Doctoral theses at NTNU, 2023:189

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Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
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Trondheim, June 2023

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ISBN 978-82-326-7080-2 (printed ver.)
ISBN 978-82-326-7079-6 (electronic ver.)
ISSN 1503-8181 (printed ver.)
ISSN 2703-8084 (online ver.)

Doctoral theses at NTNU, 2023:189

Printed by NTNU Grafisk senter

Nye angrepspunkter for målrettet behandling av aggressiv kreft

Brystkreft er en av de mest utbredte kreftformene og vi har i dag gode behandlingsformer. Hos noen brystkreftpasienter vil likevel kreftceller sprer seg fra den opprinnelige svulsten og etablere seg nye steder i kroppen. Brystkreft med spredning er vanskelig å behandle og gir dårlig prognose for pasienten.

Målet med prosjektet var å bidra til økt kunnskap om de underliggende mekanismene for aggressiv kreftutvikling. Håpet er at dette skal bidra til forbedret prognose for pasienter med aggressiv kreftutvikling.

Immunsystemet vårt er viktig for å bekjempe kreft. Noen kreftceller evner imidlertid å unnslippe immunresponsen og dermed overleve og spre seg. De nøyaktige mekanismene for hvordan kreftcellene samsnakker med immunsystemet og fremmer egen overlevelse er imidlertid uklare.

I dette prosjektet fant vi at en spesifikk immunrespons, som er viktige i kroppens forsvar mot kreft, er undertrykt i aggressive kreftsvulster. Vi fant også at et enzym kalt arginase-1 (ARG1) var sentralt i undertrykkelsen av denne responsen. ARG1 bryter ned en viktig næringskilde for kreftcellene og setter dermed i gang en næringsmobiliserende prosess, kalt autofagi inni kreftcellene. I dette prosjektet viser vi hvordan autofagi er svært viktig for å undertrykke immunresponsen og fremme aggressiv kreftutvikling.

Ved kreft er det en spesiell type immunceller som lager ARG1. Det er interessant at den samme typen immunceller og enzymaktivitet også er foreslått å spille en viktig rolle for utvikling av muskeltap hos kreftpasienter. Vi fant tegn på at immuncellene som lager ARG1 var anriket og begrenset næringstilgangen i muskel ved kreftrelatert muskeltap. Vi så at muskelceller som opplever denne næringssulten både satte i gang autofagiprosessen og fikk ytterligere skader som vil kunne redusere muskelmasse og funksjon.

Dette prosjektet gir ny innsikt i mekanismene som driver frem en aggressiv kreftutvikling og kreft-relatert muskeltap. Resultatene danner grunnlag for å vurdere ARG1-enzymet og autofagiprosessen som mulige angrepspunkter for ny, målrettet kreftbehandling.

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Forsker Miriam S. Giambelluca og Forsker Marina Vietri

Finansieringskilder: NTNU

Ovennevnte avhandling er funnet verdig til å forsvares offentlig

for graden PhD i medisinsk teknologi.

Disputas finner sted onsdag 7. juni på Laboratoriesenteret: LA21

I would like to dedicate my work to my parents, my husband, and my kids to all their sacrifices.

To the mountains, smell of the soil, food, and life of Nepal...

To everything that were left behind....

And my brother who sent me energy from heaven....

म मेरो काम मेरो आमा बुवा, मेरो श्रीमान् र मेरा बच्चाहरूलाई वहाँहरुको सबै त्यागमा समर्पित गर्न चाहन्छु।

हिमाल, पहाड, माटोको सुगन्ध, खानेकुरा, नेपालको जीवन,

पछाडी छोडिएका सबै कुराहरुलाई सम्झन चाहन्छु।

र मेरो भाइ जसले मलाई स्वर्गबाट ऊर्जा पठाइरह्यो.....

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Acknowledgements

This work was carried out at the Centre of Molecular Inflammation Research (CEMIR), at the Department of Biomedical Laboratory Science at the Faculty of Natural Sciences, Norwegian University of Science and Technology (NTNU), Trondheim. I am truly grateful for the funding from NTNU that has allowed me to do this work.

First, I would like to extend my sincere gratitude to my main supervisor Geir Bjørkøy not only for his continuous support and guidance throughout my PhD but also for his patience, motivation, enthusiasm, and immense knowledge. Your encouragement over the last years is something I sincerely appreciate.

