dr. Pnina Brodt, Ph.D.

Conceived and supervised the project, revised, and edited the thesis and manuscript, helped in design of experiments and data analysis, and obtained CIHR funding.

Stephanie Perrino, AHT, RLAT, B.Sc.

Animal Health Technician, helped with tumor cells injections, surgeries, and animal care.

Elaheh Papari, Ph.D.

Postdoctoral fellow, collaborator on the CRCLM patients' specimen analysis project.

Matthew Leibovitch, Ph.D.

Former Research Associate, helped with protein and RNA analysis experiments.

Julien Chambon, M.Sc.

Former Research Assistant, helped with surgeries and flow cytometry experiments.

Kevin Amalfi, Undergraduate Honor's trainee

Mentee and collaborator on the CXCR3/CXCL10 axis project as part of his Undergraduate honor's project in Immunology.

Sarah Lapin, B.Sc.

Mentee and collaborator on the macrophage polarization part of my project as part of her Undergraduate honor's project in Immunology.

Acknowledgements

I want to thank my thesis supervisor, Dr. Pnina Brodt, for her continuous support, guidance, and trust throughout this exciting project. She has been an inspiration for hard work and scientific dedication. I also want to thank my advisory committee composed of Dr. Ciriaco Piccirillo, Dr. Alex Gregorieff, and Dr. Elham Rahme for their insightful comments and advice.

I would also like to thank the current and past lab members of Dr. Brodt lab: Elliot Goodfellow, Stephanie Perrino, Orçun Haçariz, Kevin Amalfi, Simon Milette, Elaheh Papari, Sarah Lapin, Julien Chambon, Matthew Leibovitch, Masakazu Hashimoto, Michely Chen, John David Konda and Charles Essagian. I also would like to thank Dr. Piccirillo's, Dr. Gregorieff's, Dr. Radzioch, Dr. Ferri lab members for being welcoming to questions and for their support.

I want to thank Hélène Pagé-Veillette, Ekaterina Iourchenko and Marie-Helene Lacombe from the Immunophenotyping Core Facility, Min Fu and Shibo Feng of the Molecular Imaging Core Facility, and Fazila Chouiali of the Histopathology Core Facility, at the Research Institute of the McGill University Health Center (RI-MUHC).

Finally, funding from the CIHR (to PB) and awards from the American Association for Cancer Research (AACR), the Fiera Capital/RI-MUHC, and the Division of Experimental Medicine, McGill University to support this project are gratefully acknowledged.

List of Figures

List of abbreviations

APC	Antigen-Presenting Cell
AR	Androgen Receptor
Arg1	Arginase 1
BM	Bone-Marrow
CCL	Chemokine (C-C motif) Ligand
CCR	Chemokine (C-C motif) Receptor
CD	Cluster of Differentiation
CRC	Colorectal Carcinoma
CRCLM	Colorectal Carcinoma Liver Metastases
CTLA-4	Cytotoxic T Lymphocyte-Associated Protein 4
CXCL	Chemokine (C-X-C motif) Ligand
CXCR	Chemokine (C-X-C motif) Receptor
DC	Dendritic Cell
DHGP	Desmoplastic Histopathological Growth Pattern
DMSO	Dimethyl Sulfoxide
E1	Estrone
E2	Estradiol
E3	Estriol
ECM	Extracellular Matrix
ER	Estrogen Receptor
ET	Endocrine Therapy
FC	Flow Cytometry
FDA	U.S. Food and Drug Administration
Fulv	Fulvestrant
GI	Gastrointestinal
GITR	Glucocorticoid-Induced TNFR-Related Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GrzB	Granzyme B
H&E	Hematoxylin & Eosin
HGP	Histopathological Growth Pattern
HIC	Hepatic Immune Cells
HSC	Hepatic Stellate Cell
ICI	Immune Checkpoint Inhibitor
IF	Immunofluorescence
IFN-γ	Interferon-γ
IL	Interleukin
IME	Immune Microenvironment
iNOS	inducible Nitric Oxide Synthase
IT	Immunotherapy
KC	Kupffer Cell
LAG-3	Lymphocyte-Activation Gene 3
LM	Liver Metastases
LMP	Pancreatic Ductal Adenocarcinoma cell line
M1	Pro-inflammatory anti-tumorigenic phenotype

M2	Anti-inflammatory pro-tumorigenic phenotype
M-CSF	Macrophage Colony-Stimulating Factor
MDSC	Myeloid-Derived Suppressor Cell
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MMP	Matrix Metalloproteinase
Mo	Monocyte
mRNA	messenger RNA
Mvc	Maraviroc
NIH	National Institute of Health
NK	Natural Killer cell
NKT	Natural Killer T cell
OS	Overall Survival
OVX	Ovariectomized
PBS	Phosphate-Buffered Saline
PD-1	Programmed Death Protein 1
PD-L1	Programmed Death Ligand 1
RHGP	Replacement Histopathological Growth Pattern
ROS	Reactive Oxygen Species
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
SERD	Selective Estrogen Receptor Degrader
SERM	Selective Estrogen Receptor Modulator
SHAM	Sham-operated control
TAM	Tumor-Associated Macrophages
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-β
Tim-3	T cell immunoglobulin and mucin containing protein-3
TIMP	Tissue Inhibitor of Metalloproteinases
TLR	Toll-Like Receptor
TME	Tumor Microenvironment
TNF-α	Tumor Necrosis Factor-α
TNFR	Tumor Necrosis Factor Receptor
T reg	Regulatory T lymphocyte
VEGF	Vascular Endothelial Growth Factor

Chapter 1

Background and Introduction

Chapter 1: Background and Introduction

1.1. Preface

Liver metastasis remains an important clinical challenge. The liver's double-blood supply and unique immune-tolerant state make it an attractive niche for metastasis mainly for colorectal carcinoma, but also for other malignancies such as pancreatic ductal adenocarcinoma, melanoma, and breast cancer (1). Liver metastatic disease is particularly resistant to cancer therapy, including immunotherapy which has shown efficacy in the treatment of other malignancies (2). Moreover, similar to liver metastases, the female sex has also emerged as a factor associated with resistance to immunotherapy, although the reasons remain unclear (3). Our laboratory previously identified a sexual dimorphism in LM where estrogen promoted an immunosuppressive microenvironment (ME) via the accumulation of myeloid-derived suppressor cells (MDSCs) and regulatory T cells (T regs) (4). The goal of this thesis is to further characterize the role of estrogen in the immune microenvironment of colorectal cancer liver metastasis, by profiling the molecular mediators underlying this immunosuppressive IME and by further characterizing the phenotype and function of other key immune actors in the liver. Additionally, this work had a translational aim to assess if, and how, the addition of estrogen receptor inhibitors would maximize the therapeutic outcome of anti-PD-1 immunotherapy. A short review of the relevant background and key concepts is included as preamble to the thesis objectives.

1.2. Liver metastatic disease

1.2.1. Epidemiology

Liver metastasis (LM) is a major cause of cancer-related death and remains a major clinical challenge (5). The liver is the most common site of metastasis for tumors of the GI tract such as colorectal pancreatic ductal adenocarcinoma as well as breast cancer and melanoma. Up to 50% of colorectal cancer (CRC) patients develop liver metastases (6). The high incidence of colorectal cancer liver metastasis (CRCLM) is due mainly to the facilitated access of CRC cells to the liver through the portal circulation and also to favorable growth conditions in the liver including a unique immuno-tolerant microenvironment that evolved to dampen immunity to neoantigens entering the liver from the gastrointestinal system via the portal circulation (7, 5). Colorectal cancer is the third most prevalent cancer diagnosed worldwide, and the fourth most common cause of cancer-related deaths worldwide. In 2020, there were 1.9 million newly diagnosed CRC cases and 935,000 CRC-related death worldwide. The global incidence of CRC has been rising at an annual increase of 3.2% (8) and metastatic CRC (mCRC) is still associated with high mortality rate due to resistance to chemo and targeted therapy.

Of relevance, the incidence of CRC in the United States between 2014 and 2018 dropped by 2% each year in adults 50 years of age and older due mainly to increased screening. During the same period, however, the incidence in adults younger than 50 years rose by 1.5% annually and CRC has become the 4th most diagnosed cancer among men and women aged 30-39 years old (data from ASCO, unpublished). Additionally, patients under 45 years of age are more often diagnosed with advanced-stage disease, with rectal disease and left-sided tumors than patients between the

ages of 60 and 85. Left-sided tumors are more likely to metastasize to the liver than right-sided tumors, the latter showing a different metastatic pattern in the peritoneum (9).

1.2.2. Clinical management

The current clinical management of CRCLM requires a multi-disciplinary approach involving radiology, medical oncology, hepatic surgery, colorectal surgery, and histopathology (10). Patient management is based on the combined assessment of clinical, radiological, and molecular information (10). The prognosis of CRCLM remains poor. Curative resection and chemotherapy, involving 5-fluorouracil (5-FU) with leucovorin and oxaliplatin (FOLFLOX) in particular, are the standard of care for patients with CRCLM (11). While the goal of the standard of care is the resection of all metastases with negative histological margins while preserving sufficient functional hepatic parenchyma (12), most patients cannot undergo surgery (12). In fact, due to factors such as location and size of the tumor, unresectable disease, presence of extrahepatic disease, or patients' comorbidities, surgery is only in 10-20% of cases, with a 5-year survival rate as low as 30% (12). In addition, standard anti-cancer therapies such as chemotherapy, radiotherapy, targeted therapy, and surgery frequently result in unsatisfactory outcomes (13). Despite significant progress in the development of new chemotherapeutic drugs, CRCLM patients receiving 5-FU and chemotherapy eventually develop chemotherapy resistance (7).

1.2.3. CRCLM growth patterns and metastatic cascade

CRCLM lesions have two major histopathological growth patterns (HGPs): desmoplastic (DHGP) and replacement (RHGP) (14). Micrometastases within the liver can co-opt pre-existing vasculature for oxygenation; a phenomenon frequently observed in RHGP tumors. In contrast, DHGP tumors are dependent on neo-vascularization, most commonly via a VEGF-mediated

angiogenesis (14). The process of liver metastasis can be generally divided into four major phases – the microvascular phase, involving cancer cell intravasation and arrest in sinusoidal vessels; the extravascular pre-angiogenic phase, where type I and IV collagen and fibronectin are deposited to provide a matrix for cell migration and colonization; the angiogenic phase, for DHGP tumors, which entails neovascularization that provides oxygen and nutrients supply to cancer cells, or vessel co-option for RHGP tumors; and the growth phase during which liver metastases expand and establish clinically detectable tumours (15, 16). At each of these phases, diverse interactions occur between the cancer cells and resident liver and immune cells, as well as recruitment of host immune cells, which all participate in a complex interactive network.

1.2.4. The liver immune microenvironment

1.2.4.1. Kupffer cells

The liver-resident macrophages, known as Kupffer cells (KCs), reside in the sinusoidal vessels and play important roles in lipid metabolism and immunity. The KCs represent 80-90% of tissue macrophages in the reticuloendothelial system and account for about 15% of the total hepatic cell population (17). The resident-macrophages have a unique ontology as they are derived from the yolk sac and seed the liver early during embryogenesis. KCs are then perpetually replenished within the liver over the life span. Classically, KCs are characterized by the high expression of F4/80, combined with other markers such as $CD11b^{+}$, $CD68^{+}$, and $Clec4f^{+}$. Under steady state, KCs have been shown to play important roles in the induction of immune tolerance including liver transplant tolerance, because of their low expression of major histocompatibility complex (MHC) class I and II antigens that lead to incomplete T cell activation. Furthermore, KCs can display an anti-inflammatory M2-like phenotype, expressing interleukin (IL)-10 and transforming growth

factor (TGF)-β, which impede T cell and dendritic cell (DCs) activation (18, 19). When homeostasis is disrupted as in LM disease, KCs can induce an inflammatory response by shifting to an M1-like macrophage phenotype, expressing pro-inflammatory cytokines/chemokines such as tumor necrosis factor (TNF)-α, IL-12, IL-1β, IL-6, CX3CL1, CXCL10, CCL2, and CCL5. KCs –derived CCL2 can promote the recruitment and infiltration of $CCR2^{+}$ $Ly6C^{high}$ bone marrow-derived macrophages. Furthermore, KC-derived CCL2 and CCL5 can recruit hepatic stellate cells (HSCs) (20) and CCL5 regulates the recruitment of T cells and NK cells in liver disease (21). However, KCs are a double-edged sword in LM, as they are able to promote a favorable environment for cancer cells by indirectly promoting the production of fibronectin (22).

1.2.4.2. Monocyte-derived macrophages

In addition to the resident-macrophages, bone marrow (BM)-derived monocytes are also recruited to the liver. Under pathological conditions, these cells are recruited in response to inflammatory signaling, and invasion of metastatic cells (23). In mice, BM-derived monocytes are characterized based on high expression of CCR2 and Ly6C in addition to the markers CD11b, CD68, CX3CR1, and CSFR1 and can either lack or express low levels of F4/80. Following the response to inflammatory triggers, inflammatory monocytes have the potential to migrate to the injured site and differentiate into inflammatory monocyte (Mo)-derived macrophages and monocyte-derived DCs. Inflammatory Mo-derived macrophages have the potential to detect and phagocytose pathogens and can secrete IL-1β, TNF-α, iNOS and CCL2. At later stages of inflammation, the inflammatory Mo-derived macrophages can ultimately mature into a restorative phenotype, which contributes to tissue repair and remodelling during resolution of inflammation. These restorative Mo-derived macrophages mature in an M-CSF/CSF1R dependent manner and are characterized based on a $Ly6C^{-}$ $CCR2^{-}$ $CX3CR1^{+}$ phenotype and can secrete matrix

metalloproteases (MMPs), IL-10, and vascular-endothelial growth factor A (VEGF-A). Mo-derived macrophages are ontologically, phenotypically, and functionally distinct from KCs and can adopt an anti- or pro-inflammatory phenotype depending on the signals they encounter in the liver microenvironment (22).

1.2.4.3. Natural Killer cells

Unlike T and B cells, NK cells do not have antigen-specific receptors but are able to screen and detect changes in membrane glycoproteins on target cells (24). NK cells also preferentially kill target cells that lack or express low levels of antigen-presenting molecules, such as MHC-I, as an escape from adaptive immunity (25). When activated, NK cells can directly lyse tumor cells via the release of cytolytic granules, such as perforin and granzyme B (GrzB), and can secrete a variety of cytokines such as IFN-γ, TNF-α, and GM-CSF, which recruit and can activate immune cells, such as T cells and macrophages (26). Growing evidence show that, in the immunosuppressive tumor microenvironment, NK cells can become dysfunctional through exposure to inhibitory molecules produced by cancer cells, including a PD-1/PDL-1 interaction, leading to tumor cell escape (27, 28). In the microvascular phase of the LM cascade, liver NK cells (pit-cells) constitute a first line of defense and can release cytolytic granules and cause direct cytolysis of cancer cells arrested in the liver sinusoids (29). In CRCLM, loss of NK cells increased tumor cell growth, and enhanced NK cells activity reduced LM (30, 31).

1.2.4.4. Natural Killer T cells

Natural Killer T (NKT) cells are particularly enriched in the liver and link the innate and adaptive immune responses. These highly cytotoxic cells express TCR-αβ chains in addition to the typical NK cell markers (32). In mice, NKT cells account for up to 55% of all hepatic $CD3^+$ T cells, but

for only 1-5% of T cells in the circulation, spleen, thymus, and lymph nodes. In human, NKT cells co-express CD3 and NK receptors such as CD56, CD16, and CD161 and account for about 33% of all hepatic T cells and about 2% of peripheral T cells (24). NKT cells can be directly activated by glycolipid antigens that bind to CD1d receptors and can be indirectly activated in response to TLR agonists and other inflammatory mediators (33). Upon activation, NKT cells can kill tumor cells directly by rapidly secreting cytolytic proteins such as perforin and Granzyme B (GrzB) and can also secrete a range of cytokines/chemokines including CCL5, CCL11, MIP-1α, and MIP-1β chemokines that recruit NK cells, inflammatory neutrophils and monocytes to sites of inflammation (34). In the liver, it has been reported that CXCL16 could promote NKT cell infiltration to suppress CRCLM *in vivo* (35). Furthermore, it has been demonstrated that activation of the CCRK-CXCL1-MDSC axis could promote CRCLM via the suppression of hepatic NKT cells, whereas MDSC depletion restored hepatic NKT cells and reduced CRCLM (36). In contrast, some studies reported that NKT cells can increase LM arising from intraocular melanomas by inhibiting the anti-tumor activity of NK cells in the liver (37).

1.2.4.5. Dendritic cells

Dendritic cells (DCs) are bone-marrow derived and are the classical antigen-presenting cells of the lymphatic system. DCs take up antigens in peripheral tissues, process them to proteolytic peptides, and present these peptides in association with MHC class I and II molecules (38). DCs can then migrate to secondary lymphoid organs and present these antigens to the T cell receptor (TCR) on the surface of T lymphocytes, thus initiating antigen-specific immune responses, or immune tolerance (39). Cytotoxic T cells, expressing CD8, recognize MHCI-bound antigens, whereas helper T cells, expressing CD4, recognize MHCII-bound antigens (39). Hepatic DCs play a key role in maintaining hepatic immune homeostasis and tolerance. During

inflammation, a major expansion and maturation of hepatic DCs expressing CD11b, CD11c and MHCII occurs via CD80 stimulation (40). The role of DCs in liver metastasis is not yet fully understood, however it is known that DCs play a key role in the initiation and amplification of anti-tumor responses and in driving clinical success of immunotherapy (41). Furthermore, in liver cancers such as hepatocellular carcinoma (HCC), an insufficient crosstalk between DCs and T cells is one of the main mechanisms of immune tolerance (42).

1.2.4.6. Hepatic Stellate cells

The Hepatic Stellate cells (HSCs) constitute about 15% of the non-parenchymal hepatic cells and reside in the liver peri-sinusoidal region, known as space of Disse (15). Normally quiescent, HSCs become activated in response to liver damage and inflammation and are the main orchestrator of the fibrotic response. HSCs gain a myofibroblast-like phenotype (α-smooth muscle actin-positive HSCs) and deposit extracellular matrix (ECM) rich in type I and type IV collagen and fibronectin hence promoting fibrosis (15). These cells can also secrete cytokines and growth factors, such as macrophage inflammatory protein-2 (MIP-2), TGF-β, CCL2 and CCL5 that recruit and activate macrophages and other immune cells (43). In liver metastasis, HSCs play a pro-metastatic role the liver microenvironment, as they can induce an immunosuppressive hepatic microenvironment and promote angiogenesis via the secretion of ECM proteins, TGF-β, SDF-1 (CXCL12), and VEGF (44).

1.3. Sexual dimorphism in cancer, immunity, and therapy response

Sex is a biological factor that affects the development and progression of many diseases, such as cancer. Significant differences between males and females have been noted in terms of

cancer incidence, prognosis, and mortality. While some sex disparities are hormonally regulated by estrogen, progesterone, and testosterone levels, sex disparities can also be hormone-independent of sex hormones and arise from "sexual differentiation" involving genetic and epigenetic processes. The combined action of sex hormones and sexual differentiation factors fine tunes cancer cells metabolism, host innate and adaptive immunity, and even the response to, and toxicity from cancer therapy. In this section, the complex interactions between sex hormones and sexual differentiation will be reviewed with a focus on their effects on the tumor microenvironment, cancer initiation and progression, the immune system, metabolism, and the response to cancer therapy.

1.3.1. Cancer Incidence and Survival

Survival analyses conducted on 3,705,584 patients (44.6% males, 55.4% females) revealed that males had a lower cancer incidence (male-to-female incidence rate ratio of 0.958, $p < 0.001$) but worse survival (male-to-female hazard ratio of 1.568, $p<0.001$) compared with females (45). In particular, Kaposi sarcoma had the highest male-to-female incidence, while thyroid cancer had the highest male-to-female survival difference (45). Interestingly, in CRCLM patients, it has been reported that females have significantly lower overall survival rate compared to males and have a higher frequency of extrahepatic metastases (46, 47).

1.3.2. Immunity

The immune system plays an important role in cancer development and progression. There are various mechanisms through which cancer cells escape immune response including suppression by regulatory T cells (Treg) (48) and induction of an immunosuppressive microenvironment, such as in liver metastasis where activated antigen-specific Fas+CD8+ T cells

undergo apoptosis following their interaction with FasL+ CD11b+F4/80+ monocyte-derived macrophages (2). Sex differences in the immune system likely contribute to the sexual disparity in incidence and mortality associated with certain cancers. Immunological sex differences in the innate and adaptive immune system are due to both sex hormones and X-chromosome-linked genes. Females generally mount stronger innate and adaptive immune responses than males (49). The latter is consistent with the epidemiological studies reporting a higher prevalence of autoimmune diseases in females than in males (50, 51).

1.3.2.1. Innate Immunity

The number and activity of cells associated with innate immunity differ between sexes. Males tend to have higher NK cell frequencies than females (52), but the phagocytic activity of neutrophils and macrophages is higher in females than males (53). Female antigen-presenting cells (APCs) are more efficient antigen presenters than male APCs (54). As cancer cells can modulate antigen-presentation to escape immune destruction, notably via the secretion of tumor extracellular vesicles (TEVs) containing TGF-β (55), sexual differences in APCs function can have a considerable impact on anti-tumor immunity and response to immunotherapy. Neutrophils and lymphocytes express the estrogen receptor (ER) and the androgen receptor (AR), and sex hormones can modulate the immune system at multiple levels by controlling cytokine release. In macrophages, estrogen was shown to reduce the production of the pro-inflammatory cytokines interleukin-1 (IL-1β), IL-6 and TNF while in neutrophils, estrogen upregulates the production of nitric-oxide synthase (NOS) and nitric oxide (NO), thereby promoting their anti-inflammatory effects, and decreases their chemotactic activity (56). Thus, the overall effect of estrogen/ER signaling on these immune cells appears to be a dampening of the inflammatory responses. Estrogen is known to directly or indirectly inhibit NK cells cytotoxic activity, where the latter

function is dampened by the estrogen-induced production of the GrzB inhibitor protease inhibitor 9 (PI-9) by cancer cells (57). Another study reported that splenic NK and NKT cell numbers and IFN-γ secretion were increased with estrogen stimulation (58). Of interest, AR induces neutrophil proliferation and inhibits T cells proliferation, and a higher neutrophil/lymphocyte ratio was shown to be a poor prognosis biomarker in several types of cancer (44)(44). It has been reported that males produce higher levels of IL-6 compared to females (59). Males have a 3-5-fold higher incidence of hepatocellular carcinoma (HCC), the most common liver cancer, than females. A study by Naugler et al found that this can be attributed to sex differences in MyD88-dependent IL-6 production by Kupffer cells and has shown that IL-6 expression in KC was inhibited by estrogen. IL-6 ablation in male mice reduced the incidence of diethylnitrosamine – induced HCC in mice (60).

1.3.2.2. Adaptive Immunity

Several immune-related genes are located on the X-chromosome and are more highly expressed in females including FoxP3, expressed by T regs, and CD40L (56). Furthermore, several immune-related genes expressed in T cells have estrogen response elements in their promoters and thereby estrogen can regulate cytotoxic T cell responses in females. These genes include among others NF-κB, IFN-γ, and IL-10 (61-63). NF-κB response elements in turn have been found in the promoter regions of several cytokine genes such as IL-10, TNF-α, IL-1β, and IL-12 (64). Both estrogen receptors (ERα and ERβ) are expressed on B and T cells which allows a direct regulatory role by estrogen in these cells. After binding to the receptor, the estrogen-ERα (or –ERβ) complex translocates to the nucleus, where it bind to ER-response elements found in the gene promoter regions, regulating their transcription (65). While females $CD4^+$ T cells produce higher IFN-γ levels and have a higher proliferative rate, males $CD4^+$ produce higher levels of IL-17A, which is

known to activate angiogenesis and immunosuppressive activities and stimulate production of effectors $CD8^+$ (66). In addition to the various effects that sex hormones can have on immune cell populations, they can also affect the immune system indirectly, for example, through the gut microbiome (67). Gut microbiota composition can affect immunity, and hence cancer development, through several mechanisms that are not yet fully understood and can also affect the efficacy and toxicity of chemotherapy, the latter being greater in females (68).

1.3.3. Response to Cancer Therapies

Although a sexual dimorphism in the immune response is well documented, the effect of sex on patients' response to immune checkpoint inhibitors (ICIs) in cancer treatment, has not been adequately investigated. An evaluation of sex representation in phase I, II, and III clinical trials from January 2011 to December 2013 revealed a male recruitment prevalence in all studies, with only 4.1% of trials having equal representation of the two sexes (69). It is only since 2014 that the National Institute of Health (NIH) requested to include sex as a biological variable in all funded research (70). Growing evidence suggest that sex influences pathophysiology, clinical symptoms, response to treatment, and outcome in cancer therapy and these differences arise from the intricate interplay between sex-related factors, variable pharmacokinetic and pharmacodynamic parameters, and genetic factors important for pharmacology (71).

1.3.3.1. Chemotherapy

Sex differences have been observed in chemotherapy response including toxicity. Fluorouracil (5-FU), a pyrimidine antagonist that inhibits thymidine synthase essential for DNA synthesis, has been used in the treatment of several cancers as an effective chemotherapeutic drug. It has been reported that 5-FU has a higher clearance in males (179 $l/h/m^2$) than in female (155

l/h/m^2) (72). The lower clearance of 5-FU in females may explain the higher toxicity. The activity of dihydropyrimidine dehydrogenase (DPD), an enzyme that breaks down 5-FU and plays an important role in 5-FU toxicity, was found to be 15% lower in females than in males (73). The reduced activity of DPD is associated with higher toxicity of 5-FU chemotherapy in females who experienced side effects such as stomatitis, alopecia, leukopenia, and diarrhea more frequently than males (73). Sexual disparity was also found for the anti-cancer drug Doxorubicin-an anthracycline DNA-damaging agent that targets topoisomerase 2 activity and DNA intercalation and generates free radicals that cause DNA damage. In lymphoma or breast cancer patients, males had better doxorubicin clearance (59 l/h/m2) than female (27 l/h/m2). Signs of higher toxicity in females included cardiac abnormalities. This is thought to be due to lower levels in females of the multidrug resistance protein P-glycoprotein - a drug transporter that pumps foreign and toxic substances out of cells (73).

1.3.3.2. Immunotherapy

Immunotherapy (IT) based on the use of immune checkpoint inhibitors (ICIs) has achieved a degree of success in the treatment of advanced solid tumors in recent years. IT with ICIs enhances the anti-tumor effect of patients own immune system by reactivating the antitumor activity of exhausted T cells and NK cells (74). This can be achieved through blockade of inhibitory interactions between APCs and lymphocytes, such as T and NK cells, at immune checkpoints (anti-PD-1/PD-L1, anti-CTLA-4, anti-TIM-3, anti-LAG-3) (75). IT effectiveness is influenced by several factors, including the type of immune response, the intrinsic characteristics of the cancer cells and the tumor microenvironment (70). However, in many cancers IT provided limited or no clinical benefits to most patients, due, in part, to the immunosuppressive nature of the tumor

microenvironment (TME) (76). Some patients fail to develop an initial response to treatment with ICIs, and challenges remain in applying IT to CRC with liver metastases (12).

In 2020, a meta-analysis of 27 randomized clinical trials revealed a superior overall survival in male than in female patients receiving ICIs treatment, regardless of the protocol, the type of drug administered or the type of malignancy (3). This analysis revealed that male patients with colorectal cancer or glioblastoma multiforme treated with anti-PD-1/PD-L1 therapy had better overall survival (OS) (3). While the reasons behind this underappreciated sexual dimorphism in IT response remain unclear, it was suggested that in females cancer cells can evade immune surveillance mechanisms more efficiently and undergo a more effective immune-editing process to become metastatic (70). This renders the metastatic cells less immunogenic and more resistant to immunotherapy (70). It is also possible that the increase susceptibility of women to autoimmune diseases may increase the adverse effects associated with immunotherapy subvert an effective immune response and also require abrupt termination of treatment (77).

1.4. The estrogen axis in liver metastasis

1.4.1. The estrogen axis

Estrogens are the primary female sex hormones and are pleiotropic steroid hormones that influence multiple biological processes including development, metabolism, and immunity. The estrogens are primarily produced in the ovaries, corpus luteum, and placenta, although a small amount can also be produced by adipose tissue and non-gonadal organs, such as liver, heart, skin, and brain (78). There are three major forms of physiological estrogen in females, estrone (E1), estradiol (E2

or 17β-estradiol), and estriol (E3), with E2 being the main form of estrogen in premenopausal women (78).

1.4.1.1. Estrogens and estrogen receptors signaling

Estrogen signaling is primarily mediated through estrogen receptors (ERs). The two estrogen receptors ERα (encoded by *ESR1*) and ERβ (encoded by *ESR2*) are members of the nuclear receptor superfamily acting as transcription factors to regulate target genes transcription (78, 79). The *ESR1* and *ESR2* genes are located on different chromosomes, have different cellular distributions and can crosstalk in some cell types and tissues (80). *ESR1* expression was documented in most immune cells including B and all T lymphocytes, macrophages, neutrophils, NK cells and DCs (81, 82), while *ESR2* was reported to be expressed in some DCs, monocytes, and NK cells and to a lesser extent in mature T lymphocytes (83, 84). Notably, unlike T cells, B lymphocytes express higher ERβ than ERα levels (85).

1.4.1.2. Estrogen receptor inhibitors

Endocrine-based therapy (ET) also known as hormone/hormonal therapy includes different strategies for either enhancing or inhibiting the action of steroid hormones for treatment purposes. Direct targeting of ERα can be achieved by selective estrogen receptor modulators (SERMs) (e.g. Tamoxifen) and selective estrogen receptor degraders (SERDs) (e.g. Fulvestrant). SERMs compete with estrogen to bind to ER and can act as antagonists or agonists (86). On the other hand, SERDs bind to the ER to create an unstable protein complex that directs ER to proteasome degradation (87). Fulvestrant is a first-generation SERD approved by the FDA in 2007 for the treatment of metastatic luminal breast cancer in postmenopausal patients (88). Although effective, tamoxifen has important disadvantages, including a limited period of activity before resistance

developed and an increased risk of endometrial cancer and thromboembolism due to its partial agonist activity (89-91).

1.4.2. Previous findings and rationale for the present study

Our laboratory previously documented a sexual dimorphism in the tumor immune microenvironment of LM and identified estrogen as a central regulator of this tumor microenvironment (TME) (4). In particular, the accumulation of myeloid-derived suppressor cells (MDSC) and regulatory T cells in colon and lung carcinoma LM was found to be TNFR2-dependent in female, but not in male mice. In ovariectomized (OVX; estrogen-depleted) mice, a marked reduction in colorectal, lung, and pancreatic carcinoma LM was observed relative to SHAM-operated control (SHAM; estrogen-competent) mice, and this trend was reversible upon estradiol (E2) supplementation. Furthermore, treatment with the selective estrogen receptor modulator tamoxifen increased the number of cytotoxic $CD8^+$ T cells in the liver and reduced the number of CRCLM.

1.4.3. Aims of the thesis project

The overall goal of my research project was to gain a deeper understanding of the mechanisms underlying the regulation of the TME of CRCLM, with the long-term aim of designing novel therapies that could prevent this deadly process. The changes observed in the IME of LM in OVX mice implicated estrogen signaling in the recruitment and function of diverse immune cell types in the liver. Here we aimed to identify estrogen-regulated molecular mediators that drive this recruitment and the key immune cells that produce them. Furthermore, we aimed to assess whether targeting the immunosuppressive TME of CRCLM with estrogen receptor inhibitors would potentiate immunotherapy for mCRC. The specific research objectives were

therefore as follows: 1) evaluate the effects of estrogen depletion on the cytokine/chemokine repertoire of the liver 2) identify immune cells that produce and respond to these mediators and 3) evaluate the therapeutic effect and changes to the liver TME in mice treated with combinatorial therapy consisting of estrogen inhibitors and anti-PD-1 immunotherapy.

1.5. References

1. de Ridder J, de Wilt JH, Simmer F, Overbeek L, Lemmens V, Nagtegaal I. Incidence and origin of histologically confirmed liver metastases: an explorative case-study of 23,154 patients. Oncotarget. 2016;7(34):55368-76.
2. Yu J, Green MD, Li S, Sun Y, Journey SN, Choi JE, et al. Liver metastasis restrains immunotherapy efficacy via macrophage-mediated T cell elimination. Nat Med. 2021;27(1):152-64.
3. Ye Y, Jing Y, Li L, Mills GB, Diao L, Liu H, et al. Sex-associated molecular differences for cancer immunotherapy. Nat Commun. 2020;11(1):1779.
4. Milette S, Hashimoto M, Perrino S, Qi S, Chen M, Ham B, et al. Sexual dimorphism and the role of estrogen in the immune microenvironment of liver metastases. Nat Commun. 2019;10(1):5745.
5. Ciner AT, Jones K, Muschel RJ, Brodt P. The unique immune microenvironment of liver metastases: Challenges and opportunities. Semin Cancer Biol. 2021;71:143-56.
6. Aziz H, Ahmed Z, Lee Y, Drumm G, Saif MW. A Comprehensive Review of Management of Colorectal Liver Mets in the Current Era. Cancer Med J. 2022;5(1):46-57.
7. Zhou H, Liu Z, Wang Y, Wen X, Amador EH, Yuan L, et al. Colorectal liver metastasis: molecular mechanism and interventional therapy. Signal Transduction and Targeted Therapy. 2022;7(1):70.
8. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209-49.
9. Siegel RL, Jakubowski CD, Fedewa SA, Davis A, Azad NS. Colorectal Cancer in the Young: Epidemiology, Prevention, Management. Am Soc Clin Oncol Educ Book. 2020;40:1-14.
10. Martin J, Petrillo A, Smyth EC, Shaida N, Khwaja S, Cheow HK, et al. Colorectal liver metastases: Current management and future perspectives. World J Clin Oncol. 2020;11(10):761-808.
11. Choong ZS, Wong KH, Yang TWW, Nguyen M, Duieb Z. Complete resolution of rectal cancer with liver metastases after palliative chemoradiotherapy and unplanned surgical resection—a case report. Journal of Surgical Case Reports. 2023;2023(2).

12. Yu X, Zhu L, Liu J, Xie M, Chen J, Li J. Emerging Role of Immunotherapy for Colorectal Cancer with Liver Metastasis. Onco Targets Ther. 2020;13:11645-58.
13. Akgül Ö, Çetinkaya E, Ersöz Ş, Tez M. Role of surgery in colorectal cancer liver metastases. World J Gastroenterol. 2014;20(20):6113-22.
14. Rada M, Hassan N, Lazaris A, Metrakos P. The molecular mechanisms underlying neutrophil infiltration in vessel co-opting colorectal cancer liver metastases. Front Oncol. 2022;12:1004793.
15. Tsilimigras DI, Brodt P, Clavien P-A, Muschel RJ, D'Angelica MI, Endo I, et al. Liver metastases. Nature Reviews Disease Primers. 2021;7(1):27.
16. Milette S, Sicklick JK, Lowy AM, Brodt P. Molecular Pathways: Targeting the Microenvironment of Liver Metastases. Clin Cancer Res. 2017;23(21):6390-9.
17. Bouwens L, Baekeland M, De Zanger R, Wisse E. Quantitation, tissue distribution and proliferation kinetics of Kupffer cells in normal rat liver. Hepatology. 1986;6(4):718-22.
18. Sakai T, Lin W-L. Kupffer Cells as a Target for Immunotherapy. J. 2022;5(4):532-7.
19. Nguyen-Lefebvre AT, Horuzsko A. Kupffer Cell Metabolism and Function. J Enzymol Metab. 2015;1(1).
20. Li H, Zhou Y, Wang H, Zhang M, Qiu P, Zhang M, et al. Crosstalk Between Liver Macrophages and Surrounding Cells in Nonalcoholic Steatohepatitis. Frontiers in Immunology. 2020;11.
21. Chen L, Zhang Q, Yu C, Wang F, Kong X. Functional roles of CCL5/RANTES in liver disease. Liver Research. 2020;4(1):28-34.
22. Keirsse J, Van Damme H, Geeraerts X, Beschin A, Raes G, Van Ginderachter JA. The role of hepatic macrophages in liver metastasis. Cellular Immunology. 2018;330:202-15.
23. Kim J, Bae JS. Tumor-Associated Macrophages and Neutrophils in Tumor Microenvironment. Mediators Inflamm. 2016;2016:6058147.
24. Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in the human liver. Immunol Rev. 2000;174:5-20.
25. Crispe IN. The liver as a lymphoid organ. Annu Rev Immunol. 2009;27:147-63.
26. Paul S, Lal G. The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. Front Immunol. 2017;8:1124.

27. Quatrini L, Mariotti FR, Munari E, Tumino N, Vacca P, Moretta L. The Immune Checkpoint PD-1 in Natural Killer Cells: Expression, Function and Targeting in Tumour Immunotherapy. Cancers (Basel). 2020;12(11).
28. Cózar B, Greppi M, Carpentier S, Narni-Mancinelli E, Chiossone L, Vivier E. Tumor-Infiltrating Natural Killer Cells. Cancer Discov. 2021;11(1):34-44.
29. Brodt P. Role of the Microenvironment in Liver Metastasis: From Pre- to Prometastatic Niches. Clin Cancer Res. 2016;22(24):5971-82.
30. Bertin S, Neves S, Gavelli A, Baqué P, Brossette N, Simões S, et al. Cellular and molecular events associated with the antitumor response induced by the cytosine deaminase/5-fluorocytosine suicide gene therapy system in a rat liver metastasis model. Cancer Gene Ther. 2007;14(10):858-66.
31. Takehara T, Uemura A, Tatsumi T, Suzuki T, Kimura R, Shiotani A, et al. Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNalpha gene to mice. Int J Cancer. 2007;120(6):1252-60.
32. Bandyopadhyay K, Marrero I, Kumar V. NKT cell subsets as key participants in liver physiology and pathology. Cellular & Molecular Immunology. 2016;13(3):337-46.
33. Wu L, Van Kaer L. Natural killer T cells in health and disease. Front Biosci (Schol Ed). 2011;3(1):236-51.
34. Krijgsman D, Hokland M, Kuppen PJK. The Role of Natural Killer T Cells in Cancer-A Phenotypical and Functional Approach. Front Immunol. 2018;9:367.
35. Kee JY, Ito A, Hojo S, Hashimoto I, Igarashi Y, Tsukada K, et al. Chemokine CXCL16 suppresses liver metastasis of colorectal cancer via augmentation of tumor-infiltrating natural killer T cells in a murine model. Oncol Rep. 2013;29(3):975-82.
36. Zeng X, Zhou J, Xiong Z, Sun H, Yang W, Mok MTS, et al. Cell cycle-related kinase reprograms the liver immune microenvironment to promote cancer metastasis. Cell Mol Immunol. 2021;18(4):1005-15.
37. Yang W, Li H, Mayhew E, Mellon J, Chen PW, Niederkorn JY. NKT cell exacerbation of liver metastases arising from melanomas transplanted into either the eyes or spleens of mice. Invest Ophthalmol Vis Sci. 2011;52(6):3094-102.
38. Guermonprez P, Valladeau J, Zitvogel L, Théry C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol. 2002;20:621-67.

39. Płytycz B, Seljelid R. MHC molecules and lymphocytes: evolutionary perspective. Arch Immunol Ther Exp (Warsz). 1998;46(3):137-42.
40. Méndez-Sánchez N, Córdova-Gallardo J, Barranco-Fragoso B, Eslam M. Hepatic Dendritic Cells in the Development and Progression of Metabolic Steatohepatitis. Front Immunol. 2021;12:641240.
41. Subtil B, Iyer KK, Poel D, Bakkerus L, Gorris MAJ, Escalona JC, et al. Dendritic cell phenotype and function in a 3D co-culture model of patient-derived metastatic colorectal cancer organoids. Frontiers in Immunology. 2023;14.
42. Lurje I, Hammerich L, Tacke F. Dendritic Cell and T Cell Crosstalk in Liver Fibrogenesis and Hepatocarcinogenesis: Implications for Prevention and Therapy of Liver Cancer. Int J Mol Sci. 2020;21(19).
43. Lee Y, Friedman SL. Chapter 6 - Fibrosis in the Liver: Acute Protection and Chronic Disease. In: Kaestner KH, editor. Progress in Molecular Biology and Translational Science. 97: Academic Press; 2010. p. 151-200.
44. Kang N, Gores GJ, Shah VH. Hepatic stellate cells: partners in crime for liver metastases? Hepatology. 2011;54(2):707-13.
45. Dong M, Cioffi G, Wang J, Waite KA, Ostrom QT, Kruchko C, et al. Sex Differences in Cancer Incidence and Survival: A Pan-Cancer Analysis. Cancer Epidemiology, Biomarkers & Prevention. 2020;29(7):1389-97.
46. Hendifar A, Yang D, Lenz F, Lurje G, Pohl A, Lenz C, et al. Gender disparities in metastatic colorectal cancer survival. Clin Cancer Res. 2009;15(20):6391-7.
47. Wu Y, Xu W, Wang L. IDDF2021-ABS-0191 Gender matters: sex disparities in colorectal cancer liver metastasis survival: a population-based study. Gut. 2021;70(Suppl 2):A142-A3.
48. Jacobs JF, Nierkens S, Figdor CG, de Vries IJ, Adema GJ. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? Lancet Oncol. 2012;13(1):e32-42.
49. Klein SL, Flanagan KL. Sex differences in immune responses. Nature Reviews Immunology. 2016;16(10):626-38.
50. Whitacre CC. Sex differences in autoimmune disease. Nat Immunol. 2001;2(9):777-80.
51. Lockshin MD. Sex differences in autoimmune disease. Lupus. 2006;15(11):753-6.
52. Abdullah M, Chai PS, Chong MY, Tohit ER, Ramasamy R, Pei CP, et al. Gender effect on in vitro lymphocyte subset levels of healthy individuals. Cell Immunol. 2012;272(2):214-9.

53. Spitzer JA. Gender differences in some host defense mechanisms. Lupus. 1999;8(5):380-3.
54. Weinstein Y, Ran S, Segal S. Sex-associated differences in the regulation of immune responses controlled by the MHC of the mouse. J Immunol. 1984;132(2):656-61.
55. Del Prete A, Salvi V, Soriani A, Laffranchi M, Sozio F, Bosisio D, et al. Dendritic cell subsets in cancer immunity and tumor antigen sensing. Cellular & Molecular Immunology. 2023.
56. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. Nature Reviews Immunology. 2008;8(9):737-44.
57. Jiang X, Ellison SJ, Alarid ET, Shapiro DJ. Interplay between the levels of estrogen and estrogen receptor controls the level of the granzyme inhibitor, proteinase inhibitor 9 and susceptibility to immune surveillance by natural killer cells. Oncogene. 2007;26(28):4106-14.
58. Nakaya M, Tachibana H, Yamada K. Effect of estrogens on the interferon-gamma producing cell population of mouse splenocytes. Biosci Biotechnol Biochem. 2006;70(1):47-53.
59. Sperry JL, Friese RS, Frankel HL, West MA, Cuschieri J, Moore EE, et al. Male gender is associated with excessive IL-6 expression following severe injury. J Trauma. 2008;64(3):572-8; discussion 8-9.
60. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science. 2007;317(5834):121-4.
61. Cerillo G, Rees A, Manchanda N, Reilly C, Brogan I, White A, et al. The oestrogen receptor regulates NFkappaB and AP-1 activity in a cell-specific manner. J Steroid Biochem Mol Biol. 1998;67(2):79-88.
62. Kube D, Platzer C, von Knethen A, Straub H, Bohlen H, Hafner M, et al. Isolation of the human interleukin 10 promoter. Characterization of the promoter activity in Burkitt's lymphoma cell lines. Cytokine. 1995;7(1):1-7.
63. Fox HS, Bond BL, Parslow TG. Estrogen regulates the IFN-gamma promoter. J Immunol. 1991;146(12):4362-7.
64. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol. 1994;12:141-79.

65. Hewagama A, Patel D, Yarlagadda S, Strickland FM, Richardson BC. Stronger inflammatory/cytotoxic T-cell response in women identified by microarray analysis. Genes Immun. 2009;10(5):509-16.
66. Asadzadeh Z, Mohammadi H, Safarzadeh E, Hemmatzadeh M, Mahdian-Shakib A, Jadidi-Niaragh F, et al. The paradox of Th17 cell functions in tumor immunity. Cell Immunol. 2017;322:15-25.
67. Kim YS, Unno T, Kim BY, Park MS. Sex Differences in Gut Microbiota. World J Mens Health. 2020;38(1):48-60.
68. Özdemir BC, Csajka C, Dotto GP, Wagner AD. Sex Differences in Efficacy and Toxicity of Systemic Treatments: An Undervalued Issue in the Era of Precision Oncology. J Clin Oncol. 2018;36(26):2680-3.
69. Prakash VS, Mansukhani NA, Helenowski IB, Woodruff TK, Kibbe MR. Sex Bias in Interventional Clinical Trials. J Womens Health (Larchmt). 2018;27(11):1342-8.
70. Irelli A, Sirufo MM, D'Ugo C, Ginaldi L, De Martinis M. Sex and Gender Influences on Cancer Immunotherapy Response. Biomedicines. 2020;8(7).
71. Wang J, Huang Y. Pharmacogenomics of sex difference in chemotherapeutic toxicity. Curr Drug Discov Technol. 2007;4(1):59-68.
72. Taflin H, Odin E, Carlsson G, Tell R, Gustavsson B, Wettergren Y. Plasma deoxyuridine as a surrogate marker for toxicity and early clinical response in patients with metastatic colorectal cancer after 5-FU-based therapy in combination with arfolitixorin. Cancer Chemother Pharmacol. 2021;87(1):31-41.
73. Kim HI, Lim H, Moon A. Sex Differences in Cancer: Epidemiology, Genetics and Therapy. Biomol Ther (Seoul). 2018;26(4):335-42.
74. Koi M, Carethers JM. The colorectal cancer immune microenvironment and approach to immunotherapies. Future Oncol. 2017;13(18):1633-47.
75. Heinzerling L, de Toni EN, Schett G, Hundorfean G, Zimmer L. Checkpoint Inhibitors. Dtsch Arztebl Int. 2019;116(8):119-26.
76. Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. Nat Med. 2018;24(5):541-50.