My deepest gratitude to my co-supervisors, Kristine Pettersen, Miriam Giambelluca, and Marina Vietri for your continuous supervision. I had the opportunity to strengthen my troubleshooting and analytical thinking skills, essential for my research career. I could not have imagined having a better advisor and mentor for my thesis. Kristine, I really appreciate working together with you and I'm thankful for all the discussions over the last years. Miriam, for always guiding me and checking in on me, I appreciate it a lot. To Marina, that I met in middle of the project. I really appreciate the opportunity that you gave me to perform experiments in your lab. I admire your enthusiasm and ready to perform everything that is interesting. You are a great scientist.

I would like to mention a huge thanks to Sonja in assisting in my procedures, all the analytics, readings, corrections, and encouragement. I must say without you I would have not been able to succeed. Ida for being involved in the project specially during my absence, always having time to read and giving valuable feedbacks. To my office friend Camilla "our google queen", I value you very much, thank you for being as amazing as you are and especially for your support during the hard times, listening to me when I was stressed. Also, for reading the thesis and helping with technicalities during submission. To all past and present members of the "Autophagy group", thank you for creating a fun, inspiring and great work environment.

Special thanks to Nikolai Engedal for allowing to perform experiments in your lab. I appreciate your interest in my project and helping me with the autophagy assays and even looking into raw data closely, answering my long emails. I should say you have amazing lab members. Julia, Natalie, Christina, thank you for making me feel that I was the part of your lab. The longer lab days were much easier having so lovely people around. Nordic autophagy Society for providing the Lab exchange grant.

I would like to extend my gratitude to all my coauthors, without whom this would not have been possible. To my colleagues at CEMIR, I appreciate being part of such a diverse and inspiring research environment.

I am grateful to my family in Nepal for enduring my absence the last years and for always supporting me. To my husband Pushpa, thank you for encouraging me to always do my best. Your love and support have been very much appreciated and indispensable through this time. Thank you for always being interested in my work and at the same time delivering and bringing kids from kindergarten, making dinner every day when I was working early and late. To my little kids Prazna and Aarnav who constantly tolerated my absence.

Apsana Lamsal, February 2023

List of Papers

Paper I:

Lamsal Apsana, Andersen Sonja Benedikte, Johansson Ida, Vietri Marina, Bokil Ansooya Avinash, Kurganovs Natalie Jayne, Rylander Felicia, Bjørkøy Geir, Pettersen Kristine*, Giambelluca Miriam S*

Opposite and dynamic regulation of the Interferon response in metastatic and non-metastatic breast cancer. *Cell Commun Signal 2023 Mar 7;21(1):50*

Paper II:

Lamsal Apsana, Andersen Sonja Benedikte, Vietri Marina, Johansson Ida, Engedal Nikolai, Bjørkøy Geir, Giambelluca Miriam S*, Pettersen Kristine*

Local arginine restriction dampens IFN-I response via autophagy in breast cancer
Manuscript

Paper III:

Lamsal Apsana, Andersen Sonja Benedikte, Nonstad Unni, Bjørkøy Geir, Pettersen Kristine

A role of Arginase-1 expressing myeloid cells in cachexia

Manuscript

Paper not included in the thesis:

Bokil Ansooya A, Børkja Mathieu Le Boulvais, Wolowczyk Camilla, **Lamsal Apsana**, Prestvik Wenche S, Nonstad Unni, Pettersen Kristine, Andersen Sonja Benedikte, Bofin Anna M., Bjørkøy Geir, Hak Sjoerd*, Giambelluca Miriam S*

Discovery of new markers to identify pro-metastatic myeloid cells in breast cancer

(Submitted: NPJ Breast Cancer)

Abbreviations

AKT	Protein kinase B
ALRs	AIM2 (absent in melanoma 2) like receptors
AMPK	Adenosine monophosphate -activated protein kinase
ARG1	Arginase 1
ARG2	Arginase 2
ASL	Argininosuccinate lyase
ASS1	Argininosuccinate synthase 1
ATG	Autophagy related gene
BECN1	Beclin-1
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like
CAF	Cancer-associated fibroblast
CAR	Chimeric antigen receptor
CASTOR1	Cytosolic arginine sensor for mTORC1 subunit 1
CAT	Cationic amino acid transporters
CCL	C-C motif chemokine ligand
CCLE	Cancer cell line encyclopedia
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
cGAMP	Cyclic guanosine monophosphate adenosine monophosphate
cGAS	Cyclic GMP-AMP synthase
CLR	C-type lectin receptors
COX2	Cyclooxygenase 2
CQ	Choloroquine
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
cytDNA	Cytosolic deoxyribonucleic acid
DAMP	Damage associated molecular pattern
DFCP1	Zinc finger FYVE-type containing 1
dsDNA	Double-stranded deoxyribonucleic acid
ECM	Extracellular matrix

EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular regulated MAP kinase
ESCRT	Endosomal sorting complex required for transport
FIP200	Focal adhesion kinase family interacting protein of 200 kDa
GABARAP	γ-aminobutyric acid (GABA)-receptor associated protein
GAS	γ-activated sequences
GATOR2	GTPase-activating protein (GAP) activity toward RAGs 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCQ	Hydroxycholoroquine
HER2	Human epidermal growth factor receptor 2
HIF1A	Hypoxia inducible factor 1 subunit alpha
ICI	immune checkpoint inhibitor
IDO1	Indoleamine-pyrrole 2,3-dioxygenase 1
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3
IFN	Interferon
IFNAR	Interferon-alpha/beta receptor
IFNLR1	IFN-λ receptor 1
IL	Interleukin
iNOS	Inducible Nitric oxide synthase
IRF3	Interferon regulatory factor 3
ISGs	Interferon-simulated genes
ISREs	IFN-stimulated response element
JAK	Janus kinase
JAK/STAT	Janus kinase signal transducer and activator of transcription
LIR	LC3 interacting region
LLC	Lewis lung carcinoma
MAP1LC3	Microtubule-associated protein light chain 3
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MDSC	Myeloid deriver suppressor cells

MHC	Major histocompability complex
MMP	Matrix metalloproteinase
mtDNA	Mitochondrial deoxyribonucleic acid
mTOR	Mechanistic target of rapamycin kinase
mTORC1/2	mTOR complex 1/2
NBR1	Neighbor of BRCA1 gene 1
NDP52	Nuclear dot protein 52 kDa
NFE2L2	Nuclear factor erythroid 2-related factor 2 (also called NRF2)
NF-κB	Nuclear factor kappa-light chain enhancer of activated B cells
NK	Natural killer
NLRs	Nucleotide-binding and oligomerization domain (NOD)-like receptors
NO	Nitric oxide
NOS	Nitric oxide synthase
NRF2	Nuclear factor erythroid-derived 2-like 2
O2	Oxygen
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen-associated molecular pattern
Parp	polyadenosine diphosphate-ribose polymerase
PD-1	PD-1 Programmed cell death 1
PD-L1	PD ligand 1
PE	Phosphatidylethanolamine
PI3K	Phosphoinositide 3-kinase
PMN	Polymorphonuclear
PR	Progesterone receptor
PRRs	Pattern-recognition receptors
RAS	Rat sarcoma viral oncogene homolog
RLRs	Retinoic acid-inducible gene-I (RIG-I)-like receptors
ROS	Reactive oxygen species
S100A	S100 calcium binding protein A
SLC	Solute carrier family
SLRs	Sequestosome 1-like receptors
SQSTM1	Sequestosome 1

STAT	Signal transducers and activators of transcription
STING	Stimulator of interferon genes
TAM	Tumor-associated macrophage
TAX1BP1	Tax1-binding protein 1
TCR	T cell receptor
TLR	Toll-like receptor
TME	Tumor microenvironment
TNBC	Triple negative breast cancer
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TREX	Three Prime Repair Exonuclease
TYK2	Tyrosine kinase 2
UBA	Ubiquitin-associated domain
ULK1/2	Unc-51-like kinase1/2
UPS	Ubiquitin-proteasome system
WIPI	WD-repeat protein interacting with phosphoinositide

Abstract

To maintain homeostasis, cells need to adjust to exogenous and endogenous damage signals. Cancer cells are prone to release endogenous damage signals due to dysregulation of cell division or cell death. Both extracellular and, importantly, intracellular damage is reported to the immune system. The innate immune system responds to this damage by production of interferons and induction of autophagy. Autophagy, a "recycling station" in a cell, removes and recycles damaged proteins and organelles and delivers essential nutrients back to the cells. This process, which is important for cellular defenses, can also unfortunately cause harm. This is because autophagy can regulate inflammatory responses inside a cell, and an important example is that autophagy can suppress the IFN response by clearing these damage signals. It is acknowledged that IFNs are beneficial for cancer patients, and accordingly IFNs are usually suppressed in aggressive tumors. It is therefore interesting to gain a depth knowledge on why some tumors are difficult to treat and appear as "never healing wounds". At the same time, an array of cells like immune cells, epithelial cells, fibroblast, and extracellular matrix collectively forms a tumor niche. This plays a crucial role in shaping a tumor. Tumors with immuno-suppressing ability are favorable for the cancer itself, while tumors with immune-enhancing traits are beneficial for the patient. The objective of this work was to provide more knowledge about the interplay between the cancer cells and the immune system in facilitating tumorigenesis as well as promoting cancer related muscle loss in cachexia.