77. Tsiouda T, Sardeli C, Porpodis K, Pilikidou M, Apostolidis G, Kyrka K, et al. Sex Differences and Adverse Effects between Chemotherapy and Immunotherapy for Non-Small Cell Lung Cancer. J Cancer. 2020;11(11):3407-15.
78. Cui J, Shen Y, Li R. Estrogen synthesis and signaling pathways during aging: from periphery to brain. Trends Mol Med. 2013;19(3):197-209.
79. Levin ER. Plasma membrane estrogen receptors. Trends in Endocrinology & Metabolism. 2009;20(10):477-82.
80. Couse JF, Lindzey J, Grandien K, Gustafsson JA, Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. Endocrinology. 1997;138(11):4613-21.
81. Kovats S. Estrogen receptors regulate innate immune cells and signaling pathways. Cell Immunol. 2015;294(2):63-9.
82. Khan D, Ansar Ahmed S. The Immune System Is a Natural Target for Estrogen Action: Opposing Effects of Estrogen in Two Prototypical Autoimmune Diseases. Front Immunol. 2015;6:635.
83. Maselli A, Conti F, Alessandri C, Colasanti T, Barbati C, Vomero M, et al. Low expression of estrogen receptor β in T lymphocytes and high serum levels of anti-estrogen receptor α antibodies impact disease activity in female patients with systemic lupus erythematosus. Biol Sex Differ. 2016;7:3.
84. Dama A, Baggio C, Boscaro C, Albiero M, Cignarella A. Estrogen Receptor Functions and Pathways at the Vascular Immune Interface. Int J Mol Sci. 2021;22(8).
85. Phiel KL, Henderson RA, Adelman SJ, Elloso MM. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. Immunol Lett. 2005;97(1):107-13.
86. Hernando C, Ortega-Morillo B, Tapia M, Moragón S, Martínez MT, Eroles P, et al. Oral Selective Estrogen Receptor Degraders (SERDs) as a Novel Breast Cancer Therapy: Present and Future from a Clinical Perspective. Int J Mol Sci. 2021;22(15).
87. Liu S, Han SJ, Smith CL. Cooperative activation of gene expression by agonists and antagonists mediated by estrogen receptor heteroligand dimer complexes. Mol Pharmacol. 2013;83(5):1066-77.

88. Di Leo A, Jerusalem G, Petruzelka L, Torres R, Bondarenko IN, Khasanov R, et al. Results of the CONFIRM phase III trial comparing fulvestrant 250 mg with fulvestrant 500 mg in postmenopausal women with estrogen receptor-positive advanced breast cancer. J Clin Oncol. 2010;28(30):4594-600.
89. Hurvitz SA, Pietras RJ. Rational management of endocrine resistance in breast cancer: a comprehensive review of estrogen receptor biology, treatment options, and future directions. Cancer. 2008;113(9):2385-97.
90. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. Annu Rev Med. 2011;62:233-47.
91. Robertson JF, Lindemann J, Garnett S, Anderson E, Nicholson RI, Kuter I, et al. A good drug made better: the fulvestrant dose-response story. Clin Breast Cancer. 2014;14(6):381-9.

Chapter 2

Estrogen Receptor Blockade Potentiates Immunotherapy Response to Colorectal Carcinoma Liver Metastases by Altering the Immunosuppressive Microenvironment

[Manuscript prepared for submission,

Abstract submitted and recommended for Nature Cancer]

Chapter 2: Estrogen receptor blockade potentiates immunotherapy of colorectal carcinoma liver metastases by altering the immunosuppressive microenvironment

Yasmine Benslimane, Kevin Amalfi, Sarah Lapin, Stephanie Perino, Matthew Leibovitch, Julien Chambon and Pnina Brodt.

2.1. Abstract

Liver metastases (LM) remain a major cause of cancer-associated death and is a clinical challenge. Up to 50% of colorectal cancer (CRC) patients develop liver metastases, and CRC is increasing in the younger population. Liver metastases and the female sex were identified as predictors of a poorer response to immunotherapy, but the reasons remain unclear. We previously reported on a sexual dimorphism in the control of the tumor microenvironment (TME) of CRC liver metastases and identified estrogen as a regulator of an immunosuppressive tumor microenvironment in the liver. Here we aimed to assess the effect of estrogen deprivation on the cytokine/chemokine profile induced by CRC metastases and their effect on the innate and adaptive immune responses in the liver. Moreover, we analyzed the benefit of combining a selective estrogen-receptor degrader with immune checkpoint blockade in the treatment of CRC liver metastases. We show here that ovariectomy-mediated estrogen depletion altered the cytokine/chemokine repertoire in the TME of CRCLM, decreased macrophage polarization as reflected in reduced accumulation of M2 macrophages and increased the accumulation of $CCL5^+/CCR5^+$ $CD8^+$ effector T cells and NKT cells in the liver TME. Importantly, treatment with the SERD Fulvestrant markedly enhanced the therapeutic benefit of anti-PD1 immunotherapy by

increasing the accumulation of $CD8^+CCL5^+$, $CD8^+CCR5^+$ T cells and NK cells in the liver TME and this resulted in a significant reduction in the outgrowth of liver metastases. Taken together, our results suggest that inhibition of estrogen signaling could potentiate the inhibitory effect of immunotherapy on liver metastases in hormone-independent and IT-resistant colon cancer.

Abbreviations : **LM,** Liver Metastases; **CRCLM,** Colorectal Carcinoma Liver Metastases; **GI,** gastrointestinal; **OS,** Overall Survival; **IT**, Immunotherapy **ICI,** Immune Checkpoint Inhibitor; **TME,** Tumor Microenvironment; **NKT,** Natural Killer T cell; **NK,** Natural Killer cell; **TAM,** Tumor-Associated Macrophages; **MDSC,** Myeloid-Derived Suppressor Cell; **OVX,** Ovariectomized; **SHAM,** Sham-operated control; **ER**, Estrogen Receptor; **E2**, Estradiol; **SERD,** Selective Estrogen Receptor Degrader; **SERM,** Selective Estrogen Receptor Modulator.

2.2. Introduction

Metastasis is the primary cause of cancer mortality, and it remains a major clinical challenge. The liver is a major site of metastases for cancers of the GI tract such as colorectal carcinoma (CRC) and for non-GI cancers such as uveal melanoma and breast cancer. This is due mainly to the liver's double blood supply from the hepatic artery and the portal vein. Up to 50% of CRC patients develop liver metastases (1). In the United States, the incidence of CRC in adults of 50 years and older dropped by 2% a year between 2014 and 2018, while it rose by 1.5% annually in adults younger than 50 years old during the same period (2). CRC has consequently become the 4^{th} most diagnosed cancer among 30-39 years old men and women (2). Furthermore, male patients had a better 8-year overall survival (OS) than female patients with left or right colon cancer and with synchronous or metachronous liver metastases (3). In patients diagnosed with advanced stage CRC, the 5-year survival rate stands at 12% (4). To date, the most effective treatment option is surgical resection of liver metastases. However, only approximately 20% of patients with CRCLM

are eligible for partial hepatectomy, generally due to size, number, and location of the metastases and relapse can occur in up to 75% of resected patients (5, 6). To improve these statistics, combinatorial therapies that also target the pro-metastatic tumor microenvironment of the liver are actively being sought.

The sexual dimorphism in immunity has been well documented (7). However, the impact of sex on patients' response to cancer immunotherapy (IT) is not yet fully understood. While immune checkpoint inhibitors (ICIs) have extended patient survival across multiple cancer types, meta-analysis of 27 randomized clinical trials revealed that there is a superior overall survival in male than in female patients receiving ICI treatment, regardless of the protocol, the type of drug administered or the type of malignancy (8). Moreover, liver metastases, but not metastases to other organs, limit IT efficacy both in metastatic patients and preclinical models (9). Thus, a better understanding of the role of sex and sex hormones, particularly in regulating the response of liver metastatic disease to ICIs is essential for personalizing treatments and improving the outcome for patients of both sexes. We recently documented a sexual dimorphism in the tumor immune microenvironment of LM and identified estrogen as a central regulator of this tumor microenvironment (TME) (10). In particular, we identified an estrogen-regulated accumulation of myeloid-derived suppressor cells (MDSC) in the liver TME that impeded $CD8^+$ T cell-mediated antitumor immune reactivity. We have shown that Tamoxifen, a selective estrogen receptor modulator (SERM), could reverse immunosuppression and reduce metastatic outgrowth in the liver.

Liver resident macrophages (Kupffer cells) and recruited monocyte/macrophages can play opposing roles in the process of liver colonization by metastatic tumor cells and can either inhibit or promote metastatic expansion, depending on their phenotype (11). These properties are determined by a process of macrophage polarization on a spectrum from a pro-inflammatory anti-tumor (M1) phenotype to an anti-inflammatory pro-tumor (M2) phenotype. This process is finely regulated by cytokines, chemokines and growth factors released by the tumor cells and other immune cells in the TME (12). By definition, cytokines and chemokines can either promote or curtail tumor expansion either directly, or indirectly by regulating the recruitment and the activity of immune cells into the TME (13). Natural killer T (NKT) cells comprise a small population of αβ T lymphocytes. They bridge the innate and adaptive immune responses and mediate strong and rapid responses in pathological conditions including cancer (14). This has rendered NKT cells attractive agents for cell-based cancer therapies.

Here, we investigated the role of estrogen in macrophage polarization and NKT cytotoxicity in the TME and analyzed the effect of estrogen depletion on the cytokine/chemokine profile of the liver, as means of identifying the factors and mechanisms that may mediate the role of estrogen in their regulation. Moreover, we evaluated the effect of combining the checkpoint inhibitor anti-PD-1 antibody with the selective estrogen receptor degrader (SERD) Fulvestrant on the TME and liver metastases of colon carcinoma MC-38 cells. Overall, this work contributed to significantly show that estrogen depletion altered the chemokine/cytokine repertoire in the TME and increased the proportion of pro-inflammatory macrophages and NKT cell cytotoxicity. Moreover, the addition of Fulvestrant treatment enhanced the effect of immunotherapy leading to a reduction in CRCLM outgrowth.

2.3. Results

2.3.1. Estrogen depletion alters the M1/M2 macrophage ratio, in both the recruited and liver-resident macrophages populations, and estrogen supplementation reverses this effect.

The hepatic macrophage population consists of the liver-resident macrophages (Kupffer cells), and the monocytes/macrophages recruited from the bone marrow via the blood circulation; a process that occurs under normal physiological condition but is accelerated in response to a pathological challenge (15). Tumor associated macrophages (TAMs) constitute a major component of the TME and can play opposing roles in the process of liver metastasis (16). Kupffer cells are typically identified based on the expression of the markers CD11b, CD68 and F4/80, as well as while recruited macrophages also express high levels of the monocyte differentiation marker Ly6C and the chemokine receptor CCR2 (17, 18). Here, we investigated whether estrogen signaling plays a role in macrophage polarization in the liver TME. Estrogen-depleted (OVX) and control (SHAM) mice were euthanized 12 days post MC-38 inoculation, and macrophages were isolated and analyzed by flow cytometry and by immunofluorescence on liver frozen sections. Using the markers MHCII and CD163 to characterize M1-like and M2-like macrophages respectively, we found that estrogen depletion markedly altered the macrophage population in the TME, significantly increasing the M1/M2 macrophage ratio in the OVX as compared to SHAM-operated control mice (Fig 1.a). These results were reversed in OVX upon Estradiol (E2) supplementation and confirmed using additional M1-like macrophage markers, CD38 and TNF-α (Supp. Fig. 1a-b). This increase in M1/M2 macrophage ratio in OVX mice was observed both in the resident and recruited macrophage populations (Fig 1.b). These observations were confirmed by immunofluorescence staining, where a higher accumulation of $CD68^{+}CD163^{+}$ cells

surrounding and infiltrating CRCLM tumors was observed in SHAM-operated control compared to OVX mice (Fig 1.c).

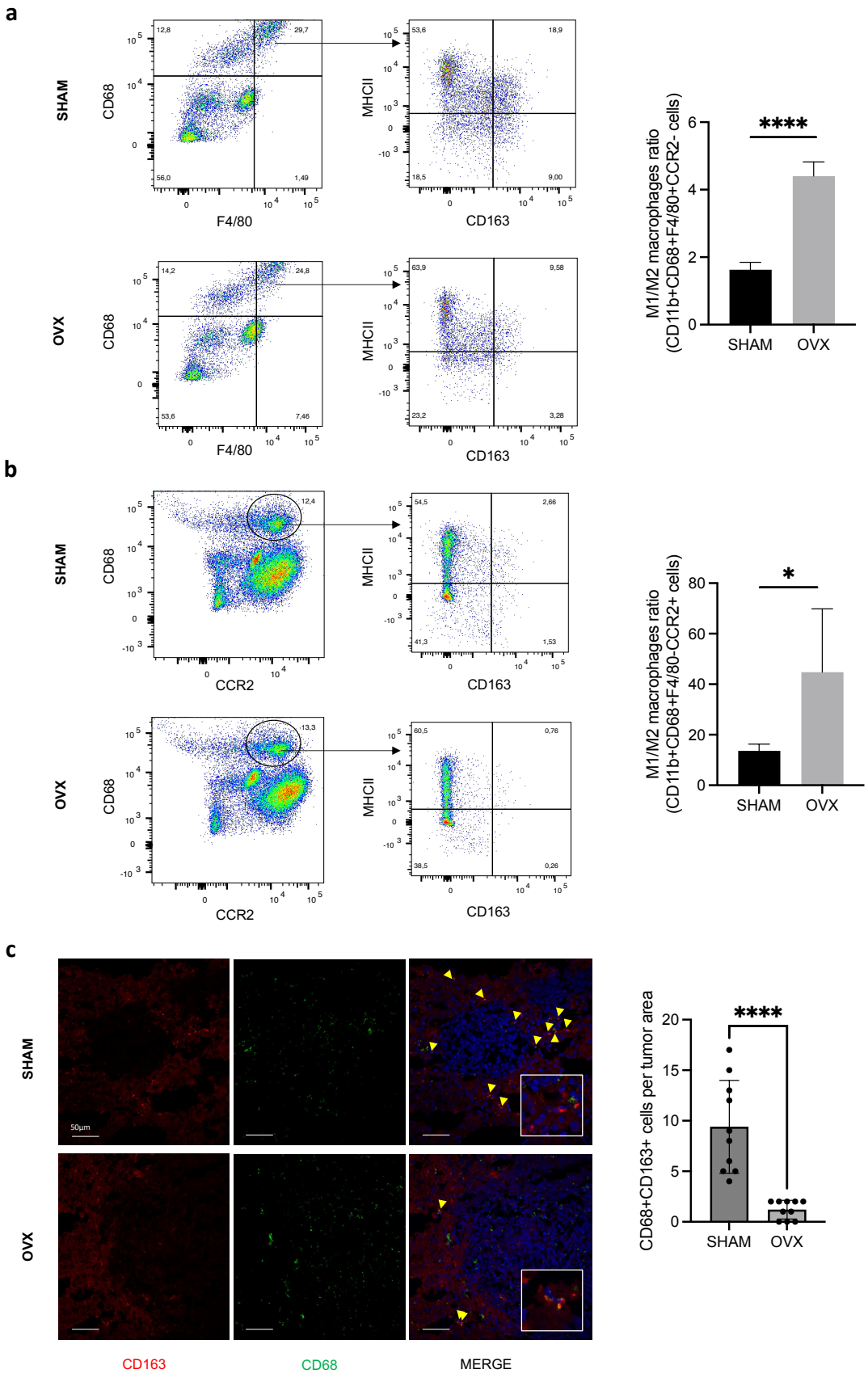


Fig. 1 Estrogen depletion increases the M1/M2 macrophage ratio in both the recruited and liver-resident macrophage populations. Flow cytometry was performed on macrophages isolated 12 days post intrasplenic/portal injection of 5 x 10^5 MC-38 cells and immunostained with the indicated antibodies. Shown in **(a)** and **(b)** are representative flow cytometric contour plots obtained for the indicated cell populations (left) and in the bar graphs (right) M1/M2 macrophages ratios (n=4). Shown on the left in **(c)** are representative confocal images from 15μm cryostat liver sections obtained 12 days post intrasplenic/portal inoculation of 5 x 10^5 MC-38 cells and immunostained with the indicated antibodies followed by Alexa Fluor 488 (green) for CD68, Alexa Fluor 647 (red) for CD163, and 4′,6-diamidino-2-phenylindole (DAPI; blue). Shown on the right in **(c)** are the number (means ± S.D.) of the indicated cells per field counted in 10 fields per group. *−p ≤ 0.05; **−p ≤ 0.01, ***−p ≤ 0.001 as determined by the Student's t-test.

2.3.2. Estrogen depletion enhances NKT cells cytotoxicity and estrogen supplementation reverses this effect.

The liver is host to the largest population of natural killer T (NKT) cells in the body (50). Liver NKT cells patrol the liver sinusoids to provide intravascular immune surveillance against foreign “invaders” such as cancer cells (19, 20). They can mount a strong anti-tumor response and have become a major focus in the development of effective cancer immunotherapy (21). Having previously observed a significant reduction in the number of liver metastases of CRC, pancreatic and lung carcinoma cells in OVX mice (10), it was of interest to determine whether estrogen depletion altered the accumulation and function of these cytotoxic cells in the TME of the liver. To this end, we isolated hepatic immune cells (HIC) from OVX and SHAM-operated control mice 7 days post intrasplenic/portal inoculation of MC-38 cells for analysis by flow cytometry (FC). As an additional control, we used a third group consisting of OVX mice in which estrogen levels were reconstituted by implanting s.c. a silastic capsule containing 18–36 μg/ml β-estradiol (OVX+E2). In OVX mice, we found almost a 2-fold increase in the proportion of $CD3^+NK1.1^+$ NKT cells (Fig 2.a) relative to SHAM-operated control and OVX+E2 mice. To determine whether estrogen

withdrawal also affected the cytotoxic activity of these cells, we developed an Incucyte®-based cytotoxicity assay that enables visualization and quantification of cell death in real time. MC-38 cells labelled with the Incucyte® Cytotox Green Dye, and co-cultured with enriched NKT cells isolated from OVX, SHAM-operated control, and OVX+E2 mice injected 6 days earlier (Fig 2.b). We observed a significantly higher number of green fluorescent (killed) MC-38 cells following co-culture with OVX-derived NKT cells as compared to those co-cultured with NKT cells derived from SHAM-operated control (Fig 2c). Furthermore, estradiol supplementation in OVX mice restored NKT cytotoxicity levels to those observed in SHAM-operated control mice (Fig 2.c) confirming the role of estrogen in regulating NKT function. This effect appears to be specific to the NKT population, as no significant difference was observed between the cytotoxicity of NK cells ($CD3^{-}NK1.1^{+}$) cells derived from OVX and SHAM-operated control mice (Supp. Fig. 2).

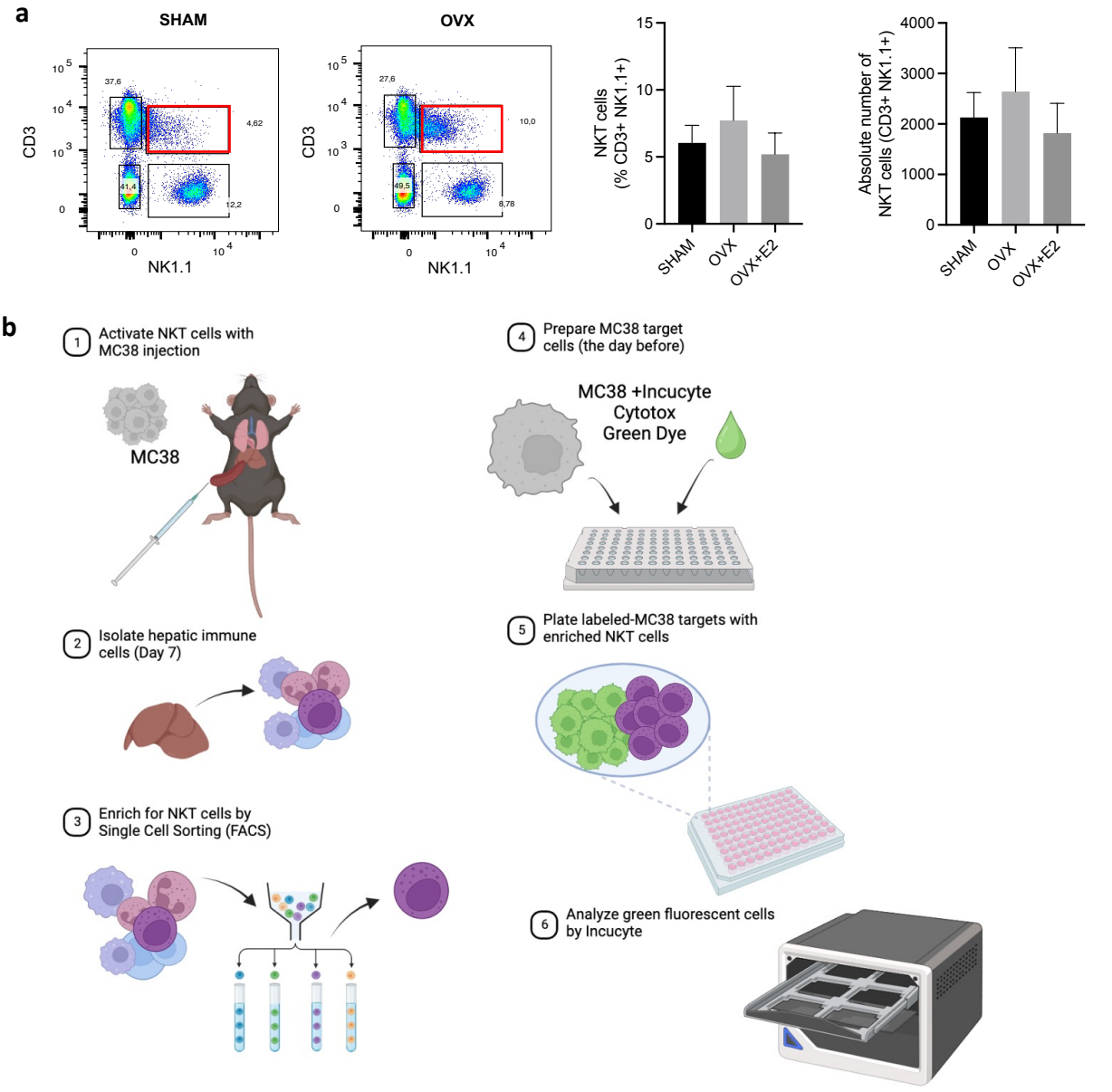


Fig. 2 Estrogen depletion enhances NKT cells accumulation and cytotoxicity in the liver TME. FC was performed on hepatic immune cells isolated 7 days post intrasplenic/portal injection of 5 x 10^5 MC-38 cells and immunostained with the indicated antibodies. Shown in **(a)** are representative flow cytometric contour plots obtained for the indicated cell populations (left) and in the bar graphs (right) the absolute cell counts (n=4). **(b)** the workflow of the NKT cytotoxicity assay where NKT cells were sorted based on CD3 (PE) and NK1.1 (APC), and MC-38 were pre-incubated with the Incucyte Cytotox Green Dye (Excitation Max 491 nm, Emission Max 509 nm). The NKT cells and MC-38 cells were co-cultured at a ratio of 5:1 for up to 30h. Show on the left in **(c)** are representative Incucyte wells images after 30h of NKT cells and MC-38 cells co-culture. Shown on the right in **(c)** is quantification of dead (green) MC-38 cells normalized to time 0h (n=3). Schematic was made using BioRender. *−p ≤ 0.05; **−p ≤ 0.01, ***−p ≤ 0.001 as determined by the Student's t-test.

2.3.3. Estrogen depletion reprograms the cytokines/chemokines repertoire in the liver TME and enhances the expression of CCL5 and its cognate receptor CCR5.

Chemokines and cytokines can affect the fate of metastatic cells either directly or indirectly by regulating the type and activities of immune cells recruited into the TME (13). Having observed differences between the TIME of LM growing in estrogen-competent and estrogen-depleted mice, it was of interest to identify the molecular mediators underpinning these shifts in immune cell accumulation in response to metastatic CRC cells. We therefore analyzed the cytokine/chemokine repertoires in livers obtained from these mice following MC-38 inoculation using a multiplex cytokine array. Intriguingly, the analysis revealed a general trend towards increased chemokine production for the majority of chemokines analyzed including CXCL10/IP-10 (4-fold), M-CSF/CSF-1 (2-fold), CCL2/MCP-1 (5-fold), CXCL9/MIG (2-fold) and CCL5/RANTES (2-fold) in OVX as compared to SHAM-operated control mice. The cytokine array results were further validated using qPCR confirming increased expression of these chemokines in the livers of estrogen depleted mice (Supp. Fig. 3).

The chemokine CCL5 has been linked to NKT recruitment and activation and associated with the M1 macrophage phenotype (22, 23). We therefore analyzed further its role in the altered microenvironment in the livers of estrogen-depleted and estrogen-competent mice. To determine the cellular source(s) of CCL5 and identify CCR5 expressing cells, we analyzed HIC isolated 7 days following MC-38 inoculation. We found increased CCR5 expression on $CD3^+CD8^+$ T cells and on $CD3^+NK1.1^+$ NKT cells derived from OVX as compared to control mice (Fig. 3b and 3d, respectively). We also found increased expression of CCL5 in $CD3^+CD8^+$ T cells (Fig 3.c), and moreover CCL5 production in NKT cells derived from OVX mice was significantly increased (Fig 3.e), consistent with the increased activity of these cells in the estrogen-depleted mice. These results were similarly obtained in the pancreatic tumor model FC1199 (Supp. Fig. 4). Maraviroc (Mvc) is a small molecule CCR5 antagonist that is in clinical use for the treatment of CCR5-tropic HIV-1 infection (24). To ascertain the role of CCL5/CCR5 signaling in reducing the growth of metastases in OVX mice, we treated these mice with 5 or 10 mg/kg Maraviroc (or vehicle) daily for 6 days from day 2 onward post injection of $1x10^5$ MC-38 via the intrasplenic/portal route. We found that Mvc treatment significantly increased the number of CRCLM in OVX mice, in a dose-dependent manner (Fig 3.f-g) and a more modest increase was seen in SHAM-operated control mice treated with Mvc, suggesting that in OVX mice CCL5 inhibits tumor growth by reprograming the TME.

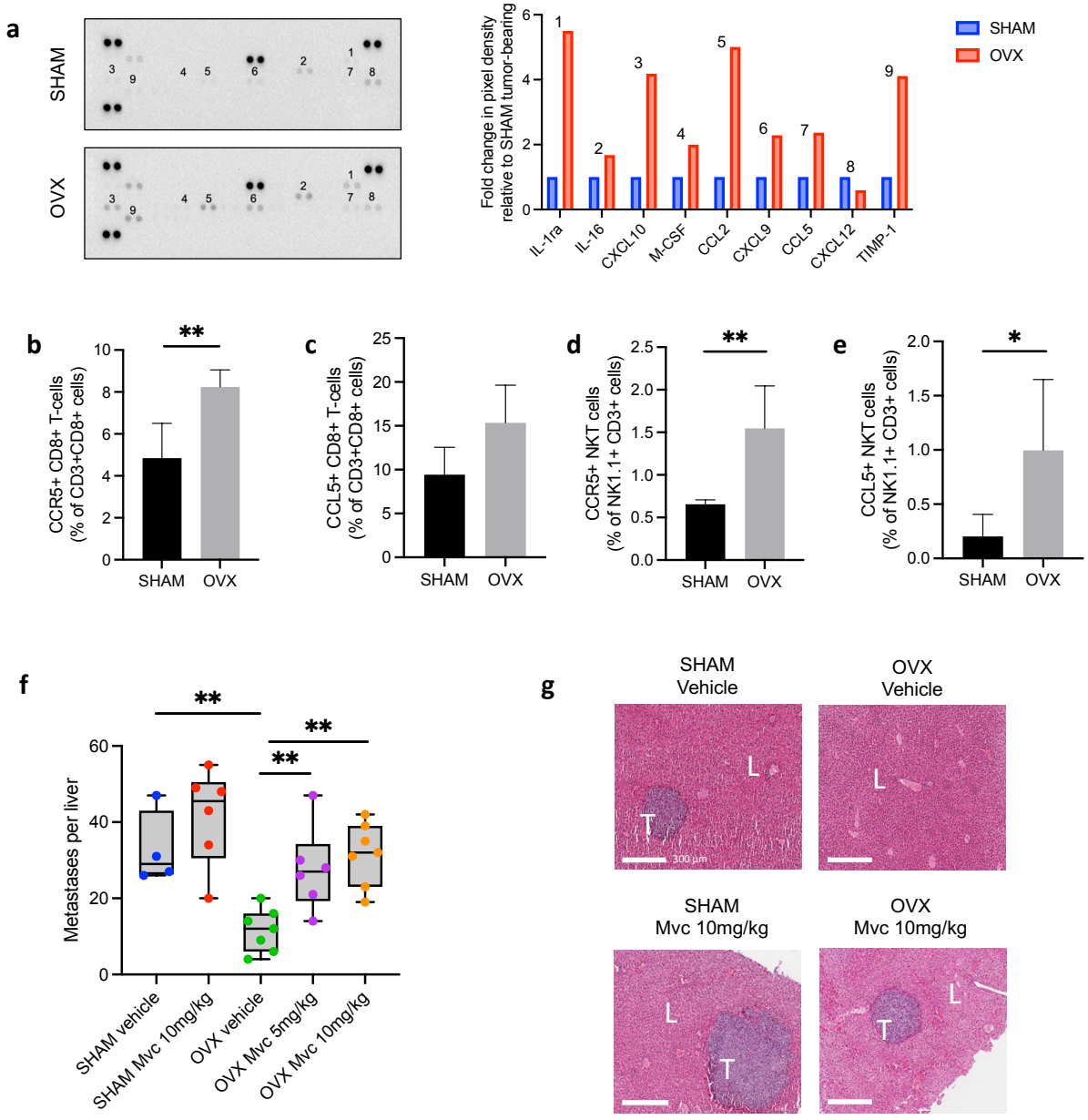
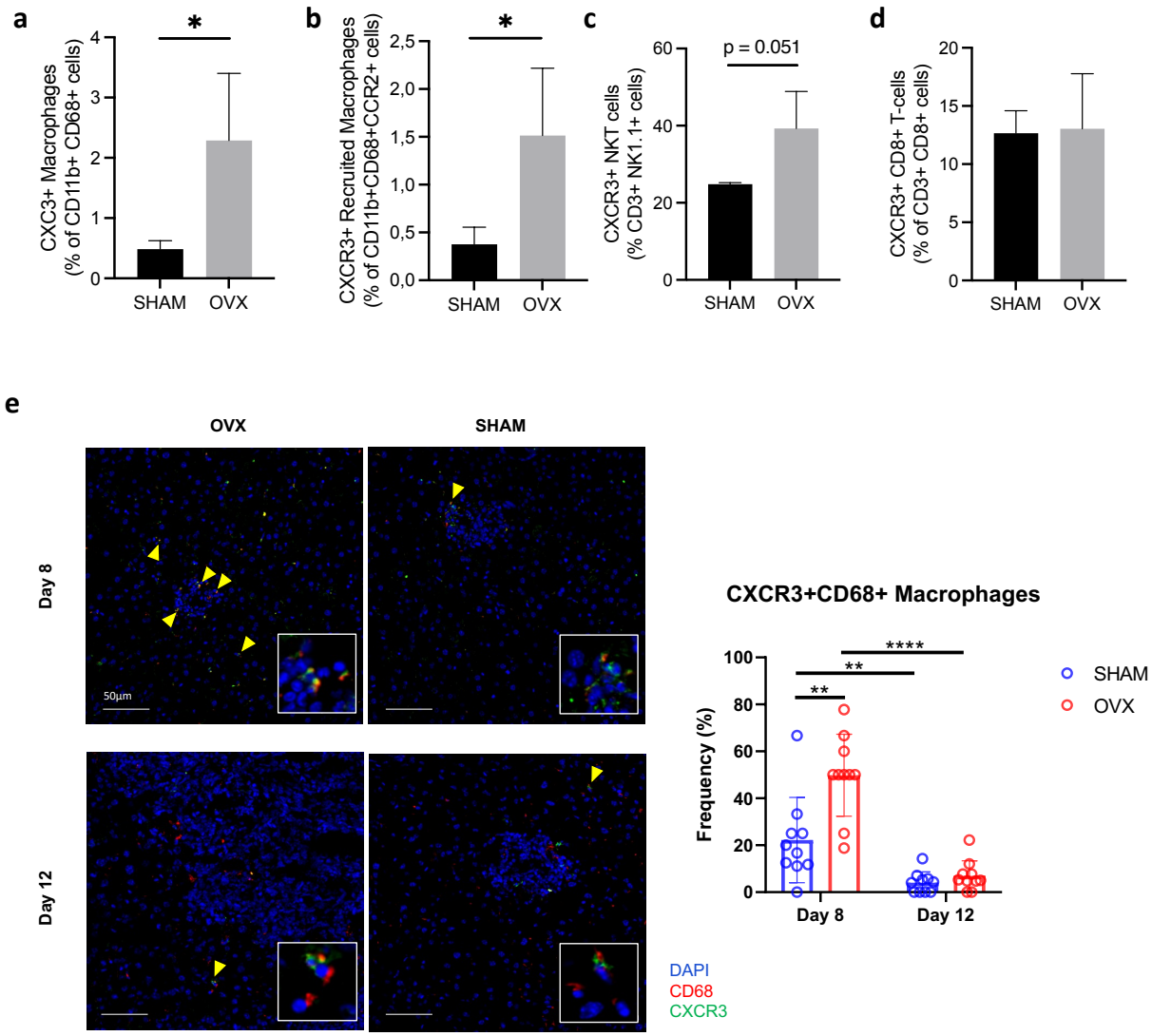


Fig. 3 Estrogen depletion modifies the cytokines/chemokines repertoire and increases expression of CCL5 / CCR5 in NKT and CD8+ T cells in the liver TME. (a) Cytokine array analysis was performed on whole liver protein lysates derived from MC-38 tumor-bearing SHAM and OVX mice livers. The results are based on a pool of four livers per group, and pixel densities were normalized to non-tumor bearing values. Shown in **(b-e)** are FC results obtained for the indicated cell populations (n=4). Experimental LM were generated by inoculation of 1 x 10^5 MC-38 colon carcinoma cells via intrasplenic/portal route. Intraperitoneal injections of Maraviroc or

vehicle (PBS + DMSO) were administered the day after MC-38 injection, for 6 consecutive days. Mice were sacrificed 18 days post tumor inoculation and LM enumerated. Shown in **(f)** are number of metastases counted per liver (n = 4-7; horizontal bars denote median values). **(g)** Representative H&E-stained liver sections for each treatment groups. *−p ≤ 0.05; **−p ≤ 0.01, ***−p ≤ 0.001 as determined by the Student's t-test (Flow cytometry) and Mann–Whitney test (metastasis assay).

Furthermore, consistent with the increase in CXCL10 and CXCL9, both ligands of the CXCR3 receptor, we found an increase in CXCR3 levels in OVX compared to SHAM-operated control mice. We found CXCR3 to be particularly increased on hepatic macrophages (Fig 4.a). More specifically, a significant increase of CXCR3 was observed on the recruited $CD11b^{+}CCR2^{+}CD68^{+}$ macrophage phenotype (Fig 4.b) and occurred early (Day 8) compared to late (Day 12) in the metastatic process (Fig 4.e). This macrophages populations correspond to the highly M1-polarized state, indicating a possible M1-polarizing effect via CXCR3 signaling. The CXCR3 expression was slightly higher in NKT cells (Fig 4.c), and not different on CD8 T cells (Fig 4.d) in OVX mice compared to SHAM-operated control mice. Additionally, CXCL10 was found to be increased in NK cells (Fig 4.f) and $CD68^{+}$ macrophages (Fig 4.g) in OVX relatively to SHAM, and this increase was more important at earlier timepoint.



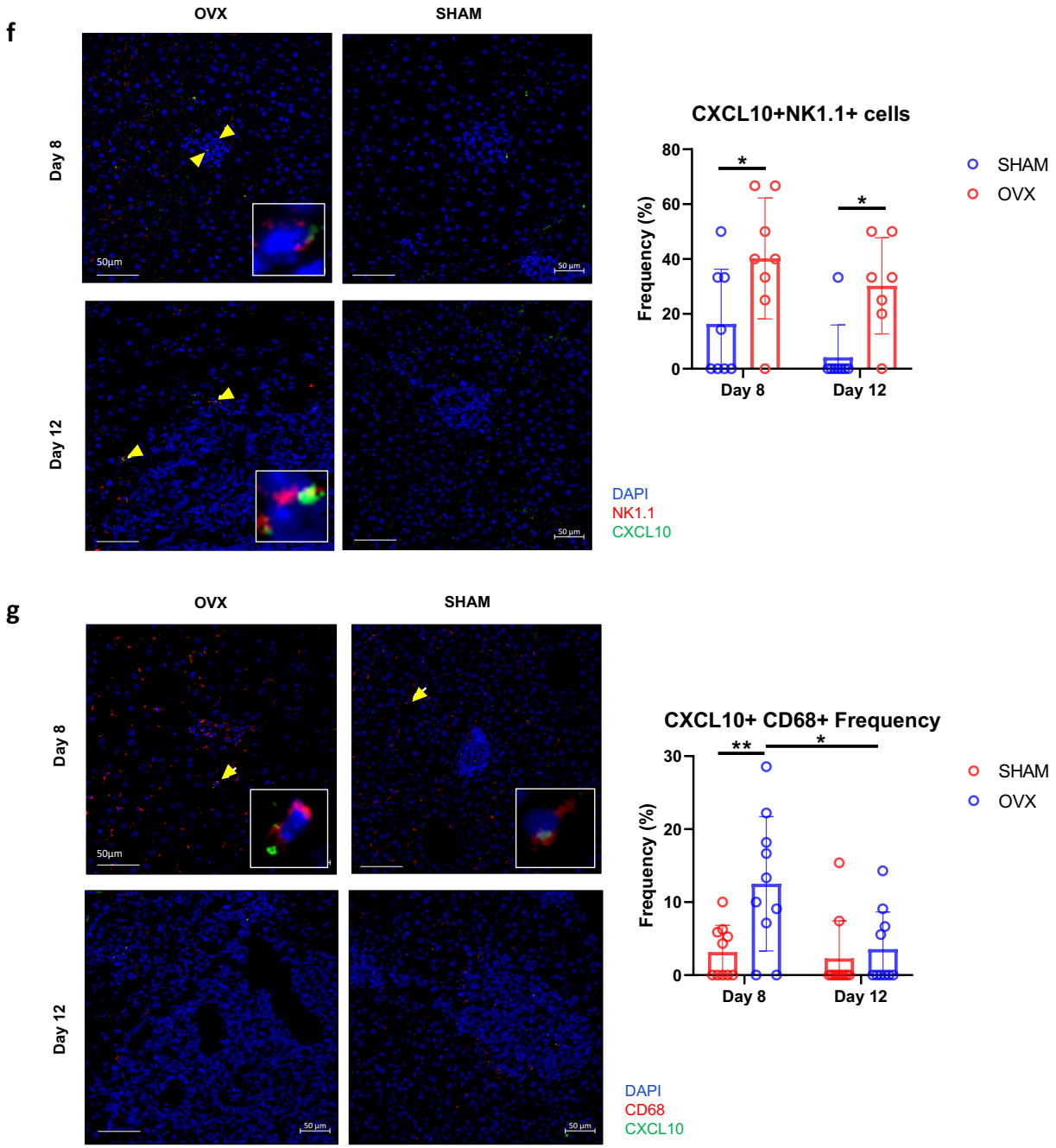


Fig. 4 Expression of CXCR3 and its ligand CXCL10 increase upon estrogen depletion on hepatic macrophages early in the metastatic process. Shown in **(b-d)** are FC results obtained for the indicated cell populations (n=3-4). Shown on the left in **(e)** are representative confocal images from 10µm cryostat liver sections obtained 8- and 12-days post intrasplenic/portal inoculation of 5 x 10^5 MC-38 cells and immunostained with the indicated antibodies followed by

Alexa Fluor 488 (green) for CXCR3, Alexa Fluor 568 (red) for CD68, and 4′,6-diamidino-2-phenylindole (DAPI; blue). Shown on the right in **(e)** are the number (means ± S.D.) of the indicated cells per field counted in 10 fields per group. *−$p \leq 0.05$; **−$p \leq 0.01$, ***−$p \leq 0.001$ as determined by the Student's t-test.

2.3.4. Fulvestrant - a selective estrogen receptor degrader - reprograms the immune microenvironment of the liver and inhibits CRCLM outgrowth.

Selective estrogen receptor degraders (SERDs) represent a class of molecules that effectively inhibit ER signaling by forming unstable SERD–ER complexes that can be targeted for proteasomal degradation (25). Unlike selective estrogen receptor modulators (SERMs) such as Tamoxifen, SERDs have no agonistic effects. Fulvestrant (Fulv) is a FDA-approved SERD presently in clinical use for the management of hormone-receptor-positive advanced breast cancer (26). We analyzed the effect of Fulvestrant on CRCLM to assess the potential translational implication of anti-estrogen therapy in the management of hepatic metastases. Female mice injected with MC-38 cells were administered Fulv (5 or 20 mg/kg) or vehicle (sunflower seed oil + DMSO) subcutaneously, three times weekly. In Fulv-treated mice, we observed a significant and dose-dependent reduction in the number and size of CRCLM as compared to controls (Fig 5a-c). Moreover, we found a dose-dependent increase in NK and NKT cell frequencies relative to vehicle-injected mice (Fig 5.d), essentially recapitulating the effect of surgical depletion of estrogen in OVX mice. To rule out the direct effect of Fulv on MC-38 growth inhibition, Fulv was tested *in vitro* on MC-38 cells an no growth inhibition effect was observed compared to MCF-7 cell line which is sensitive to Fulv (Fig 5.e) (27).

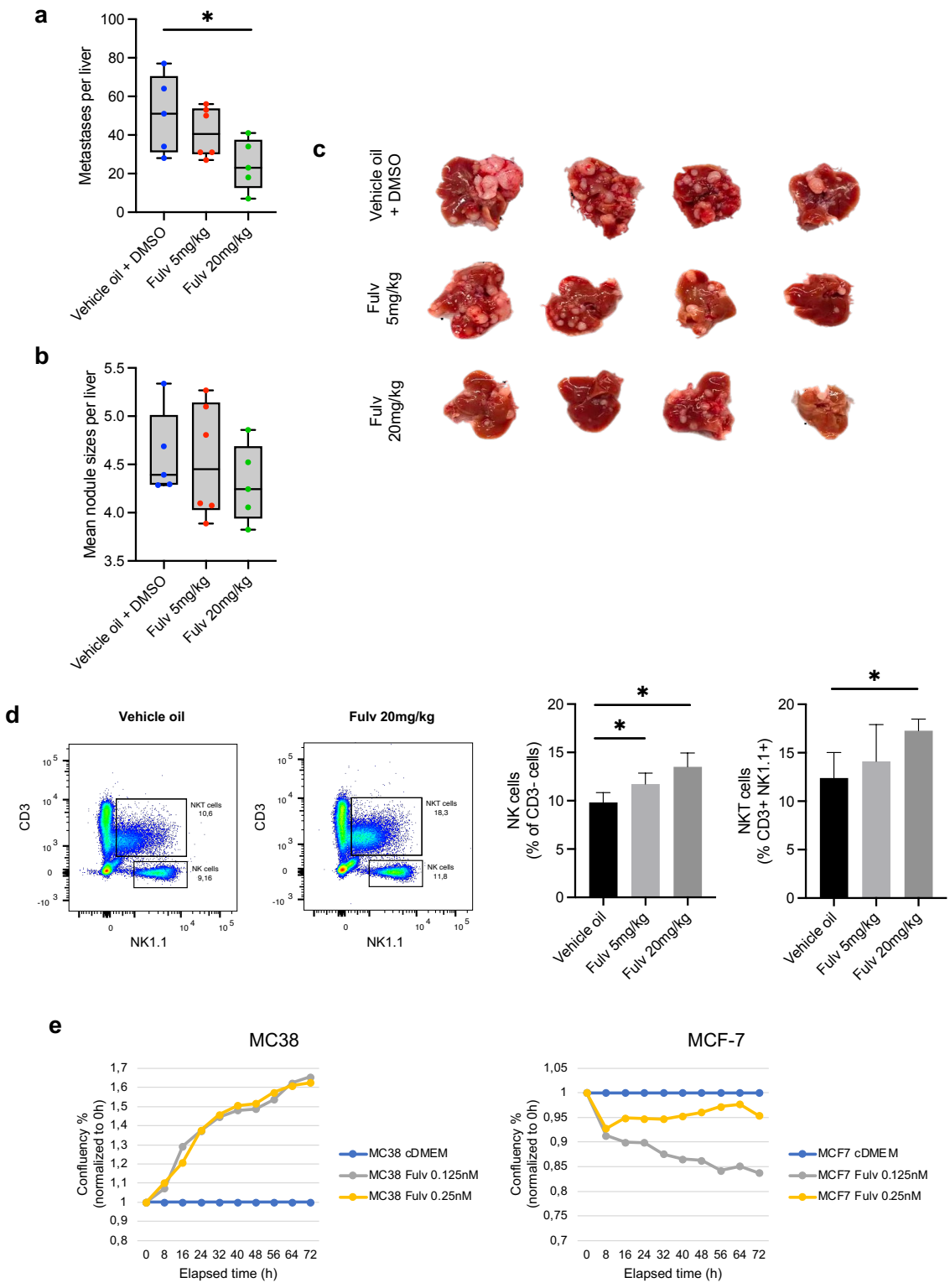
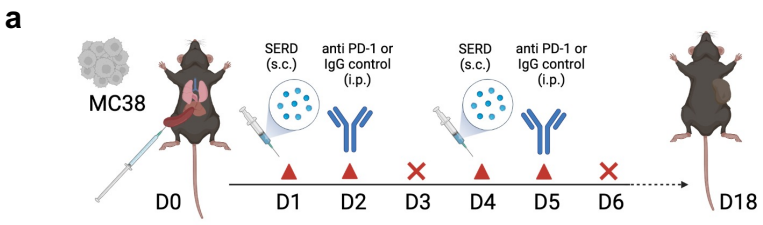


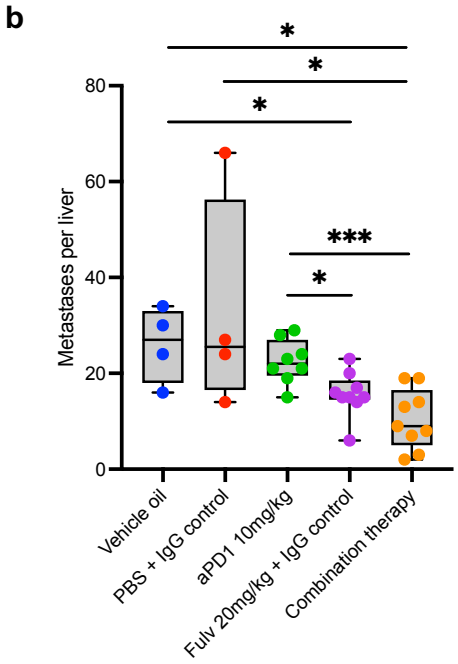
Fig. 5 Fulvestrant inhibits CRCLM outgrowth and reprograms the liver TME in a dose-dependent manner. Experimental LM were generated by inoculation of 1 x 10^5 MC-38 colon carcinoma cells via intrasplenic/portal route. Subcutaneous injections of Fulvestrant or vehicle oil + DMSO were administered on alternate days for 16 days. Mice were sacrificed 21 days post tumor inoculation and LM enumerated. **(a)** Numbers of visible metastases counted per liver (n = 5-6; horizontal bars denote median values). **(b)** Mean nodule sizes of visible metastases (n = 5-6; horizontal bars denote median values). **(c)** Representative livers. Show in **(d)** are representative flow cytometry contour plots of HIC isolated 7 days post injection of 5 x 10^5 MC-38 cells from mice treated with Fulvestrant 20mg/kg (or vehicle oil + DMSO) on alternate days and stained with the indicated antibodies. Show in **(e)** are the results of Incucyte phase assay performed on the indicated cells using the Fulv concentrations shown. Control cells were treated with complete media only. Shown are confluency normalized to time 0h. results of *−$p \leq 0.05$; **−$p \leq 0.01$, as determined by the Mann–Whitney test (metastases) or Student's t-test (FC). Box and whiskers graphs: the box extends from the 25th to 75th percentiles, the middle line denotes the median and the whiskers extend from the minimum to the maximum value.