In paper I, using transcriptomics in a model of aggressive, triple negative breast cancer (TNBC), we found that 11 IFN-I related genes (IFN-I signature) were constitutively active in the metastatic breast cancer cells when grown in culture. In contrast, this IFN-I signature was significantly dampened when these cells formed a primary mammary tumor in immunocompetent mice. This IFN-I gene signature was validated in TNBC patients using meta-analysis in a publicly available database. We observed that a lower IFN-I signature correlated with poor overall and relapse free survival in TNBC patients, supporting the importance of the IFN-I response in breast cancer prognosis. These data led us to hypothesize that immunosuppressive cells present in an aggressive tumor might be key players in suppressing the IFN-I response in the tumor. In paper II, we found that metastatic-competent primary tumors have elevated arginase-1 (ARG1) levels compared to non-metastatic primary tumors. It is known that ARG1 is produced by myeloid cells and that it exerts an immunosuppressive role in multiple cancer types by limiting arginine availability. Restricting the metastatic breast cancer cells with the essential amino acid arginine, dampened the IFN-I response and induced the autophagy process. Thus, we have showed that induction of autophagy upon arginine restriction regulates the IFN-I signaling in aggressive breast cancer cells. We also identified that autophagy targets the upstream signals of the cGAS-STING pathway involved in IFN-I production.

We have also provided data supporting that these immunosuppressive cells are not only confined to the tumors, but also have a role outside the tumor in the severe, systemic muscle wasting condition called cachexia. The role of autophagy and immunosuppressive cells is emerging in the ongoing process of understanding the mechanisms of cachexia development. In paper III, we found that weight loss in cancer patients positively correlated with ARG1 levels in the plasma, indicating ARG1 as an important prognostic marker. Arginine restriction in skeletal muscle cells and cardiomyocytes severely impaired mitochondrial functions and induced the autophagy process. These three studies and our unpublished data identify ARG1 and autophagy both as potential therapeutic targets for treatment of aggressive breast cancer and reversal of muscle loss in cachexia.

1 Introduction

1.1 Epithelial cancers

Epithelial tissues that serve as a barrier between the body and outer surroundings are an important site for cancer development. Around 90% of human cancers emerge from the epithelial tissue and are termed carcinomas [1]. The most common carcinomas include skin, breast, kidney, liver, lungs, pancreas, prostate gland, head, and neck carcinoma. In breast tissues, the cancer especially arises from the luminal epithelial cells lining both the milk-producing lobules and ducts. Occasionally, breast cancer can also form sarcomas rising from the stromal components of the breast [2]. Here, only epithelial breast cancer will be discussed further.

1.2 Breast Cancer

Female breast cancer is estimated to be the most diagnosed cancer worldwide with approximately 2.3 million new cases annually and 685,000 deaths, thus being the leading cause of death in women and a true global health challenge [3]. The incidence and mortality rate of female breast cancer is higher in western parts of the world with higher human development index. This is partly due to high prevalence of reproductive, hormonal and lifestyle associated risk factors along with higher mammographic screening [4]. In Norway, 4023 new cases were registered in 2021, being the highest number registered to date [5, 6]. The Norwegian cancer registry was established in 1951 and the screening program "BreastScreen" was started in 1996 [5]. In this breast cancer screening program, all women between the ages of 50 and 69 are offered screening mammography every other year. The overall 5-year survival rate for breast cancer patients in Norway increased from 91.4% to 92.3% from 2016 to 2021. Despite this, 591 women and 10 men died of breast cancer in 2021 in Norway [5, 6].

1.2.1 Classification

Before the launching of the 8th edition of the American Joint Committee on Cancer (AJCC) staging manual, breast cancer was commonly classified by an anatomical staging method [7]. Based on clinical and pathological examinations, tumors were classified by primary tumor size (T), nodal involvement (N), and metastasis (M), also known as TNM classification. Later, advancement in the understanding of gene expression patterns and prognostic biomarkers allowed oncologists and clinicians to realize that patients with similarly staged cancers can have different outcomes [8]. Based on the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor2 (HER2) and the proliferation marker Ki-67, five intrinsic molecular subtypes have been classified [9, 10]. These include triple-negative/basal-like, HER2 enriched, luminal A, luminal B, and normal-like (Figure 1). Even if there are apparent overlap between the Luminal A and the Normal-like subtypes, extensive expression profile analyses confirm that these are indeed separate subtypes and should not be integrated for treatment[11, 12].