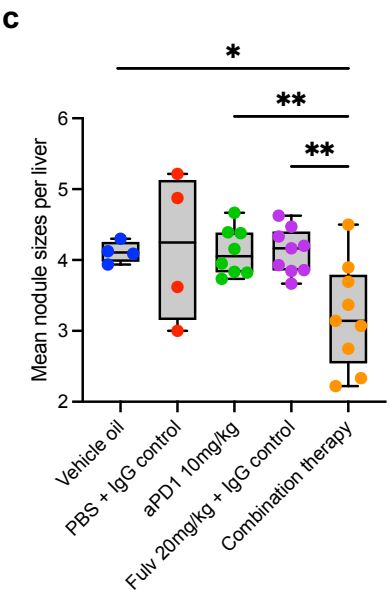
2.3.5. Fulvestrant enhances therapeutic efficacy of anti-PD-1 immunotherapy against CRCLM

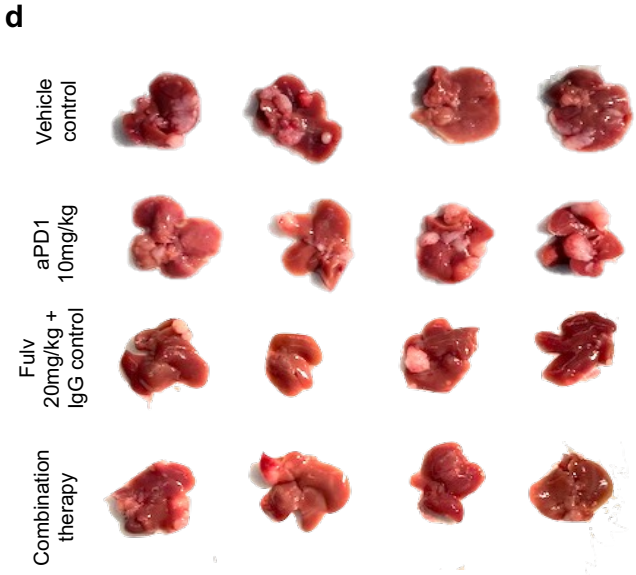
It has become increasingly clear that for most cancer, the efficacy of immunotherapy can be enhanced by combining immune checkpoint inhibitors with other drugs that target immunosuppressive elements within the TME (28). Female sex was identified as one of five variables with significant negative association to response to anti-PD-1 therapy (29). Furthermore, our work and others have identified estrogen as a promoter of immunosuppressive mechanisms within the TME (30). To determine if, and to what extent, SERD can potentiate the therapeutic efficacy of immunotherapy, female mice were injected with 1x10^5 MC-38 cells via intrasplenic/portal route and randomized to the following treatment groups: control 1 (Fulv vehicle (sunflower seed oil + DMSO)); control 2 (anti-PD-1 vehicle (PBS + IgG Isotype control)); 10mg/kg anti-PD-1; Fulv 20 mg/kg + IgG isotype control; and 20mg/kg Fulv + 10 mg/kg anti-PD-

1 (combination therapy) administered on alternate days. A detailed treatment regimen is outlined in Fig 6.a. Treatment continued for a total of 5 Fulv and anti-PD-1 injections. We found that the combination of Fulv and anti-PD-1 significantly decreased the number and size of liver metastases relative to the control groups or anti-PD-1 therapy alone (Fig. 6 b-e). As expected, treatment with Fulv alone also significantly reduced the number of LM, but the addition of anti-PD-1 caused a further reduction in both their number and size. Notably, we also observed a significant reduction in CRCLM counts in Fulv-treated mice comparatively to anti-PD-1 treated mice (Fig 6.b).









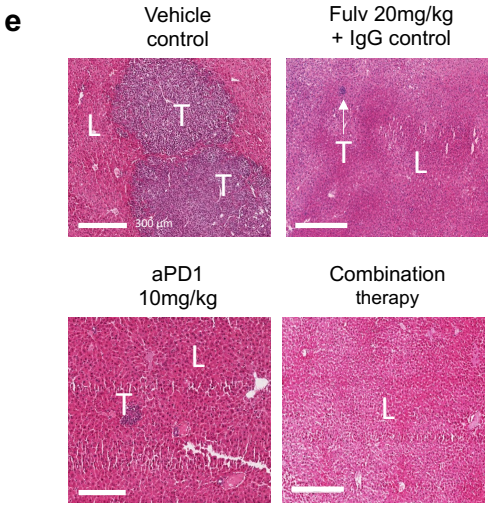


Fig. 6 Fulvestrant enhances therapeutic efficacy of anti-PD-1 immunotherapy against CRCLM. Experimental LM were generated by inoculation of 1 x 10^5 MC-38 colon carcinoma cells via the intrasplenic/portal route. Shown in **(a)** is the treatment regimen which consists in MC-38 cells inoculation on Day 0, Fulv 20mg/kg or vehicle (sunflower seed oil + DMSO) s.c. administration on Day 1, followed by i.p. administration of either anti-PD-1 therapy 10mg/kg or IgG isotype control 10mg/kg on Day 2, and no treatment on Day 3. This sequence of treatment repeated until Day 15, and mice were sacrificed on Day 18. **(b)** Numbers of visible metastases counted per liver (n = 4-9; horizontal bars denote median values). **(c)** Mean nodule sizes per liver (n = 4-9; horizontal bars denote median values). **(d)** Representative livers (where white masses = metastases). **(e)** Representative H&E-stained liver sections for each treatment groups. *−$p \leq 0.05$; **−$p \leq 0.01$, ***−$p \leq 0.001$ as determined by the Mann–Whitney test. Box and whiskers graphs: the box extends from the 25th to 75th percentiles, the middle line denotes the median and the whiskers extend from the minimum to the maximum value. Schematic was made using BioRender.

To elucidate the changes in the TIME that underlined the marked reduction in metastatic outgrowth caused by the combinatorial therapy, hepatic lymphocytes and macrophages were isolated from the livers of mice at termination of the treatment protocols described above and analyzed by flow cytometry. We found a significant increase in $CD8^+$ T cells, NKT and NK cells in mice treated with the combination therapy relative to controls; an effect that was specific to this treatment group (Fig 7a-c). Similar to our findings in OVX mice, CCL5 and CCR5 levels were also increased in combination therapy-treated mice. In particular, we observed a significant increase in $CCL5^+$ and $CCR5^+$ $CD8^+$ T cells and in $CCR5^+CCL5^+$ NKT cells in the combination therapy-treated mice as compared to other treatment groups (Fig 7 d-g). Changes were also found in the myeloid compartment of the TME. In combination therapy-treated mice, we observed a higher accumulation of $CD11b^+CD11c^+$ dendritic cells (DCs) suggesting increased recruitment of these cells following treatment (Fig 7.h). Additionally, a decrease in $CD11b^+Ly6C^+Ly6G^+$ MDSCs was also observed in these mice (Fig 7.i). Consistent with results in OVX mice, the M1/M2

macrophages ratio was founds significantly higher in combination therapy-treated mice in both the $CD11b^{+}CD68^{+}F4/80^{+}$ resident macrophage (Fig 7.j) and the $CD11b^{+}CD68^{+}CCR2^{+}$ recruited macrophage (Fig 7.k) compartments. Moreover, RNA isolated from the livers of these mice and analyzed by RT-qPCR revealed a significant reduction in the expression of TGF-b accompanied by a significant increase in IFN-g mRNA levels in mice treated with the combination therapy relative to controls (Fig 7.l). These data suggest that Fulvestrant potentiated anti-PD-1 efficacy against CRCLM by reprogramming the TME engendered by the liver metastases.

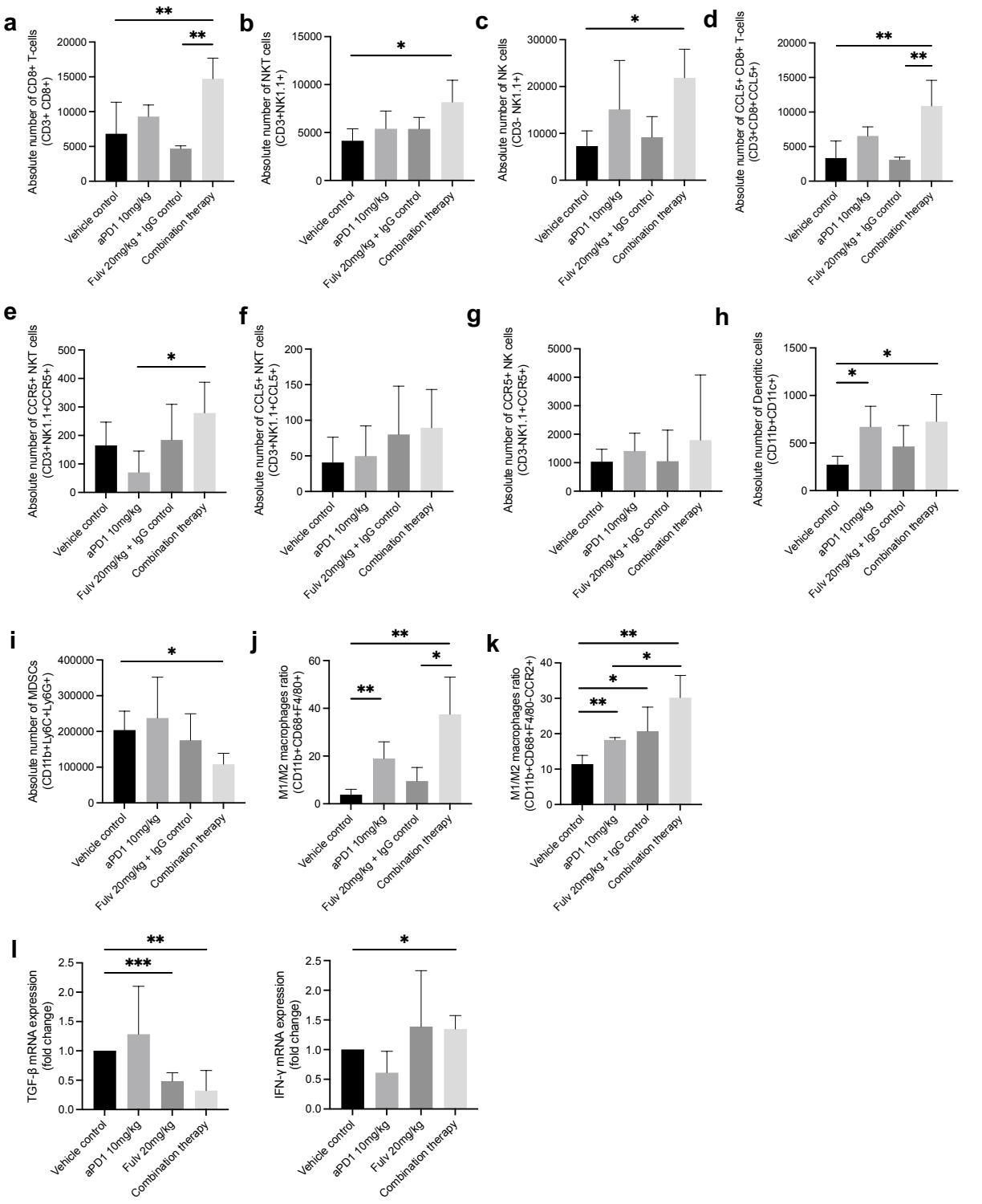


Fig. 7 Combination of Fulvestrant and anti PD-1 therapy modulates the immune architecture of CRCLM microenvironment. (a-k) FC analysis results of HIC isolated at the end of the

treatment schedule shown in Figure 5.a from mice treated with Fulvestrant 20mg/kg alone, anti-PD-1 10mg/kg alone, vehicle, or combination therapy and stained with the indicated markers. Shown in **(l)** are results of RT-qPCR performed on whole liver RNA obtained from the mice of the same experimental groups described above. The results for each of the indicated transcripts are based on livers obtained from three mice and expressed as means (±s.d.) relative to vehicle-treated mice that were assigned a value of 1, normalized to GAPDH. *−p ≤ 0.05; **−p ≤ 0.01, ***−p ≤ 0.001 as determined by the Student's t-test.

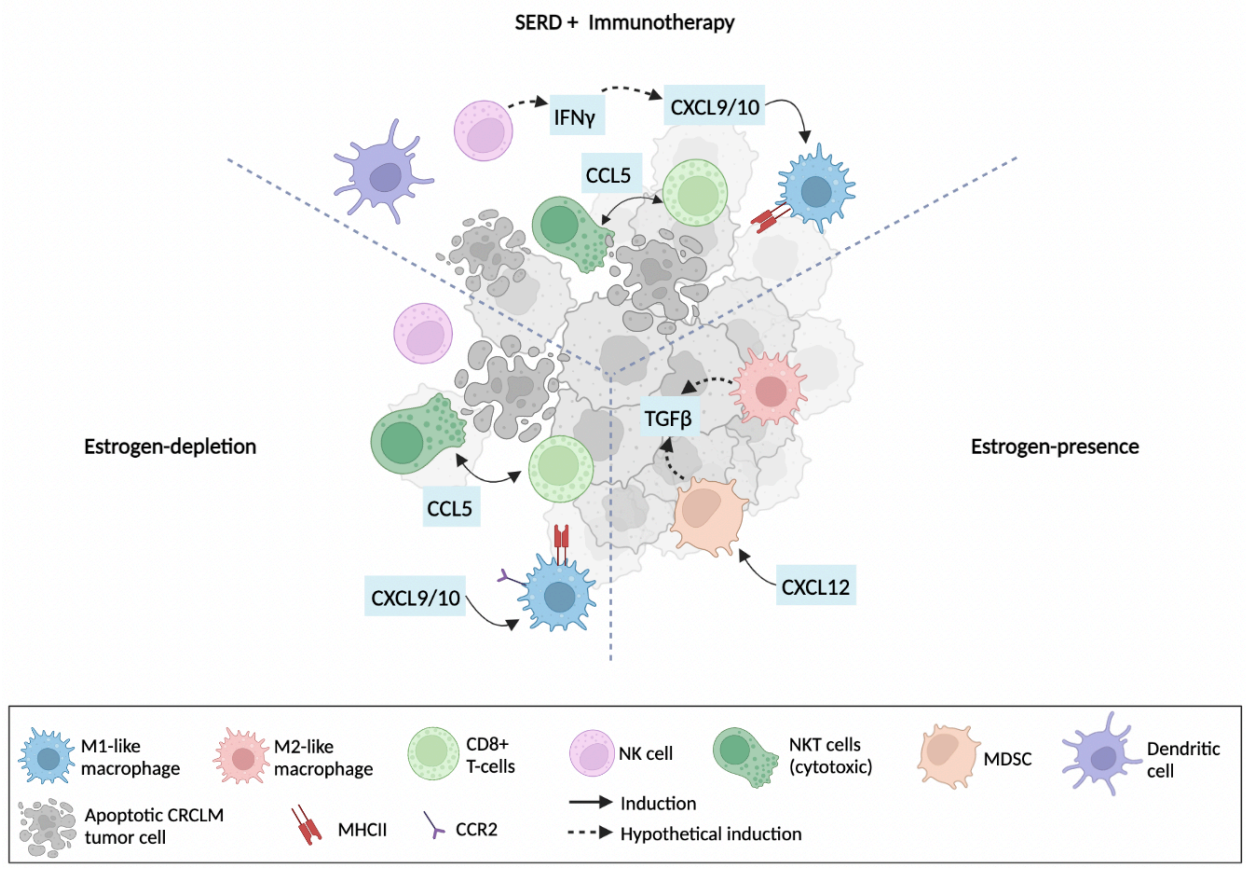


Fig. 8 The role of estrogen in the regulation of the TME in CRCLM. Shown is a diagrammatic representation of postulated mechanisms that may be underlying the observed estrogen-mediated regulation of immunotherapy response and of an immunosuppressive TME in the CRCLM liver. The ER/E2 signaling pathway can promote CRCLM expansion via the recruitment of MDSCs and preferential macrophages polarization to the M2-phenotype. ER/E2 signaling attenuation resulting from ovariectomy and Fulvestrant treatment promotes accumulation of NK/NKT cells and M1-

like macrophages. Fulvestrant treatment degrades ER and potentiates immunotherapy response to improve tumor cell elimination.

2.4. Discussion

The myeloid and lymphoid immune cells that are recruited and/or activated within the TME such as macrophages, NK, NKT, and $CD8^+$ T cells determine tumor development and anti-cancer immunity. Here, we observed a significant increase in the pro-inflammatory M1 (anti-tumorigenic)/ M2 (pro-tumorigenic) macrophages ratio in estrogen-depleted compared to estrogen-competent mice. This was noted in both the recruited and liver-resident macrophage populations; with a higher M1/M2 ratio in the recruited macrophage compartment. In parallel, estrogen depletion enhanced liver NKT cell accumulation and cytotoxicity, and both could be reversed upon E2 replacement. Furthermore, in the livers of OVX mice, we observed an increase in CCL5/RANTES, CCR5, CXCL10/IP-19, CXCL9/MIG, and CCL2/MCP-1 levels. While the role of CCL5/CCR5 axis in cancer development has been a subject of conflicting results, our findings of increased CRCLM outgrowth following inhibition of CCR5 with Maraviroc in OVX mice suggest that in the context of CRCLM, CCL5 plays an anti-tumorigenic, host protective role. Interestingly, a higher proportion of $CCL5^+CCR5^+$ $CD8^+$ T and NKT cells was found in OVX mice, suggesting an increase in CCR5/CCL5 signaling in these immune cells when estrogen is depleted. Concomitantly, we observed a higher proportion of $CXCR3^+CD68^+CCR2^+$ recruited macrophages, suggesting that CXCR3 plays a role in retaining macrophages in the M1-polarized state. Finally, we found a marked decrease in CRCLM outgrowth following combinatorial SERD/anti-PD-1 therapy, as compared to SERD or anti-PD-1 therapy alone. This was accompanied by an increase in $CD8^+$ T cells, NK and NKT cells accumulation in the liver. Similarly, to results in OVX, $CCL5^+$ and $CCR5^+$ $CD8^+$ T cells as well as NKT cells were

increased in mice treated with combination therapy. Additionally, dendritic cell accumulation and M1/M2 macrophages ratios were higher and MDSCs accumulation was lower, in mice treated with the combination therapy. Of note, we also found reduced numbers of metastases in response to anti-PD-1 therapy alone and this may be related to MC-38 cell immunogenicity, which is a common characteristic of murine CRC cell lines (31).

Little is presently known about how estrogen regulates macrophage function within the tumor microenvironment. Consistent with our findings, Chakraborty and colleagues, recently reported that ER signaling promoted melanoma growth in mice by skewing macrophage polarization toward the immunosuppressive M2 phenotype that promoted CD8+ T cells exhaustion and immune checkpoint blockade resistance (16). We found increased M1-like macrophage accumulation in the livers of estrogen-depleted mice, where CXCL10 and CCL2 were found to be elevated. Consistent with our findings, CXCL10 was identified as a macrophage chemoattractant, and CXCL10/CXCR3 signaling was shown to enhance macrophage proliferation and maintain their pro-inflammatory M1 phenotype in pancreatic cancer (32).

We also observed an increase in CCL2 protein levels in the livers of OVX as compared to SHAM-operated mice. While the contribution of CCL2 to macrophage polarization appears to be context dependent, several reports suggest that CCL2 favored M1 pro-inflammatory macrophage polarization as it increased TNFα and decreased Arg1 levels in the macrophage cell line RAW264.7 (33) , increased TNFα expression in peritoneal macrophages (34) and upregulated IL-1β, iNOS, and IL-6 mRNA levels in murine alveolar and bone-marrow derived macrophages (35) . *In vivo* CCL2 knockout mice displayed an M2 phenotype, evidenced by elevated expression

of Arg1, Tm1, and TGFβ in the livers of lipoatrophic diabetic A-ZIP-Tg mice (36). A recent study using scRNA sequencing and spatial transcriptomics, showed that in human CRCLM the highly metabolically activated *MRC1*$^{+}$*CCL18*$^{+}$ M2-like macrophages accumulated in metastatic sites, and this correlated with progressive disease (37). While our results suggest that upon estrogen-depletion M1 macrophage polarization may be orchestrated via CXCR3 signaling, it remains to be determined whether macrophage polarization is regulated by estrogen directly or indirectly through crosstalk with other cells in the TME. Interestingly, consistent with our previously published results on reduced MDSCs accumulation in OVX mice (10), we found an increase in CXCL12/SDF-1 in SHAM as compared to OVX mice. It has been demonstrated that the CXCL12/CXCR4 axis promotes migration and survival of MDSCs and dampens the response to anti-PD-1 therapy (38). Furthermore, reducing CXCL12 availability in the TME decreased liver metastasis (39) and activated hepatic stellate cells were shown to regulate MDSC migration through the CXCL12/CXCR4 axis (40).

Natural killer T (NKT) cells comprise a small population of αβ T lymphocytes that bridge the innate and adaptive immune responses. NKT cells have the capacity to mount strong and rapid responses in various pathological conditions including cancer, which has made them attractive agents for cell-based cancer therapies (14). In our study, we found a significantly higher NKT cells cytotoxicity, and increased M1 macrophages accumulation in the liver of OVX and Fulvestrant-treated mice. Interestingly, it has been reported that CRC patients have high NKT cell infiltration levels that are prognostic for overall survival (41). We also observed an increase in CCL5 and its cognate receptor CCR5 in T lymphocytes NKT cells in OVX mice, and enhanced CRCLM outgrowth upon CCR5 inhibition. In a recent study by Massalha et al., single cell sequencing

showed that T cells expressing CCL5 displayed a cytotoxic phenotype in CRCLM (42). Interestingly, another study revealed a cooperation between CXCL9/10 and CCL5 in recruiting effector T cells into human solid tumors (43). The macrophage-NKT crosstalk is not yet fully understood. Both cell types were shown to be mobilized by the chemoattractant CCL2 (44-46), suggesting that their recruitment into the TME can be coordinately regulated.

An explorative case study in Sweden on 23,154 patients diagnosed with LM has shown that ~15% of all female patients diagnosed with LM were younger than 50 years old, with colorectal cancer LM accounting for 21.2% of LM diagnosed in this age group (47). We have shown a marked decrease in CRCLM outgrowth following SERD and anti-PD-1 combination therapy, as compared to control or SERD and anti-PD-1 therapy alone. Similarly, to our results in OVX mice, the accumulation $CCL5^{+}CCR5^{+}$ $CD8^{+}$ T and NK/NKT cells, as well as dendritic cells and M1/M2 macrophages ratio, were increased in mice treated with the combination therapy. In a study by Böttcher et al., the accumulation of conventional type 1 dendritic cells (cDC1) in mouse tumors was shown to depend on NK cells that produce CCL5 and the NK cells/CCL5/cDC1 axis was found to be associated with patient survival (48). Taken together, our findings provide a rationale for the use of SERDs to enhance anti-PD-1 therapy efficacy in premenopausal female patients (age <50) and suggest that this may be beneficial in the clinical management of other hormone independent cancers with a propensity to metastasize to the liver.

2.5. Material and Methods

2.5.1. Animals. All mouse experiments were carried out in strict adherence to the guidelines of the Canadian Council on Animal Care (CCAC) "Guide to the Care and Use of Experimental Animals" and under the conditions and procedures approved by the Animal Care Committee of McGill University (AUP number: 5260). Mouse experiments were performed mainly in 6-12 weeks old female C57BL/6 mice bred in the animal facility of the RI-MUHC (Glen Site) ad all control mice (sham, and vehicle-treated) were age-matched to treated mice in all the experiments.

2.5.2. Cells. The murine colorectal carcinoma MC-38 and FC1199 cells are syngeneic to the C57BL/6 strain. Their origins and metastatic properties have been described in detail previously (49). MC-38 cells were originally from an NCI repository and were obtained as a kind gift from Dr. Shoshana Yakar (New York University, NY). They were recently authenticated by Didion and colleagues using single nucleotide polymorphism profiling. The murine pancreatic cancer line KPC FC1199, referred to here as FC1199, was generated in the Tuveson laboratory (Cold Spring Harbor Laboratory, New York, USA) from PDA tumor tissues obtained from KPC mice of a pure C57BL/6 background, and was a generous gift from the Tuveson laboratory. The cells were routinely tested for common murine pathogens and mycoplasma contamination, as per the McGill University Animal Care Committee and the McGill University Biohazard Committee guidelines. All cells were maintained as a frozen stock and generally cultured *in vitro* for up to 4 weeks only, prior to use in the in vivo experiments, to minimize genetic drifts and changes to their metastatic phenotypes. They were cultured in DMEM medium (Wisent), supplemented with 4 mM L-glutamine, 4.5 gL−1 glucose, 100 Uml−1 penicillin, and 100 μgml−1 streptomycin solution

(Sigma) containing also 2 gL−1 sodium pyruvate and 10% fetal bovine serum (FBS; Wisent) and incubated at 37°C in a humidified incubator with 5% CO_2.

2.5.3. Ovariectomy procedure. Mouse ovariectomy was performed according to the McGill University Standard Operating Procedures (SOP N°206.01) and with the approval of the Animal Care Committee of McGill University and the Research Institute of the McGill University Health Center. Briefly, female mice aged 5-7 weeks were administered carprofen (Rimadyl®; 20 mg/kg; s.c.) and Buprenorphine (Chiron Compounding Pharmacy Inc., 1 mg/kg; s.c. slow release), 30 min prior to surgery and anaesthetized using isoflurane. Animals were placed in sternal recumbence; their backs shaved and then disinfected using 70% ethanol and a 2% chlorhexidine solution. For each ovary, a single 0.5 cm dorsal flank incision was made, penetrating the abdominal cavity. The exposed ovaries were then removed by cauterization. The incisions in the peritoneal wall were sutured, and incisions in the skin were closed with metal clips. Administration of carprofen (s.c.) and buprenorphine (s.c.) provided postoperative analgesia. In control, sham-operated mice, two 0.5 cm dorsal flank incisions penetrating the abdominal cavity were made, but the ovaries were not removed.

2.5.4. Hormone replacement. β-Estradiol (Sigma) was thoroughly mixed in sterile sesame oil (Sigma) at a concentration of 18–36 μg/ml−1. Placebo capsules were filled with sesame oil only. Capsules were prepared from silastic tubing and plugged with 3 mm wooden applicator sticks. The capsules were filled with the hormone solution, capped, and incubated overnight in the remaining hormone/oil solution to equilibrate, then implanted s.c. in female mice that were ovariectomized 14 days earlier.

2.5.5. Immunostaining and confocal microscopy. C57BL/6 female mice were injected via the intrasplenic/portal route with 5×10^5 MC-38 cells and the livers perfused at the time intervals indicated, first with 3 ml PBS and then with 4 ml of a 4% paraformaldehyde solution. The perfused livers were placed in 4% paraformaldehyde for 48 h and then in 30% sucrose for an additional 48 h, before they were stored at −80 °C until used. For immunostaining, 15 μm cryostat sections were prepared, incubated first in a blocking solution (1% bovine serum albumin and 1% FBS in PBS) and then for 1 h each with the primary antibodies, used at the indicated dilutions, and the appropriate Alexa Fluor conjugated secondary antibodies, all at room temperature (RT). The sections were mounted in the Prolong Gold antifade reagent (Molecular Probes, Eugene, Oregon, USA) and confocal images were captured with a Zeiss LSM-780 microscope with a spectrum detection capability. Immunostained cells were quantified blindly in at least 10 images acquired per section, per experimental group.

2.5.6. Hepatic lymphocytes isolation protocol. To analyze early changes in the TME, mice were injected with 5×10^5 tumor cells via the intrasplenic/portal route, and the livers removed 6-9 days later (as indicated). Liver homogenates were prepared in cold PBS and filtered through a stainless-steel mesh using a plunger. The filtrates were centrifuged at 60 g to separate the hepatocytes, the supernatants containing the nonparenchymal cell fraction centrifuged at 480 g and the pellets resuspended in 10 ml of a 37.5% Percoll solution in HBSS containing 100 Uml−1 heparin and centrifuged at 850 g for 30 min to obtain the immune cell-rich fraction. Prior to Flow cytometry (FC), red blood cells were removed using the ACK (ammonium–chloride–potassium) solution and 1×10^6 cells were immunostained with the indicated antibodies (antibodies list in Annex). Data

acquisition was performed with a BD FACS Diva software and the data analyzed using the FlowJo software. For flow cytometric experiments on hepatic leukocytes, single cells were gated based on size (forward scatter), granularity (side scatter) and viability using an eFluor™ 450 fixable dye (eBioscience™, ThermoFisher).

2.5.7. Hepatic macrophages isolation protocol. To analyze macrophages polarization in the TME, mice were injected with 5 x 10^5 MC-38 cells via the intrasplenic/portal route, and the livers removed 10 to 12 days later (as indicated). Livers were minced, enzymatically digested using 0.1% Collagenase IV (from *Clostridium histolyticum*) in cRPMI for 30 min at 37°C. The liver homogenates were filtered through a 74µm mesh to remove debris and undigested tissue, the filtrate centrifuged for 5 min at 300 g in a 4°C centrifuge and the cells washed twice in cRPMI. Hepatocytes were removed by a 3 min centrifugation at 50 g and the supernatants containing the macrophages centrifuged at 300 g for 5 min at 4°C. Prior to FC, red blood cells were removed using the ACK (ammonium–chloride–potassium) solution and 1 × 10^6 cells were immunostained with the indicated antibodies (antibodies list in Annex). The cells were then stained and analyzed as described above. Data acquisition was performed with a BD FACS Diva software and the data analyzed using the FlowJo software. For flow cytometric experiments on hepatic macrophages, single cells were gated based on size (forward scatter), granularity (side scatter) and viability using an eFluor™ 450 fixable dye (eBioscience™, ThermoFisher).

2.5.8. NK and NKT cells Cytotoxicity assay. To analyze hepatic NKT and NK cells cytotoxicity, mice were injected with 5 × 10^5 tumor cells via the intrasplenic/portal route, and the livers removed 7 days later. One day prior immune cells isolation, MC-38 cells were seeded with the Incucyte®

Cytotox Green Dye. As per manufacturer's notice, addition of the Incucyte® Cytotox Dyes to normal healthy cells is non-perturbing to cell growth or morphology and won't yield in fluorescence/fluorescence increase. Entry and DNA-binding of Incucyte® Cytotox Dye occurs in damaged cells. The day after MC-38 target cells seeding, liver homogenates were prepared as per the hepatic lymphocyte isolation protocol (described above). NK and NKT cells from OVX, SHAM, and OVX+E2 mice were isolated and sorted based on the CD3-PE and NK1.1-APC markers and co-cultured with MC-38 target cells at a ratio 5:1 (NK/NKT: MC-38 cells) in triplicates. The co-cultures were analyzed for 30 hours using Incucyte and green fluorescent/dead MC-38 cells were reported and normalized to time 0h.

2.5.9. Multiplex cytokine array. A cytokine array assay was performed on livers from mice injected with $5x10^5$ MC-38 cells via the intrasplenic/portal route and sacrificed on day 12. Liver tissue was lyophilized and lysed using the RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1%Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate) supplemented with the Protease Inhibitor Cocktail (Roche cOmplete Mini, Sigma Aldrich Canada, Oakville, Ontario) for 30 min at 4 °C. Total protein lysates were clarified by centrifugation at 13,000 g for 20 min. Protein was collected and expression profiles of cytokines and chemokines were analyzed with the Proteome Profiler Mouse Cytokine Array Panel A (R&D systems, Minneapolis, MN, USA), as per the manufacturer's instructions. Pixel densities from non-tumor bearing mice were used to normalize the values.

2.5.10. RNA extraction and qPCR. Total cellular RNA was extracted from snap-frozen liver fragments using the TriZol reagent (Life Technologies, Inc., Burlington, Ontario, Canada),

according to the manufacturer's instructions. Two μg of total RNA were reverse transcribed and the cDNA used for qPCR analysis using the primer sets listed in the Annex.

2.5.11. Experimental LM. Experimental LM were generated by intrasplenic/portal injections of 10^5 tumor cells, followed by splenectomy as we previously described (10). Animals were euthanized 18–21 days later, and visible metastases on the surfaces of the livers were enumerated and sized without prior fixation. Where indicated, fragments of the livers were also fixed in 10% phosphate buffered formalin, paraffin embedded, and 7 μm sections stained with H&E.

2.5.12. Fulvestrant treatment. Experimental liver metastases were generated by intrasplenic/portal injections of 10^5 tumor cells, followed by splenectomy. Mice were inoculated s.c. three times weekly with the indicated concentration of Fulvestrant in sterile sunflower seed oil (Sigma) or with sunflower seed oil + DMSO as vehicle control. Fulvestrant was reconstituted in DMSO as per manufacturer's instructions. Mice were sacrificed on day 21 post-MC-38 injection.

2.5.13. Fulvestrant and anti-PD-1 treatment. Experimental liver metastases were generated as described above (day 0). On day 1, 20mg/kg Fulvestrant (Sigma) reconstituted in DMSO and injected in sterile sunflower seed oil or sunflower seed oil/DMSO were administered subcutaneously. The following day 10mg/kg anti-PD-1 or 10mg/kg IgG isotype control (BioXcell) were administered intraperitoneally. This was repeated on days 4 and 5, respectively and thereafter weekly until day 15. The mice were sacrificed on day 18 post MC-38 injection.

2.5.14. Statistical analyses. The nonparametric Mann–Whitney test was used to analyze all metastasis data and a two-tailed Student's t-test was used to analyze ex vivo and in vitro data and the IF results.

2.6. References

1. Zhou H, Liu Z, Wang Y, Wen X, Amador EH, Yuan L, et al. Colorectal liver metastasis: molecular mechanism and interventional therapy. Signal Transduction and Targeted Therapy. 2022;7(1):70.
2. Siegel RL, Jakubowski CD, Fedewa SA, Davis A, Azad NS. Colorectal Cancer in the Young: Epidemiology, Prevention, Management. American Society of Clinical Oncology Educational Book. 2020(40):e75-e88.
3. Wu Y, Xu W, Wang L. IDDF2021-ABS-0191 Gender matters: sex disparities in colorectal cancer liver metastasis survival: a population-based study. Gut. 2021;70(Suppl 2):A142-A3.
4. Miller KD, Nogueira L, Mariotto AB, Rowland JH, Yabroff KR, Alfano CM, et al. Cancer treatment and survivorship statistics, 2019. CA Cancer J Clin. 2019;69(5):363-85.
5. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012;62(1):10-29.
6. Tauriello DV, Calon A, Lonardo E, Batlle E. Determinants of metastatic competency in colorectal cancer. Mol Oncol. 2017;11(1):97-119.
7. Gal-Oz ST, Shay T. Immune Sexual Dimorphism: Connecting the Dots. Physiology. 2022;37(2):55-68.
8. Ye Y, Jing Y, Li L, Mills GB, Diao L, Liu H, et al. Sex-associated molecular differences for cancer immunotherapy. Nat Commun. 2020;11(1):1779.
9. Cortese N, Marchesi F. Liver metastases "siphon" off immunotherapy response. Hepatobiliary Surg Nutr. 2021;10(4):526-9.
10. Milette S, Hashimoto M, Perrino S, Qi S, Chen M, Ham B, et al. Sexual dimorphism and the role of estrogen in the immune microenvironment of liver metastases. Nat Commun. 2019;10(1):5745.
11. Zhang Y, Zhao Y, Li Q, Wang Y. Macrophages, as a Promising Strategy to Targeted Treatment for Colorectal Cancer Metastasis in Tumor Immune Microenvironment. Front Immunol. 2021;12:685978.
12. Boutilier AJ, Elsawa SF. Macrophage Polarization States in the Tumor Microenvironment. Int J Mol Sci. 2021;22(13).

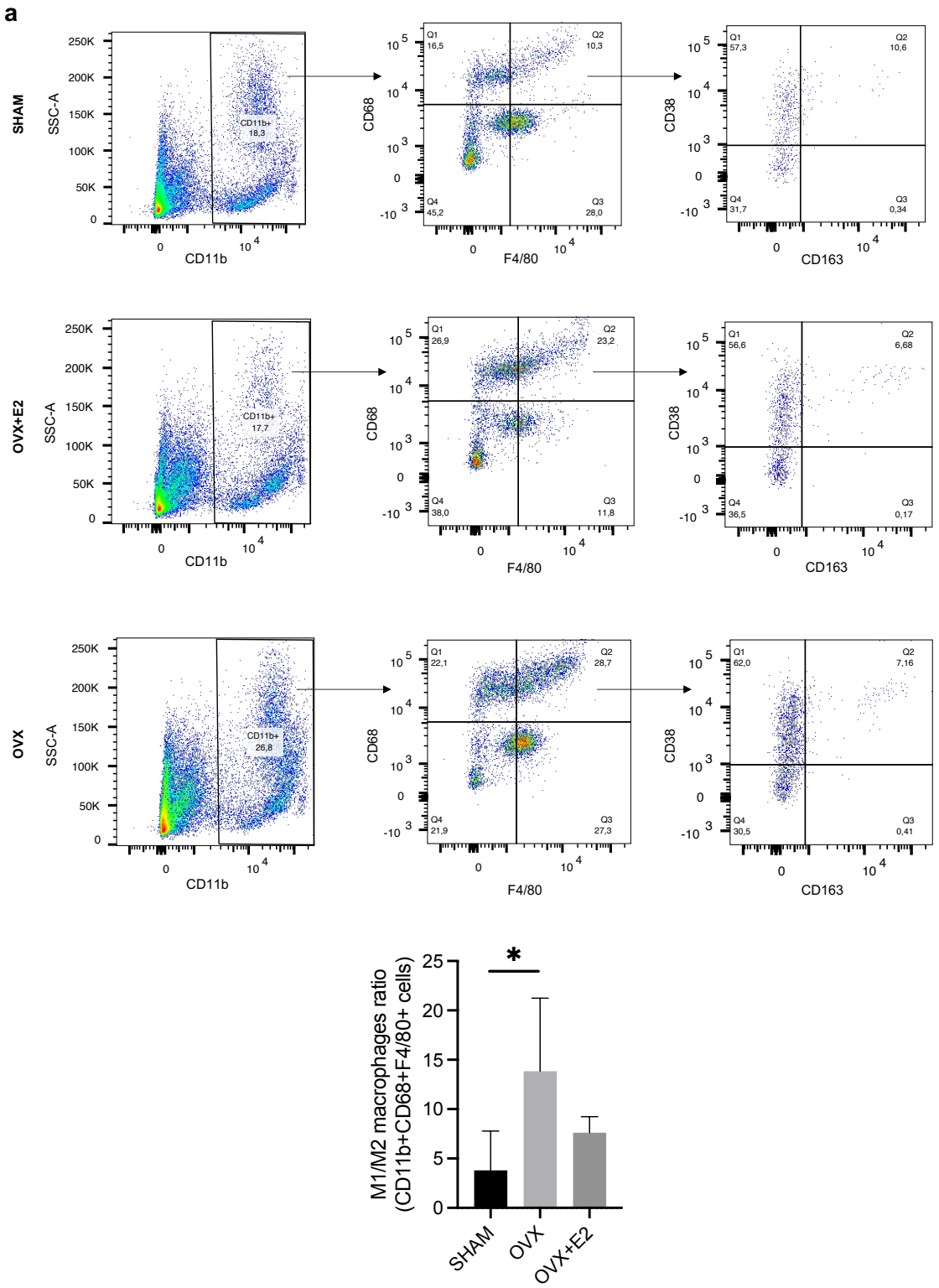
13. Elsawa SF, Novak AJ, Ziesmer SC, Almada LL, Hodge LS, Grote DM, et al. Comprehensive analysis of tumor microenvironment cytokines in Waldenstrom macroglobulinemia identifies CCL5 as a novel modulator of IL-6 activity. Blood. 2011;118(20):5540-9.
14. Li Z, Lee D, Zeng S, Yang L. Chapter 4 - Successes and challenges of NKT cell immunotherapy: Breaking tolerance to cancer resistance. In: Bonavida B, Jewett A, editors. Successes and Challenges of NK Immunotherapy: Academic Press; 2021. p. 63-80.
15. Klein I, Cornejo JC, Polakos NK, John B, Wuensch SA, Topham DJ, et al. Kupffer cell heterogeneity: functional properties of bone marrow–derived and sessile hepatic macrophages. Blood. 2007;110(12):4077-85.
16. Chakraborty B, Byemerwa J, Shepherd J, Haines CN, Baldi R, Gong W, et al. Inhibition of estrogen signaling in myeloid cells increases tumor immunity in melanoma. The Journal of Clinical Investigation. 2021;131(23).
17. Endo-Umeda K, Nakashima H, Komine-Aizawa S, Umeda N, Seki S, Makishima M. Liver X receptors regulate hepatic F4/80+CD11b+ Kupffer cells/macrophages and innate immune responses in mice. Scientific Reports. 2018;8(1):9281.
18. Cotechini T, Atallah A, Grossman A. Tissue-Resident and Recruited Macrophages in Primary Tumor and Metastatic Microenvironments: Potential Targets in Cancer Therapy. Cells. 2021;10(4).
19. Bandyopadhyay K, Marrero I, Kumar V. NKT cell subsets as key participants in liver physiology and pathology. Cellular & Molecular Immunology. 2016;13(3):337-46.
20. Geissmann F, Cameron TO, Sidobre S, Manlongat N, Kronenberg M, Briskin MJ, et al. Intravascular Immune Surveillance by CXCR6+ NKT Cells Patrolling Liver Sinusoids. PLOS Biology. 2005;3(4):e113.
21. Webb TJ, Yuan W, Meyer E, Dellabona P. Editorial: NKT Cells in Cancer Immunotherapy. Front Immunol. 2020;11:1314.
22. Karlmark KR, Wasmuth HE, Trautwein C, Tacke F. Chemokine-directed immune cell infiltration in acute and chronic liver disease. Expert Rev Gastroenterol Hepatol. 2008;2(2):233-42.

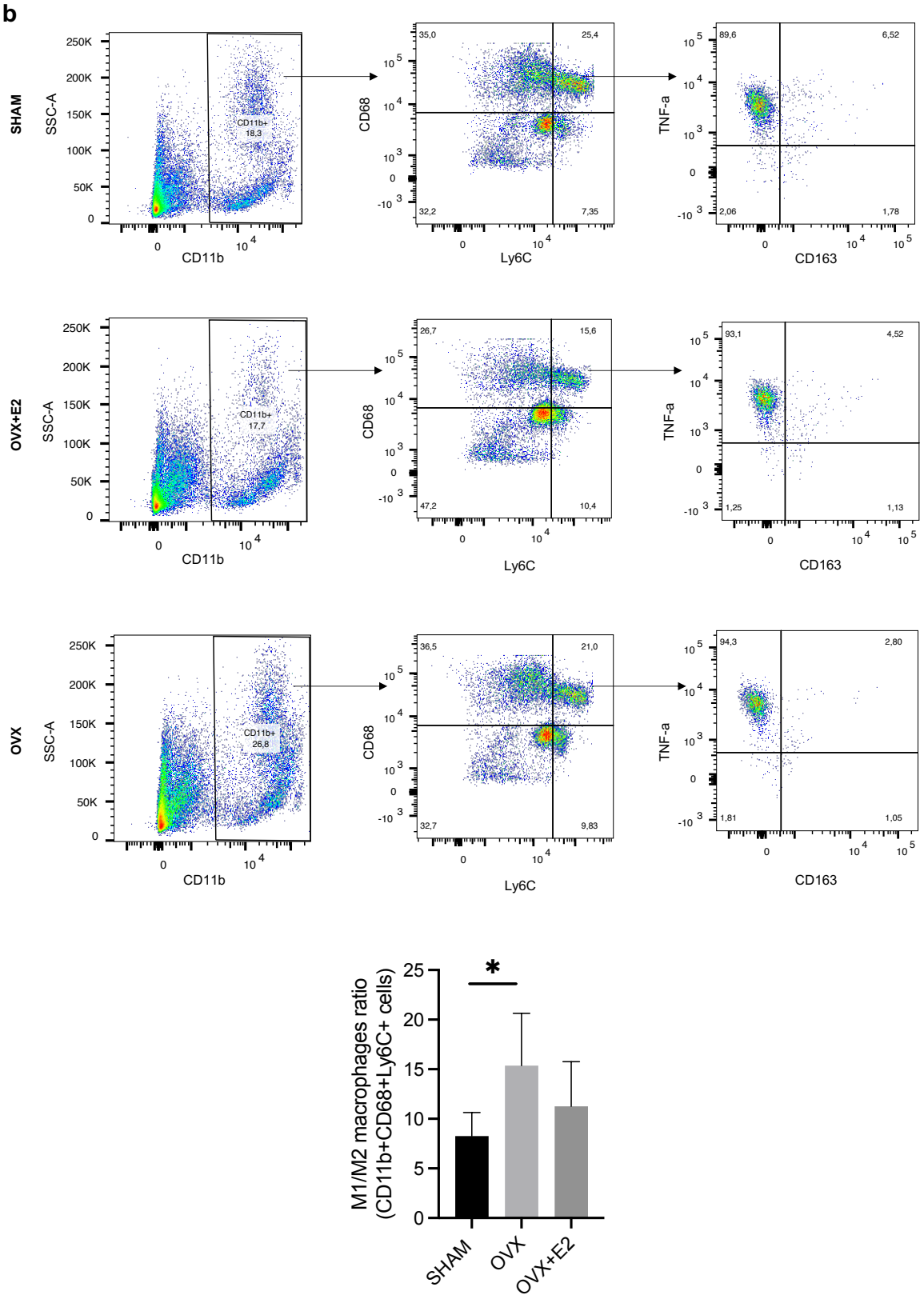
23. Tarique AA, Logan J, Thomas E, Holt PG, Sly PD, Fantino E. Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. Am J Respir Cell Mol Biol. 2015;53(5):676-88.
24. Woollard SM, Kanmogne GD. Maraviroc: a review of its use in HIV infection and beyond. Drug Des Devel Ther. 2015;9:5447-68.
25. Rocca A, Maltoni R, Bravaccini S, Donati C, Andreis D. Clinical utility of fulvestrant in the treatment of breast cancer: a report on the emerging clinical evidence. Cancer Manag Res. 2018;10:3083-99.
26. Boér K. Fulvestrant in advanced breast cancer: evidence to date and place in therapy. Ther Adv Med Oncol. 2017;9(7):465-79.
27. Nukatsuka M, Saito H, Noguchi S, Takechi T. Estrogen Down-regulator Fulvestrant Potentiates Antitumor Activity of Fluoropyrimidine in Estrogen-responsive MCF-7 Human Breast Cancer Cells. In Vivo. 2019;33(5):1439-45.
28. Varayathu H, Sarathy V, Thomas BE, Mufti SS, Naik R. Combination Strategies to Augment Immune Check Point Inhibitors Efficacy - Implications for Translational Research. Front Oncol. 2021;11:559161.
29. Tumeh PC, Hellmann MD, Hamid O, Tsai KK, Loo KL, Gubens MA, et al. Liver Metastasis and Treatment Outcome with Anti-PD-1 Monoclonal Antibody in Patients with Melanoma and NSCLC. Cancer Immunol Res. 2017;5(5):417-24.
30. Rothenberger NJ, Somasundaram A, Stabile LP. The Role of the Estrogen Pathway in the Tumor Microenvironment. Int J Mol Sci. 2018;19(2).
31. Efremova M, Rieder D, Klepsch V, Charoentong P, Finotello F, Hackl H, et al. Targeting immune checkpoints potentiates immunoediting and changes the dynamics of tumor evolution. Nat Commun. 2018;9(1):32.
32. Pandey V, Fleming-Martinez A, Bastea L, Doeppler HR, Eisenhauer J, Le T, et al. CXCL10/CXCR3 signaling contributes to an inflammatory microenvironment and its blockade enhances progression of murine pancreatic precancerous lesions. Elife. 2021;10.
33. Wang Q, Ren J, Morgan S, Liu Z, Dou C, Liu B. Monocyte chemoattractant protein-1 (MCP-1) regulates macrophage cytotoxicity in abdominal aortic aneurysm. PLoS One. 2014;9(3):e92053.