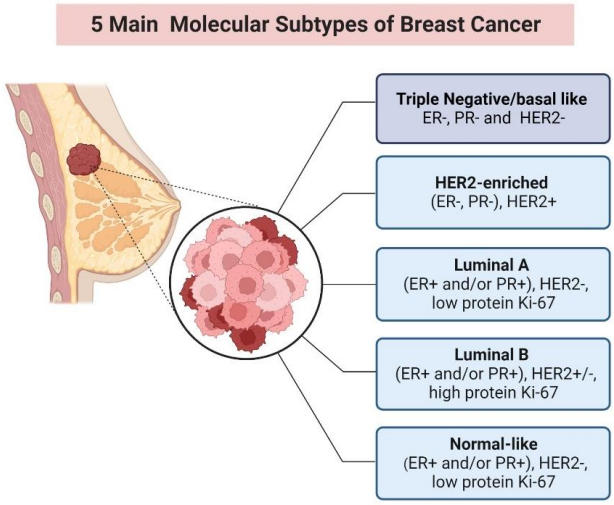

Figure 1. Breast cancer molecular subtypes. The five main molecular subtypes of breast cancer (ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor 2, Ki-67: proliferation index marker). This figure is created in biorender.com. with modifications from [13].

High throughput gene expression studies using patient biopsies emerged in the past few years have identified distinct gene expression signatures. These signatures provide improved classification of the patient groups, beyond the distinction between the five subtypes listed above, and assist in developing specific treatment strategies. Several gene expression signatures like Mammaprint, Oncotype DX, Nanostring producing Prosigna (PAM50), Breast Cancer Index and Curebest are already in use in clinics [14-18].

1.2.2 Metastasis and current treatment strategy in breast cancer

Metastasis is a primary cause of death in all cancer patients including breast cancer [19]. Metastasis is an inefficient process where most cancer cells released from the primary tumor die, and only very few cells form distant metastases [20]. During metastasis, cancerous cells of the primary tumor acquire an epithelial-to-mesenchymal transition (EMT) phenotype, enabling them to break through the basal membrane, invade neighboring tissues, reach the bloodstream or lymphatic system, and disseminate into other organs [21]. Primary breast cancer cells tend to metastasize to lymph nodes, lungs, liver, bones, brain, and pleura [22, 23]. It is also known that primary breast cancer cells establish a premetastatic niche pivotal for metastasis development, aiding support for the “seed and soil” theory introduced by Sir Stephen Paget in 1889 [24]. Metastases can stay dormant for several years, making them clinically undetectable until they are reactivated and difficult to treat.

For the last 150 years and until 1980s, most women with breast cancer underwent complete removal of the breast tissue. Surgery is still a common practice, but other treatments such as adjuvant chemotherapy, radiotherapy and immunotherapy are all used in the clinic today [25, 26]. The German Society of Radiation Oncology (DEGRO) published guidelines for breast cancer treatment using radiotherapy. The panel recommends that surgery should be followed up by radiotherapy to wipe out the remaining cancer cells within the tissue. Systemic neoadjuvant therapy should be given to primarily unresectable and inflammatory breast cancer, followed by surgery and radiotherapy [27]. Systemic therapy, including hormone therapy, chemotherapy, immunotherapy, and molecular target therapy is important for improving disease-free survival, overall survival, and risk of metastasis. Adjuvant systemic therapy is associated with prolonged survival by treating latent micro-metastases [16]. Hormone therapy is exclusively given to the patient based on the hormone receptor expression according to the breast cancer subtypes (Figure 1). For example, Trastuzumab, an anti-HER2 target therapy, in combination with adjuvant chemotherapy is recommended for patients with HER2-positive tumors [28]. Adjuvant therapy using aromatase inhibitor that blocks production of estrogen or blocks action of estrogen on the receptors is mostly used in post-menopausal women [29-31]. Neoadjuvant chemotherapy is used in early and locally advanced breast cancer patients [32]. Randomized control trials in patients have shown that chemotherapy can also be administered prior to surgery to attain beneficial effects [33].

Since tumors can escape immune surveillance and resist the cytotoxic effect of host T cells, immunotherapy using checkpoint inhibitors have now emerged, allowing proper immune recognition and execution of cancer cells [34]. Patients with triple negative breast cancer do not respond to conventional hormone therapy, but a fraction of these patients benefit from immune checkpoint inhibitors targeting the programmed cell death-1 (PD-1) receptor or PD ligand 1(PD-L1) [35, 36]. Recent studies have highlighted the combination therapy of anti-PD-L1 and polyadenosine diphosphate-ribose polymerase (PARP) inhibitor, an enzyme involved in DNA repair, as a novel therapeutic approach in breast cancer treatment [37]. Chimeric antigen receptor (CAR) T cell therapy is a new form of immunotherapy with promising results. This involves isolating T cells from the patient and further engineering them *ex vivo* to express synthetic receptors that recognize tumor-associated antigens, and then infuse these back into the patients to provide a strong immune response. There are several ongoing and completed clinical trials of CART cell therapy in multiple breast cancer subtypes [38, 39]. Despite the clear advances in both diagnosis and treatment of breast cancer, for the tumors that have metastasized or for specific breast cancer subtypes there are still limited efficient treatment options. This strongly emphasizes the demand for further research.