34. Sodhi A, Biswas SK. Monocyte chemoattractant protein-1-induced activation of p42/44 MAPK and c-Jun in murine peritoneal macrophages: a potential pathway for macrophage activation. J Interferon Cytokine Res. 2002;22(5):517-26.
35. Mu J. RhoA signaling in CCL2-induced macrophage polarization. Journal of Allergy and Clinical Immunology. 2018;141(2):AB114.
36. Nio Y, Yamauchi T, Iwabu M, Okada-Iwabu M, Funata M, Yamaguchi M, et al. Monocyte chemoattractant protein-1 (MCP-1) deficiency enhances alternatively activated M2 macrophages and ameliorates insulin resistance and fatty liver in lipoatrophic diabetic A-ZIP transgenic mice. Diabetologia. 2012;55(12):3350-8.
37. Wu Y, Yang S, Ma J, Chen Z, Song G, Rao D, et al. Spatiotemporal Immune Landscape of Colorectal Cancer Liver Metastasis at Single-Cell Level. Cancer Discov. 2022;12(1):134-53.
38. Jiang K, Li J, Zhang J, Wang L, Zhang Q, Ge J, et al. SDF-1/CXCR4 axis facilitates myeloid-derived suppressor cells accumulation in osteosarcoma microenvironment and blunts the response to anti-PD-1 therapy. International Immunopharmacology. 2019;75:105818.
39. Xu Y, Fang F, Jiao H, Zheng X, Huang L, Yi X, et al. Activated hepatic stellate cells regulate MDSC migration through the SDF-1/CXCR4 axis in an orthotopic mouse model of hepatocellular carcinoma. Cancer Immunol Immunother. 2019;68(12):1959-69.
40. Benedicto A, Romayor I, Arteta B. CXCR4 receptor blockage reduces the contribution of tumor and stromal cells to the metastatic growth in the liver. Oncol Rep. 2018;39(4):2022-30.
41. Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H, et al. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. Clin Cancer Res. 2005;11(20):7322-7.
42. Massalha H, Bahar Halpern K, Abu-Gazala S, Jana T, Massasa EE, Moor AE, et al. A single cell atlas of the human liver tumor microenvironment. Mol Syst Biol. 2020;16(12):e9682.
43. Dangaj D, Bruand M, Grimm AJ, Ronet C, Barras D, Duttagupta PA, et al. Cooperation between Constitutive and Inducible Chemokines Enables T Cell Engraftment and Immune Attack in Solid Tumors. Cancer Cell. 2019;35(6):885-900.e10.
44. Jin J, Lin J, Xu A, Lou J, Qian C, Li X, et al. CCL2: An Important Mediator Between Tumor Cells and Host Cells in Tumor Microenvironment. Front Oncol. 2021;11:722916.
45. Slauenwhite D, Johnston B. Regulation of NKT Cell Localization in Homeostasis and Infection. Frontiers in Immunology. 2015;6.

46. Van Acker HH, Beretta O, Anguille S, De Caluwé L, Papagna A, Van den Bergh JM, et al. Desirable cytolytic immune effector cell recruitment by interleukin-15 dendritic cells. Oncotarget. 2017;8(8):13652-65.
47. de Ridder J, de Wilt JH, Simmer F, Overbeek L, Lemmens V, Nagtegaal I. Incidence and origin of histologically confirmed liver metastases: an explorative case-study of 23,154 patients. Oncotarget. 2016;7(34):55368-76.
48. Böttcher JP, Bonavita E, Chakravarty P, Blees H, Cabeza-Cabrerizo M, Sammicheli S, et al. NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. Cell. 2018;172(5):1022-37.e14.
49. Ham B, Wang N, D'Costa Z, Fernandez MC, Bourdeau F, Auguste P, et al. TNF Receptor-2 Facilitates an Immunosuppressive Microenvironment in the Liver to Promote the Colonization and Growth of Hepatic Metastases. Cancer Res. 2015;75(24):5235-47.
50. Wang H, Yin S. Natural killer T cells in liver injury, inflammation and cancer. Expert Rev Gastroenterol Hepatol. 2015;9(8):1077-85.

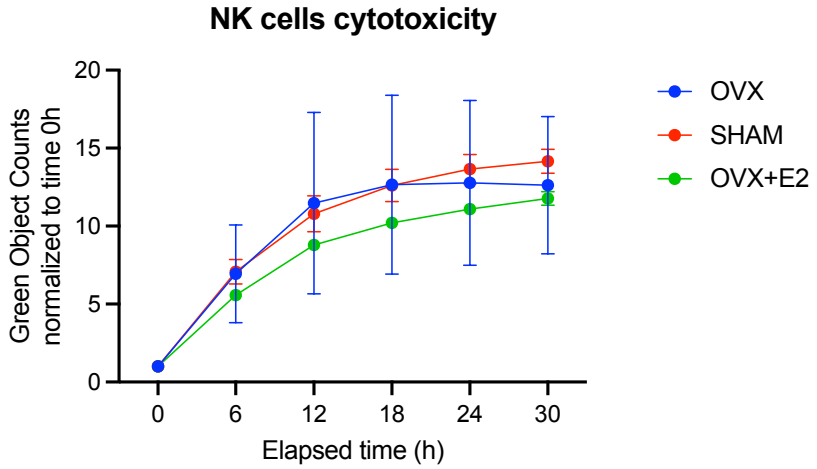
2.7. Supplementary Figures



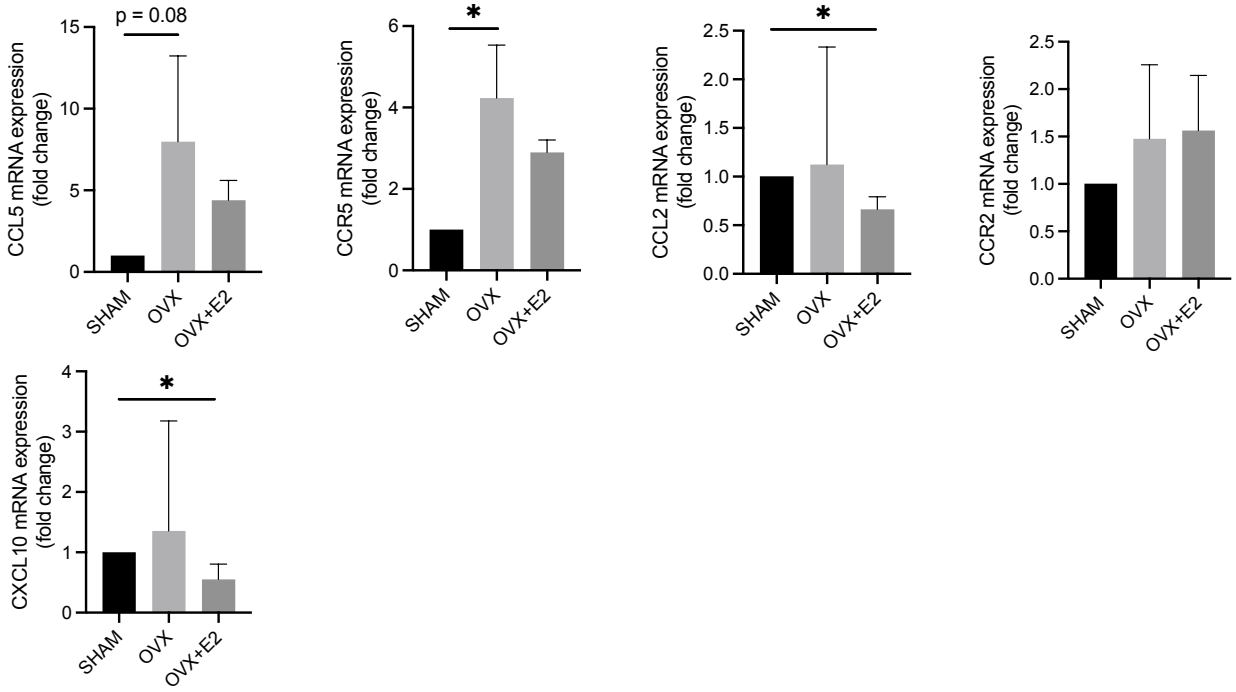


Supplementary Fig 1. Validation of M1/M2 macrophages results using additional markers. FC was performed on hepatic macrophages isolated on Day 12 following injections of 5 x 10^5 MC-

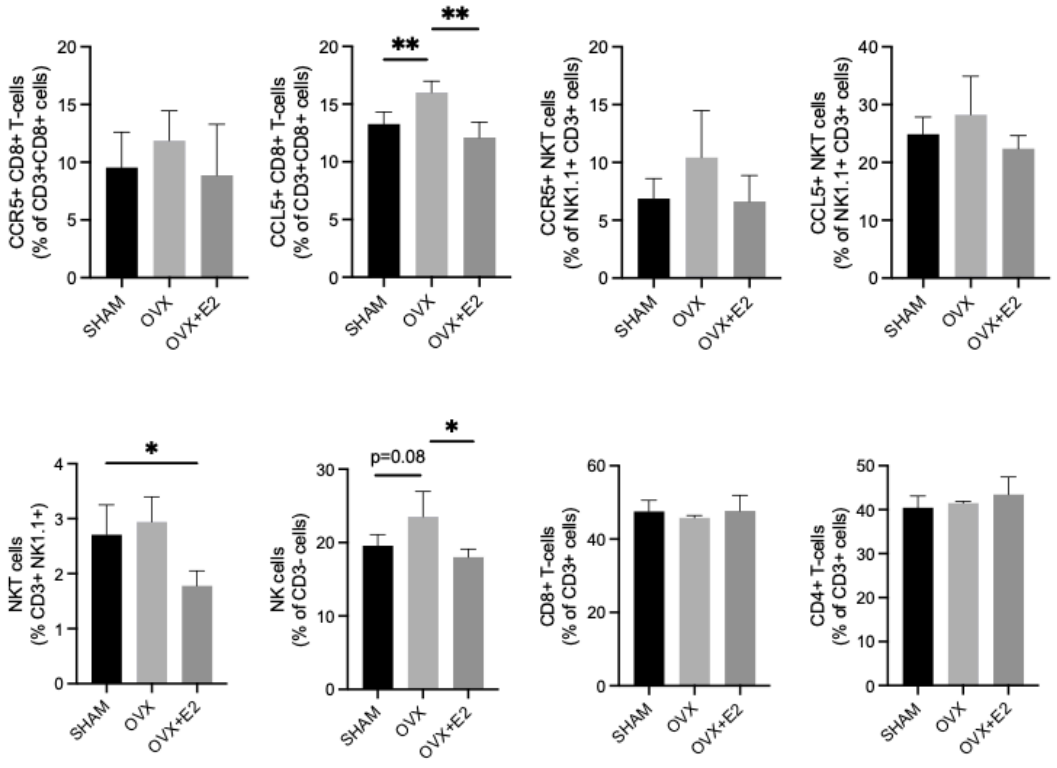
38 cells via intrasplenic/portal route in OVX, SHAM, and OVX+E2 mice. Additional Ly6C, CD38 and TNF-α markers were used to validate M1/M2 macrophages ratio findings.



Supplementary Fig 2. NK cells cytotoxicity is not significantly higher in estrogen-depleted compared to estrogen-competent mice. Quantification of dead (green) MC-38 cells normalized to time 0h (n=3).



Supplementary Fig 3. Validation of multiplex cytokine array results at the RNA level. RNA was isolated from the same livers used for the multiplex cytokine array, and cDNA was synthesized. RT-qPCR was performed looking at the mRNA expression levels of the indicated genes (n=3).



Supplementary Fig 4. Lymphocytes analyses in FC1199 pancreatic liver metastasis tumor model. FC was performed on hepatic lymphocytes isolated on Day 8 following injections of 5 x 10^5 FC1199 cells via intrasplenic/portal route in OVX, SHAM, and OVX+E2 mice (n=4).

Foreword

Results described in Chapter 2 showed that estrogen plays a critical role in the regulation of the liver immune microenvironment in CRCLM, via the accumulation of M2-like macrophages and reduction of NKT cells cytotoxicity. We therefore sought to determine whether estrogen is equally involved in the immune microenvironment of colorectal liver metastasis in human samples. The next Chapter of this thesis (Chapter 3) presents preliminary validation findings in a set of CRCLM patients' liver resections. Analyses on a larger sample size are still ongoing.

Chapter 3

Validation Findings on the Role of Estrogen in Human CRCLM Surgical Resections

Chapter 3: Validation Findings on the Role of Estrogen in Human CRCLM Surgical Resections

This work was performed in collaboration with Dr. Elaheh Papari (Postdoctoral fellow, Brodt Lab) and Fazila Chouiali (Histopathology Core Facility, RI-MUHC). In addition to my main thesis project, I have contributed to this project by optimizing the experimental techniques and protocols, analysing the preliminary results, and coordinating the experiments with the Core Facility. The FFPE liver samples were a kind gift from Dr. Jason Sicklick, Division of Surgical Oncology Moores Cancer Center, La Jolla, CA 92093.

3.1. Abstract

Liver metastases (LM) occur in almost 50% of patients with colorectal carcinoma (CRC) and CRC is increasing in the younger population, being the 4th most diagnosed cancer among men and women aged 30-39 years old. LM and female sex have been identified as poor predictors to immunotherapy (IT) response, but the reasons remain unclear. Although a sexual dimorphism in the immune response is well documented, the effect of sex on patients' response to immune checkpoint inhibitors (ICIs) in cancer treatment, has not been adequately investigated. To determine whether our results in the mouse model of CRC liver metastasis were relevant clinically, we began to analyze immune cell subpopulation in surgical CRCLM specimens using FFPE sections. Here preliminary results obtained on a small number of specimens are described. We observed a higher proportion of $CD68^{+}CD163^{+}$ and $CD68^{-}CD163^{+}$ M2-like macrophages in the young female patient comparatively to the older female, the latter having a higher propensity of $CD68^{+}CD163^{-}$ M1-like macrophages. Analyses on a larger sample size are currently ongoing.

3.2. Introduction

Liver metastases (LM) occur in almost 50% of patients with colorectal carcinoma (CRC). The incidence of CRC in the United States between 2014 and 2018 dropped by 2% each year in adults 50 years of age and older due mainly to increased screening. However, during the same period, the incidence in adults younger than 50 years rose by 1.5% annually and CRC has become the 4th most diagnosed cancer among men and women aged 30-39 years old (data from ASCO, unpublished). Furthermore, male patients had a better 8-year overall survival (OS) than female patients with left or right colon cancer and with synchronous or metachronous liver metastases (1). In patients diagnosed with advanced stage CRC, the 5-year survival rate stands at 12% (2). To date, the most effective treatment option is surgical resection of liver metastases. However, only approximately 20% of patients with CRCLM are eligible for partial hepatectomy, generally due to size, number, and location of the metastases and relapse can occur in up to 75% of resected patients (3, 4). To improve these statistics, combinatorial therapies that also target the pro-metastatic tumor microenvironment of the liver are actively being sought.

Immune checkpoint inhibitors (ICIs) enhance the anti-tumor effect of patients own immune system by reactivating the antitumor activity of exhausted T cells and NK cells (5). Compared with previous standards of care (including chemotherapy, radiotherapy, and surgery), immunotherapy (IT), which is based on the use of ICIs, has brought significant improvements for cancer patients' survival and quality of life (6). LM and female sex have been identified as poor predictors to IT response, but the reasons remain unclear (7, 8). Although a sexual dimorphism in the immune response is well documented, the effect of sex on patients' response to immune

checkpoint inhibitors (ICIs) in cancer treatment, has not been adequately investigated. In the context of CRCLM, the roles of patients' sex and age on the immune microenvironment and response to ICI have not been examined. Interestingly, recent studies have linked M2-like macrophage accumulation with increased liver metastasis in CRCLM patients (9). In mice, we found an increase in M2-like macrophages accumulation in estrogen-competent CRCLM-bearing mice which was accompanied by an increase in CRCLM outgrowth, compared to the estrogen-depleted counterpart. To determine whether our results in the mouse model of CRC liver metastasis were relevant clinically, we began to analyze immune cell subpopulation in surgical CRCLM specimens using FFPE sections. Here, preliminary results obtained on a small number of specimens are described. Analyses on a larger sample size are currently ongoing.

3.3. Results

3.3.1. Immunohistochemistry analyses on CRCLM resections show variability in tumor-associated NK cells and M1/M2 ratios.

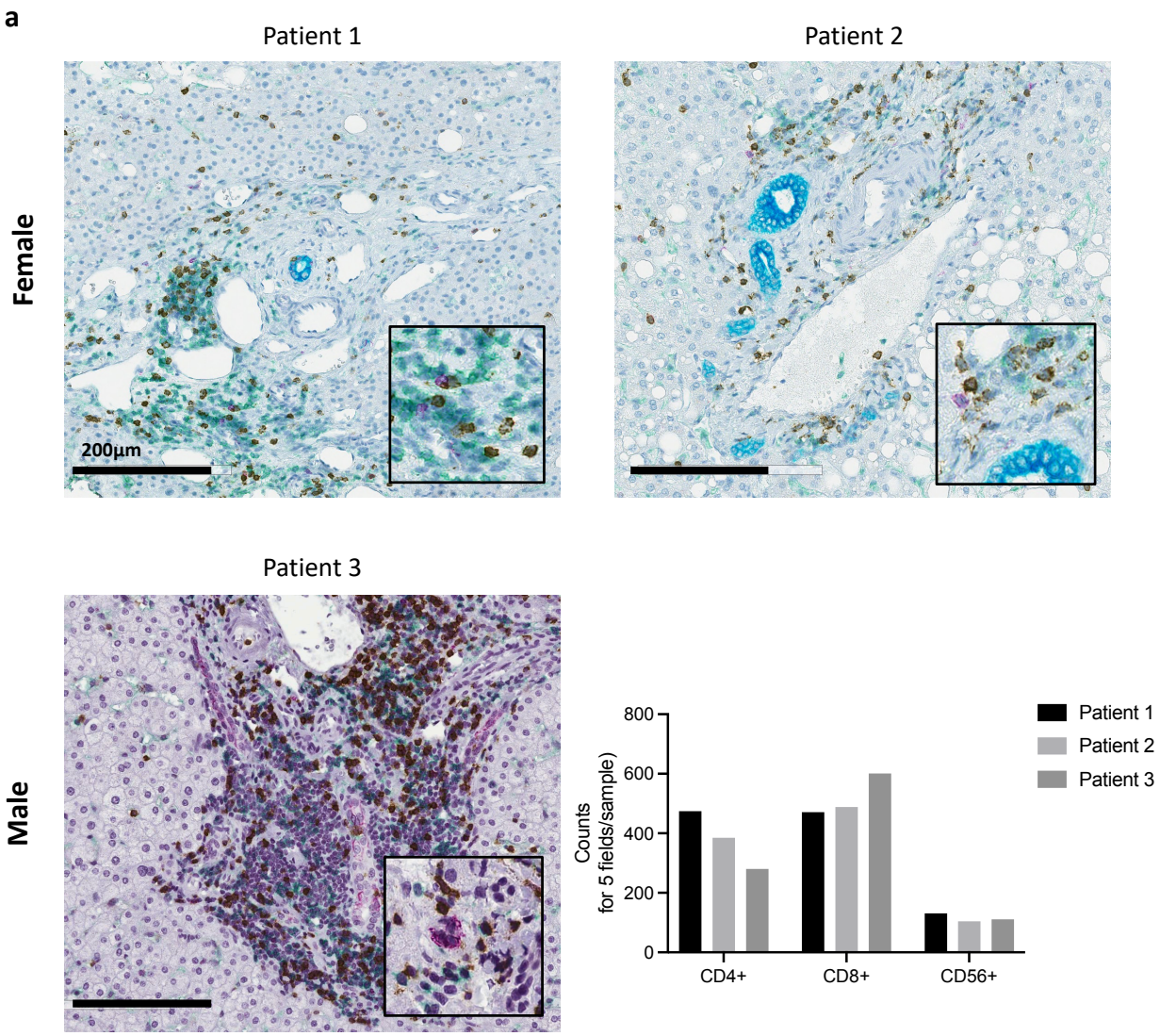
Formalin-Fixed Paraffin-Embedded (FFPE) liver resections available from a total of 6 patients were used (see information in Table 1 below). Samples from patients 1-3 were immunostained with antibodies to cytokeratin (CK) 19, CD8, CD4, and CD56 and the staining intensity scored using a + - ++++ score. Results are shown in Table 1 and Figure 9. Of interest, we observed the highest score for $CD4^+$ T cells and $CD56^+$ NK cells in the younger female patient while $CD8^+$ T cells had the highest score in the male patient. Additional specimens were stained for CD163, CD68, and estrogen receptors (ER). We observed a marked reduction in $CD163^+$ cells in sections derived from the older female (83 years old (y.o)) and male (65 y.o) patients as compared to their younger counterparts. While these preliminary results on a limited sample size

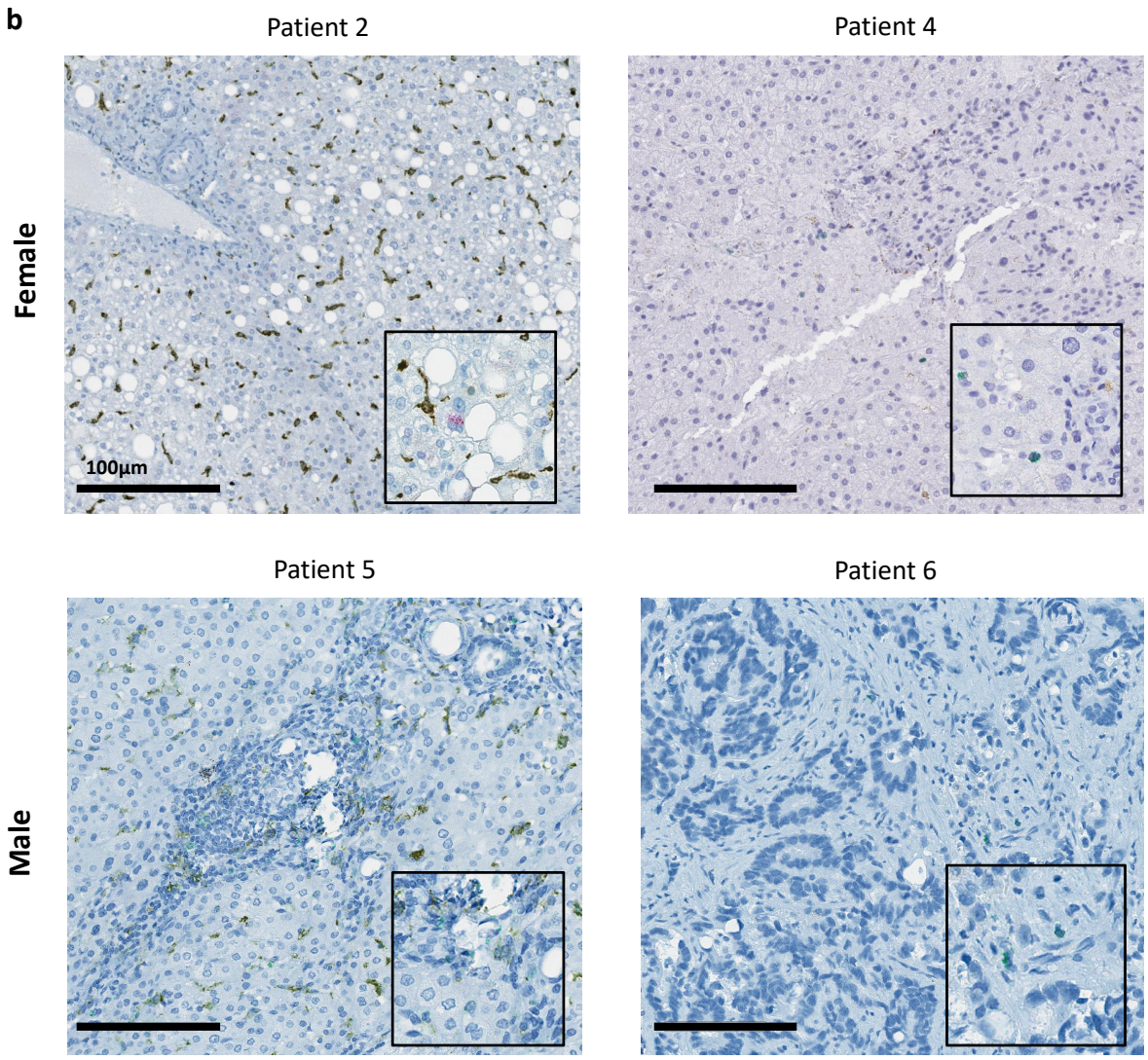
are suggestive of an age-related difference in immune cell infiltration, confirmation on a larger patient cohort (work now in progress) will be crucial to obtaining conclusive data.