1.3 Tumor microenvironment

Tumorigenesis requires the cancer cells to obtain different functional capabilities to form malignant tumors, as described in the recent update of the classical "Hallmarks of Cancer" by Hanahan [40]. A total of ten hallmarks have been defined, and in addition two "emerging" hallmarks and two "enabling characteristics" have been described [40, 41] (Figure 2).

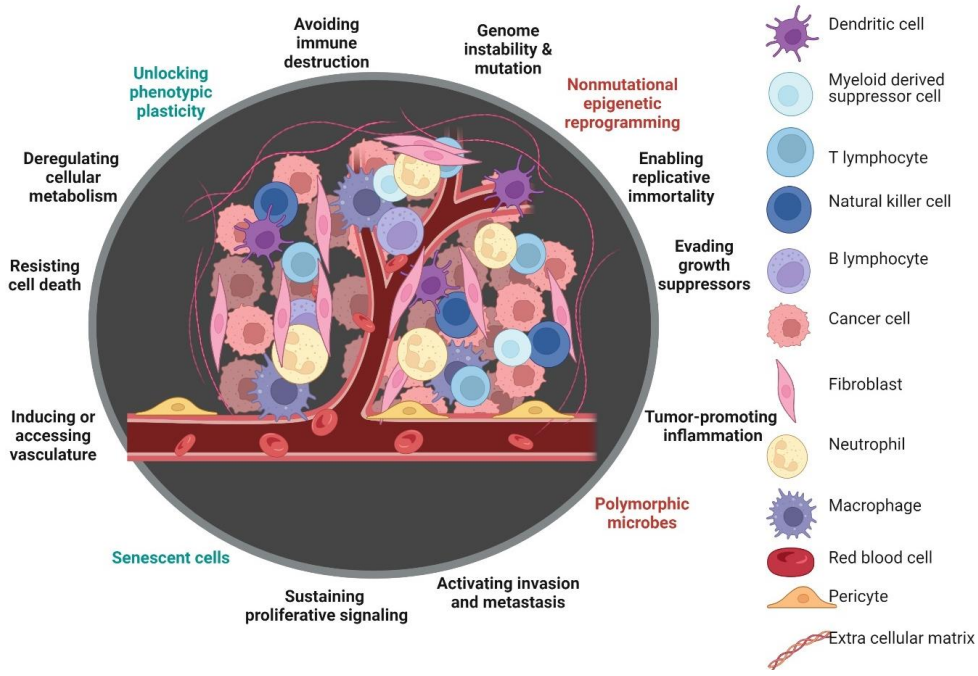

Figure 2. Hallmarks of cancer and the tumor microenvironment. The illustration shows several cell types along with cancer cell inside a tumor, together forming a tumor microenvironment (TME). The complex process of tumor formation involves multiple changes in the cancer cells, enabling them to obtain a plethora of functional capabilities needed to form a malignant tumor. The hallmarks of cancer currently include ten hallmarks (in black) plus two "emerging" hallmarks (in green) and two "enabling characteristics" (in red). Created with biorender.com with adaptations from [40-43].

In a solid tumor, transformed cancer cells collaborate with multiple players, including fibroblasts, endothelial cells, infiltrating immune cells, blood vessels, signaling molecules, dendritic cells, secreted factors, and extracellular matrix collectively comprising the tumor microenvironment (TME) (Figure 2). All the cells within the TME are embedded into the extracellular matrix that assists in the attachment and communication of different cells [44]. The TME also contributes with physiological components such as oxygen, metabolic products, growth factors and nutrients that the cancer cells can utilize [45]. The intercommunication between cancer cells and the other TME constituents, greatly impacts tumor development by influencing processes that can lead to either tumor eradication or tumor progression and metastasis [46-48]. The TME is an essential determinant for cancer cell survival and metastasis, as

it influences response to therapies, development of local resistance and immune escape [49]. Thus, developing TME-targeting therapies that can be combined with conventional cancer therapy will be an important strategy to achieve better clinical outcomes.

1.3.1 Tumor-infiltrating immune cells

The immune infiltration of tumors is closely linked to patient prognosis and clinical outcome [50]. This depends on both the type and number of immune cells found in the tumor. The immune cells are recruited and polarized by secreted factors in the TME, resulting in a diverse cell population with both anti-tumorigenic and pro-tumorigenic properties [51]. Thus, the immune cells that could recognize the transformed cancer cells are edited to support tumor progression and survival. Avoiding immune destruction is now considered a core hallmark of cancer [40]. The secretion of immunomodulatory compounds from cancer cells and other cells in the TME influence the function of the recruited immune cells, and the dynamic composition allows the TME to shift between an immunosuppressive and an immune-promoting state [52]. Due to the plasticity of immune cells in response to the shifting TME, it is challenging to classify the different immune cell types as either tumor-promoting or tumor-suppressive [52]. However, numerous studies have shown that it is favorable to have a high lymphocyte-to-myeloid cell ratio [53, 54]. The good prognosis is predominantly seen in tumors highly infiltrated by cytotoxic and/or memory T cells, while infiltration of regulatory T cells correlates with a poorer prognosis [55]. In addition to the T cell infiltration, the cancer cells themselves also exploit the natural regulation of T cells by upregulating receptors to inhibit their activation and survival [56, 57]. This led to the discovery and use of immune checkpoint inhibitors (ICIs). ICIs block the immunosuppressive receptors and revive the exhausted T cells, including CD8+T cells [58, 59].

The impact of B cells in tumorigenesis has long been underestimated, but recent studies have revealed a prominent role for this population [60]. B cells are found in smaller quantities than T cells in tumors, but the number of antibodies and cytokines they produce can have a large impact on the TME. B cells can produce specific cytokines like TNF, CCL3, GM-CSF, IL-6, IL-17, and IL-2 that can boost effector and memory T cell functions. At the same time, B cells can also suppress inflammatory immune response via IL-10 and IL-35 production [61, 62]. B cells can further have regulatory function via PD-1 or PD-L1 expression and suppress the T cell function by CD39 and CD73 expression [63-65]. NK cells, on the other hand, are quite known for their anti-tumor properties. Through release of cytotoxic granules, NK cells are involved in a prompt intervention against cancer cells and thus correlate with a good prognosis [66, 67]. However, NK cells can also adopt pro-tumorigenic traits in response to the TME's attempt to evade the immunosurveillance of these immune cells [68].

Also innate immune cells such as neutrophils, dendritic cells and macrophages are essential components in the TME. Due to their heterogeneity and plasticity, these cells can polarize into different phenotypes.

Macrophages are classified as M1 (classically activated) when they exert anti-tumorigenic properties, and M2 (alternatively activated) when they act pro-tumorigenic [69, 70]. However, this reductionistic view of polarization does not grasp the complexity of the acquired phenotypes of these cells. In cancer, increased neutrophil expression is usually associated with poor prognosis, and neutrophils are thought as a potential target in cancer treatment [71]. However, the intratumoral neutrophils have shown different patient outcome; positive correlation in gastric cancer [72], negative in renal cancer and melanoma or no correlation in lung cancer [73-75].

1.3.1.1 Myeloid-derived suppressor cells (MDSCs)

Another immunosuppressive cell population, myeloid derived suppressor cells (MDSCs), is the heterogenous population of immature myeloid cells infiltrating the tumor [76]. In healthy individuals, MDSCs are present at low levels but their number increases during cancer development and other chronic inflammations [77-79]. MDSCs are recruited from bone marrow to peripheral lymphoid organs and tumor sites by chemokines and inflammatory proteins such as C-C motif chemokine ligand (CCL) 2 and 3, chemokine (C-X-C motif) ligand (CXCL) 1, 2 and 5, and S100 calcium binding protein A (S100A) 8/9 [80-88]. MDSCs can be classified into polymorphonuclear (PMN-MDSC) and monocytic (Mo-MDSC), depending on the similarities to either recruited neutrophils or monocytes, respectively [89].