Table 1. Patients sex, age, and immunohistochemistry staining scores

Patient	Sex	Age	CD68+ CD163-	CD68+ CD163+	CD68- CD163+	ER+	CD56+	CD8+	CD4+
1	Female	41					++	++	+++
2	Female	51	+	++	++++	+++	+	++	++
3	Male	54					+	+++	+
4	Female	83	+++	+	++	+			
5	Male	43	++++	++++	+++	+			
6	Male	65	++	+	+	+			

**areas in light gray mean that no staining was done on these particular samples*





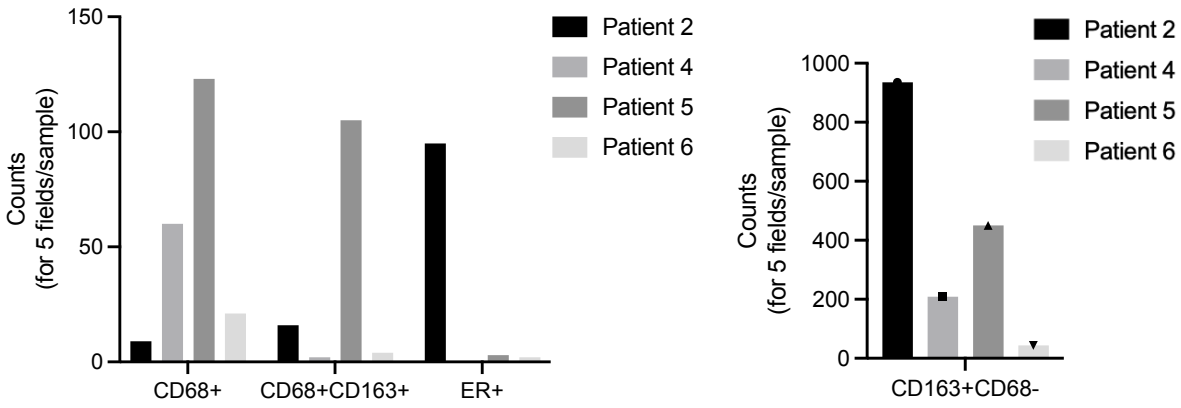


Fig. 9 Multiplex immunohistochemistry analyses of CRCLM patients' liver sections. Representative Multiplex immunohistochemistry (mIHC) was performed using the Ventana Discovery Ultra automated system on FFPE liver sections obtained CRCLM patients **(a)** Analysis of lymphocytes was done on tissue from Patient 1 (Female, 41 y.o), Patient 2 (Female, 51 y.o), and Patient 3 (Male, 54 y.o) where Cytokeratin (CK) 19 (teal), CD8 (DAB), CD4 (green), and CD56 (purple) were detectable **(b)** Analysis of macrophages was done on tissue from Patient 2 (Female, 51 y.o), Patient 4 (Female, 83 y.o), Patient 5 (Male, 43 y.o), and Patient 6 (Male, 65 y.o) where CD163 (DAB), CD68 (green), and estrogen receptors/ER (purple) were detectable. Slides were scanned using the Leica Aperio AT Turbo digital pathology scanner. Counts were done on 5 fields per section and shown in respective panels.

3.4. Discussion

Several reports demonstrate differential immune cells accumulation in CRCLM compared to healthy liver or primary CRC tumor, and between treatment-responsive or non-responsive patients (9, 10). In fact, He et al. reported that $CD56^+$ NK cells were more commonly found in primary CRC tumors than in liver metastases, while $FoxP3^+$ regulatory T cells and $CD163^+$ macrophages were more enriched in liver metastases ($p<0.05$) (10).

Here we show a differential accumulation in lymphocytes and macrophages in a small number of CRCLM patients of different sexes and ages. We observed a marked decrease in $CD163^+$ and $CD68^+CD163^+$ M2-like macrophages and ER expression in the 83 y.o female patient compared to the 41 y.o patient, indicative of a potential estrogen-dependent M2-like macrophages accumulation. These preliminary results are in line with our findings in the mouse model. Similarly, but to a lesser extent, we observed a lower count in these M2-like macrophages in the 65 y.o male patient as compared to the 43 y.o male patient. Single-cell RNA sequencing and spatial transcriptomic analyses revealed an important enrichment in *MRC1*$^+$ (coding for CD206) *CCL18*$^+$

SPP1^{+} M2-like macrophages in livers harboring CRCLM as compared to primary CRC tumors (9). Moreover, these M2-like macrophages accumulated in patient that had the worse response to neoadjuvant chemotherapy. In the same study, the accumulation of CXCL10^{+} M1-like macrophages and CCL5^{+} T cells was documented in CRCLM, consistent with our findings in mice. We also found a greater accumulation of CD4^{+} and CD56^{+} lymphocytes in the 41 y.o. female patient as compared to the 51 y.o. female and male patients. Additionally, the accumulation of CD8^{+} cells was highest in the male patient. It was reported that CRC and melanoma patients with liver metastases exhibited an accumulation of polymorphonuclear (PMN)-MDSCs and a paucity of NKT cells compared to healthy liver transplantation donors, and this was associated with a hyperactivation of hepatic CCRK/NF-κB/CXCL1 signaling (11). Furthermore, T cell expansion has been observed in CRCLM enriched with Th17 CD4^{+} lymphocytes and this has been correlated to CRCLM patients' survival (12). Kryceczek et al. demonstrated that IL-17A and IFN-γ secreted by Th17 cells act synergistically to stimulate CXCL9 and CXCL10 production to recruit effector T cells to the TME (13). In contrast, increased frequencies of CD4^{+} T cells and decreased frequencies of CD8^{+} T cells were observed in CRCLM when compared to adjacent liver (14).

Of note, acquisition of additional data on a larger patient cohort is now in progress in our laboratory and this will include additional analyses of M1 and M2-like macrophages, NKT cells and MDSCs. Our preliminary results show infiltration of macrophages, M2-like macrophages, NK cells, CD4+ T cells, and CD8+ T cells into the CRCLM and demonstrate variability which may be related to age, possibly related (in females) to differential estrogen levels. Current treatments including systemic, radical, and localized therapies have achieved a limited success in CRCLM patients, which is further disadvantaged by high recurrence rate. A better understanding

of the underlying mechanisms governing the metastasis-permissive liver immune microenvironment will open new immuno-oncology avenues for CRCLM management.

3.5. Material and Methods

3.5.1. Patients' samples. The FFPE liver samples were a kind gift from Dr. Jason Sicklick, Division of Surgical Oncology Moores Cancer Center, La Jolla, CA 92093, and were given as tissue sections.

3.5.2. Automated multiplex immunohistochemistry. Multiplex immunohistochemistry (mIHC) was performed at the RI-MUHC Histopathology Core Facility using the fully automated Ventana Discovery Ultra automated slide preparation system and using the Core Facility optimized antibodies. Slides were scanned using the Leica Aperio AT Turbo digital pathology scanner.

3.6. References

1. Wu Y, Xu W, Wang L. IDDF2021-ABS-0191 Gender matters: sex disparities in colorectal cancer liver metastasis survival: a population-based study. Gut. 2021;70(Suppl 2):A142-A3.
2. Miller KD, Nogueira L, Mariotto AB, Rowland JH, Yabroff KR, Alfano CM, et al. Cancer treatment and survivorship statistics, 2019. CA Cancer J Clin. 2019;69(5):363-85.
3. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012;62(1):10-29.
4. Tauriello DV, Calon A, Lonardo E, Batlle E. Determinants of metastatic competency in colorectal cancer. Mol Oncol. 2017;11(1):97-119.
5. Koi M, Carethers JM. The colorectal cancer immune microenvironment and approach to immunotherapies. Future Oncol. 2017;13(18):1633-47.
6. Esfahani K, Roudaia L, Buhlaiga N, Del Rincon SV, Papneja N, Miller WH, Jr. A review of cancer immunotherapy: from the past, to the present, to the future. Curr Oncol. 2020;27(Suppl 2):S87-s97.
7. Ye Y, Jing Y, Li L, Mills GB, Diao L, Liu H, et al. Sex-associated molecular differences for cancer immunotherapy. Nat Commun. 2020;11(1):1779.
8. Yu X, Zhu L, Liu J, Xie M, Chen J, Li J. Emerging Role of Immunotherapy for Colorectal Cancer with Liver Metastasis. Onco Targets Ther. 2020;13:11645-58.
9. Wu Y, Yang S, Ma J, Chen Z, Song G, Rao D, et al. Spatiotemporal Immune Landscape of Colorectal Cancer Liver Metastasis at Single-Cell Level. Cancer Discov. 2022;12(1):134-53.
10. He Y, Han Y, Fan Ah, Li D, Wang B, Ji K, et al. Multi-perspective comparison of the immune microenvironment of primary colorectal cancer and liver metastases. Journal of Translational Medicine. 2022;20(1):454.
11. Zeng X, Ward S, Zhou J, Cheng A. Liver Immune Microenvironment and Metastasis from Colorectal Cancer-Pathogenesis and Therapeutic Perspectives. Cancers. 2021;13:2418.
12. Kroemer M, Turco C, Spehner L, Viot J, Idirène I, Bouard A, et al. Investigation of the prognostic value of CD4 T cell subsets expanded from tumor-infiltrating lymphocytes of colorectal cancer liver metastases. J Immunother Cancer. 2020;8(2).

13. Kryczek I, Banerjee M, Cheng P, Vatan L, Szeliga W, Wei S, et al. Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. Blood. 2009;114(6):1141-9.
14. Liang F, Nilsson LM, Byvald F, Rezapour A, Taflin H, Nilsson JA, et al. A Fraction of CD8+ T Cells from Colorectal Liver Metastases Preferentially Repopulate Autologous Patient-Derived Xenograft Tumors as Tissue-Resident Memory T Cells. Cancers (Basel). 2022;14(12).

Chapter 4
General Discussion

Chapter 4: General Discussion

4.1. Overview of data and Contribution to Knowledge

The clinical management of liver metastases remains a challenge. The resistance of liver metastatic disease to standard of care therapies and the finding that female patients respond more poorly to immunotherapy warrant the development of a more personalized approach for prevention and management of liver metastatic disease. This study examined the role of estrogen in regulating the immune microenvironment of liver metastases. It also provides proof-of-concept data in support of the conclusion that immunotherapy efficacy for CRCLM may limited, at least in part, due to an estrogen regulated immunosuppressive TME. We demonstrated herein a marked reduction in CRCLM outgrowth, in both number and size, in the experimental group treated with the combination of Fulvestrant and anti-PD-1 immunotherapy. This beneficial therapeutic outcome was accompanied by an increase in $CD8^+$ T, NK and NKT cells, the M1/M2 macrophage ratio, increased dendritic cell activation, increased accumulation of $CCR5^+CCL5^+$ cells and a decrease in MDSCs in the liver TME. Consistent with our data, Cui et al. reported that a decrease in M1/M2 macrophage ratio was positively correlated with the development of CRCLM (1). Furthermore, in our studies, mRNA analyses revealed an increase in IFN-γ and a decrease in the pro-tumorigenic cytokine TGF-β in the livers of combination therapy-treated mice and in addition, we found that blockade of the CCR5/CCL5 signaling pathway using Maraviroc promoted CRCLM outgrowth, indicating that this pathway may be anti-tumorigenic. Current reports indicate that CCL5 is a double-edged sword; it may promote antitumor immunity by recruiting anti-tumor T cells and dendritic cells to the TME (2) or can promote metastasis via the recruitment of regulatory T cells (3). In our study, we found CCR5 and CCL5 to be expressed by $CD8^+$ T cells and NKT

cells; two major cytotoxic immune cells in the liver TME. This was also observed in a second mouse model, namely following injection of pancreatic ductal adenocarcinoma FC1199 cells suggesting that it has broader relevance. Additionally, we investigated the cellular sources of CXCL10 and CXCR3, and identified NK cells, NKT cells, and macrophages as the major sources of CXCL10. In CRCLM, it was recently shown that STING signaling downstream of CXCL10, promoted NLRP3-mediated IL-18 and IL-1β production by macrophages and subsequent anti-tumor response of NK cells, hence suppressing CRCLM (4).

In ovariectomized mice, we found an increased expression of CXCR3 on macrophages at early time points and this coincided with the expression of CCR2 on these cells. Interestingly, the $CCR2^{+}$ macrophages were also the most polarized to the M1-phenotype, when comparing to $CCR2^{-}$ macrophages. Together, these results indicate that CXCR3 expression on recruited macrophages may promote their polarization to the M1-like phenotype, via the action of one of its ligands CXCL10. Moreover, using an Incucyte®-based cytotoxicity assay that enables visualization and quantification of cell death in real time, we found a significantly higher NKT cell cytotoxicity in estrogen-depleted compared to estrogen-competent mice, and this was reversed upon E2 supplementation. While the effect of E2 on NKT cells has not been previously described, it was shown that E2 reduced NK cells cytotoxicity, while progesterone exerted an opposite effect, in cervical cancer (5). We also optimized a macrophage isolation protocol to evaluate macrophage polarization profiles. Using this protocol, we have identified a significant increase in M1/M2-like macrophage ratio in estrogen-depleted compared to estrogen-competent mice, and this was reversed upon E2 supplementation. We also show in a few human CRCLM tissue samples a decrease in M2-like macrophages in an older female as compared to younger female patients, and

this trend was similar in male patients. While we still need to confirm these findings in a larger patients' cohort, these preliminary results combined with our findings in the mouse models highlight the importance of evaluating the immune infiltrate in male and female patients of different ages as both the sex and the age of the patient may affect the type and frequency of innate and adaptive immune cells.

Taken together, our findings warrant further investigating the potential use of SERD to enhance the efficacy of anti-PD-1 therapy in premenopausal female patients (age <50) but may also be of useful for postmenopausal female patients, as estrogen production still slightly occurs mainly in adipose tissue (6). This combinatorial therapy may be beneficial for the clinical management CRCLM and other hormone-independent cancers with a propensity to metastasize to the liver.

4.2. Limitations and Future Studies

Our results provide further evidence for the effect of estrogen on the immune microenvironment of CRCLM. Future investigations should complete the functional characterization of the M1-like and M2-like macrophages, investigate the cell-cell communication pathways, and assess the broader relevance of the findings in a large cohort of CRCLM patients. Furthermore, validation of the cellular sources of CCL5, CXCL10 and other cytokines of interest should be assessed at the mRNA level. These validation analyses are currently ongoing using in Situ Hybridization (RNAscope). Observations of lymphocytes frequencies and CCL5/CCR5 cellular expression were similar in the pancreatic liver metastasis FC1199 model. Additionally, in this tumor model, validation of macrophages polarization and of the effect of Fulvestrant and anti-PD-1 treatment

are being undertaken. While the major estrogen form E2 has been supplemented in ovariectomized mice in this study, it would be interesting to evaluate the effect of other ovarian hormones, such as E1, E3, progesterone and androgens, on the immune microenvironment and CRCLM outgrowth. There are a number of potential mechanisms through which estrogen deprivation could lead to the chemokine production and immune cell phenotype modulation. Estrogen deprivation has been associated with alterations in the expression of multiple genes, which may reflect the diverse potential cellular pathways mediating the effect on chemokine secretion (7). For instance, E2 has been shown to repress mRNA expression of *CCL5* in a mouse model of autoimmune encephalomyelitis (8). The underlying mechanisms are poorly understood, but type 1 interferon production was shown to be controlled by estrogen (9). Alternatively, estrogen deprivation may also have a more direct effect on immune cells. ERα is expressed on most immune cells, including NKT cells (10) and macrophages (11); hence, some of the immune effects seen in this study may be the result of direct regulation of immune cells by estrogen. The latter may in turn regulate chemokines production and be responsible of the differences observed in chemokine production.

Moreover, as prospective studies, it would be of interest to investigate the potential of Fulvestrant to enhance the therapeutic efficacy of other immunotherapeutic agents such as anti-CTLA4, anti-PDL-1, anti-Lag3 and other immune checkpoint inhibitors of this class. Furthermore, it is worth considering future investigation of the efficacy of new emerging technologies, such as PROTACS (Proteolysis targeting chimeras) which are designed to target selectively receptor degradation, in combination with immunotherapeutic agents (12). For example, ARV-471, an ER degrader currently in phase 3 clinical trials for patients with ER+/HER2- locally advanced or metastatic breast cancer was identified as a more potent ER degrader than Fulvestrant and other SERDs (12).

Finally, testing anti-androgen receptor drugs in combination with immunotherapy is of interest for future work. Evaluation of the effect of estrogen in macrophage polarization *in vitro* can also be but would only give insight on the direct estrogenic effect and not about the immune microenvironment role on this process. Using multiplexed analyses of liver tissues or cell suspension, using high-throughput technologies such as imaging mass cytometry, could bring an integrated overview of the different spatiotemporal interactions between immune actors and CRCLM tumor and could provide a way to monitor the polarization spectra of macrophages.

4.3. Conclusion

In this study, we demonstrate that estrogen exert pro-metastatic effects in the liver microenvironment by promoting immunosuppressive macrophages accumulation, reducing the cytotoxic potential of NKT cells, and limiting anti-PD-1 immunotherapy efficacy. Our results provide additional insight into the underlying mechanisms of sexual dimorphisms in immunity and immunotherapy response and thereby provide a rationale for further considering the role of hormone-receptor antagonists for personalizing immunotherapy for management of hormone-independent severe malignancies.

4.4. References

1. Cui YL, Li HK, Zhou HY, Zhang T, Li Q. Correlations of tumor-associated macrophage subtypes with liver metastases of colorectal cancer. Asian Pac J Cancer Prev. 2013;14(2):1003-7.
2. Aldinucci D, Borghese C, Casagrande N. The CCL5/CCR5 Axis in Cancer Progression. Cancers (Basel). 2020;12(7).
3. Tan MC, Goedegebuure PS, Belt BA, Flaherty B, Sankpal N, Gillanders WE, et al. Disruption of CCR5-dependent homing of regulatory T cells inhibits tumor growth in a murine model of pancreatic cancer. J Immunol. 2009;182(3):\1746-55.
4. Sun Y, Hu H, Liu Z, Xu J, Gao Y, Zhan X, et al. Macrophage STING signaling promotes NK cell to suppress colorectal cancer liver metastasis via 4-1BBL/4-1BB co-stimulation. J Immunother Cancer. 2023;11(3).
5. Godoy-Pacheco A, García-Chagollán M, Ramírez-De-Arellano A, Hernández-Silva CD, Villegas-Pineda JC, Ramírez-López IG, et al. Differential modulation of natural killer cell cytotoxicity by 17β-estradiol and prolactin through the NKG2D/NKG2DL axis in cervical cancer cells. Oncol Lett. 2022;24(2):288.
6. Cui J, Shen Y, Li R. Estrogen synthesis and signaling pathways during aging: from periphery to brain. Trends Mol Med. 2013;19(3):197-209.
7. Hazlett J, Niemi V, Aiderus A, Powell K, Wise L, Kemp R, et al. Oestrogen deprivation induces chemokine production and immune cell recruitment in in vitro and in vivo models of oestrogen receptor-positive breast cancer. Breast Cancer Research. 2021;23(1):95.
8. Matejuk A, Adlard K, Zamora A, Silverman M, Vandenbark AA, Offner H. 17 beta-estradiol inhibits cytokine, chemokine, and chemokine receptor mRNA expression in the central nervous system of female mice with experimental autoimmune encephalomyelitis. J Neurosci Res. 2001;65(6):529-42.
9. Dragin N, Nancy P, Villegas J, Roussin R, Le Panse R, Berrih-Aknin S. Balance between Estrogens and Proinflammatory Cytokines Regulates Chemokine Production Involved in Thymic Germinal Center Formation. Sci Rep. 2017;7(1):7970.
10. Gourdy P, Araujo LM, Zhu R, Garmy-Susini B, Diem S, Laurell H, et al. Relevance of sexual dimorphism to regulatory T cells: estradiol promotes IFN-gamma production by invariant natural killer T cells. Blood. 2005;105(6):2415-20.

11. Vishnyakova P, Poltavets A, Nikitina M, Midiber K, Mikhaleva L, Muminova K, et al. Expression of Estrogen Receptor α by Decidual Macrophages in Preeclampsia. Biomedicines. 2021;9(2).
12. Békés M, Langley DR, Crews CM. PROTAC targeted protein degraders: the past is prologue. Nature Reviews Drug Discovery. 2022;21(3):181-200.

Annex

qPCR Primer Sequences

Gene	Forward primer sequence	Reverse primer sequence
CCL5	GCTCCAATCTTGCAGTCG	GACCGAGTGGGAGTAGGG
CCR5	GAGGTGAGACATCCGTTCCC	TTGGCAGGGTGCTGACATAC
CCL2	GGCTGGAGAGCTACAAGAGG	GGTCAGCACAGACCTCTCTC
CCR2	AAGACCAGAAGAGGGCATTG	CGTAGTCATACGGTGTGGTG
CXCL10	TCCGCTGCAACTGCATCCATA	CAGGATAGGCTCGCAGGGAT
GAPDH	ACCCAGAAGACTGTGGATGG	ACACATTGGGGGTAGGAACA
TGF-β	GGACTCTCCACCTGCAAGAC	CTGGCGAGCCTTAGTTTGGA
IFN-γ	AGCAAGGCGAAAAAGGATGC	TCATTGAATGCTTGGCGCTG

Flow Cytometry Antibodies

Name	Company	Catalog Number
CD3 PE	EBioscience	12-0031-81
CD11b PE	BD Bioscience	557397
NK1.1 APC	Invitrogen	17-5941-82
CD8a BV650	Biolegend	100742
CD68 AF488	Biolegend	137012
F4/80 BV786	Biolegend	123141
CCR2 BV510	Biolegend	150617
Ly6C PerCP-Cy5.5	Invitrogen	45-5932-82
CD163 APC	Biolegend	155306
MHCII AF700	Invitrogen	56-5321-82
TNF-α APC-Cy7	Biolegend	506343
CD38 PE-Cy5	Abcam	ab25043
CD11c AF647	Biolegend	117312
CCL5 PE-Cy7	Biolegend	149105

CCR5 BV605	BD Bioscience	743697
Ly6G PE-Cy7	Invitrogen	25-9668-82
CXCR3 PE-Cy7	Invitrogen	25-1831-82
CD8b	BD Bioscience	567737