MDSCs exert immunosuppressive functions in tumors. This is mainly because MDSCs control T cell activity through several mechanisms including ROS production, elevated arginase (ARG) and inducible nitric oxide synthase (iNOS) expression, IL-10 production etc. [90]. MDSCs metabolically target T cells by competing for specific amino acids. For example, cysteine is an essential amino acid for T cell function and proliferation, but it cannot be synthesized *de novo* by T cells. This is because T cells do not express the enzyme cystathionase needed for cysteine synthesis, and they also lack the xCT chain of a heterodimeric cysteine-glutamate antiporter making them unable to take up the oxidized cysteine-dimer cystine, which normally is reduced back to cysteine intracellularly [91, 92]. Through the expression of ARG and iNOS, MDSCs can reduce the level of the semi-essential amino acid arginine, and arginine depletion is destructive for T cell function and survival [77, 93-97]. ARG depletes the arginine level by converting it to ornithine and urea. iNOS on the other hand, converts arginine to nitric oxide (NO) and citrulline [98]. The mechanism behind the biological effects of arginine depletion is that intracellular arginine levels regulate numerous metabolic pathways including glycolysis and oxidative phosphorylation. A mass spectrometry-based metabolomic and proteomic study in T cells showed that arginine was essential for both activated CD4+ and CD8+ T cells, but that the downstream metabolite NO had no effect on T cell survival [96]. However, several other studies have shown that elevated NO has a potential role in suppression of T cell proliferation, as well as a role in regulating differentiation and activation of T cells [99-101].

Various studies have shown the correlation between intratumoral MDSC frequency, tumor stage and metastasis, highlighting the clinical significance of these cells [95, 102, 103]. MDSCs are key constituents of the premetastatic niches in distant organs that mediate metastasis formation in combination with decreased NK cell cytotoxicity and T cell anergy [104-106]. In the 3LL lung metastasis mouse model, MDSC depletion was also associated with increased CD8+ T cell and effector NK cell population, increased interferon-gamma (IFN- γ) production and reduced IL-10 secretion [107, 108]. Alternative enzymes involved in MDSC-mediated immunosuppression include cyclooxygenase 2 (COX2) and indoleamine 2,3-dioxygenase (IDO) [109-111]. MDSCs are also known to produce matrix metalloproteinase 9 (MMP9) in premetastatic lungs for increased ECM remodeling to promote angiogenesis [104].

1.3.1.2 Arginase in TME

Arginase can be expressed as two isoforms, ARG1 and ARG2, and ARG1 is a cytosolic enzyme while ARG2 is located to mitochondria. While ARG2 has ubiquitous expression, the expression of ARG1 is limited to liver [112] and in cells of the myeloid lineage [113]. In mice, almost all myeloid cells express ARG1, but in humans ARG1 is mainly produced by neutrophils and MDSCs [114, 115]. Elevated ARG expression has been reported in many human cancers, and several studies have demonstrated that the presence of ARG in the TME can influence on tumorigenesis [116-118]. Many cancer cells can produce elevated levels of either ARG1 or ARG2, thus causing depletion of arginine in the TME [116]. Also, cancer cells can produce myeloid growth factors and inflammatory mediators dysregulating myeloid cell differentiation [119-124]. Thus, a heterogenous group of tumor-infiltrating myeloid cells, including monocytes, macrophages, neutrophils, dendritic cells and MDSCs, are recruited to the tumor [125]. The recruited cells can create a tumor-promoting, immunosuppressive TME, by producing a variety of factors like ROS, cytokines and PD-L1. Many of these cells, like the MDSCs, can produce ARG1, and this can be released either directly to the TME or in extracellular vesicles enabling ARG1 to exert effects more distally [126]. An interesting difference between human and mouse MDSCs is that in human they release ARG1 in the circulation and thus deplete arginine levels systemically, while murine MDSCs deplete arginine by increased uptake and intracellular degradation [127]. Also, macrophages, especially M2 macrophages in mice with pro-tumorigenic functions, are an important source of ARG1 in the TME, while there has yet not been reported that human macrophages can make ARG1 [125, 128, 129]. Human neutrophils are another important source for ARG1, and while they do not metabolize arginine themselves since ARG1 is localized in gelatinase granules, they can exocytose these granules together with azurophilic granules ensuring enzymatic activity[130-132].

ARG1/2 can influence on tumorigenesis in several ways. As described above, arginine depletion, initiated either by the cancer cells themselves or by cells in the TME, will profoundly influence on the function of T cells, thereby leading to impaired anti-tumor responses. It has also been shown that cancer

cells with increased ARG1/2 can have elevated activation of different central constituents of important signaling cascades, like AKT, ERK, mTORC1 and STAT3, which promote cancer cell viability, proliferation, adhesion, and migration [116]. Accordingly, overexpression of ARG1/2 is anticipated as an unfavorable prognostic factor in several cancer variants, including ovarian, colorectal, pancreatic ductal adenocarcinoma, head, and neck cancer [126, 133-135]. Regarding breast cancer, several studies support that ARG1 and/or ARG2 is implicated in the pathogenesis and development of the disease, they demonstrate increased enzymatic activity in tissue and blood from breast cancer patients and this correlates with the severity of the disease [136-139].

1.3.2 Hot and cold tumors in immunotherapy

Tumor heterogeneity can cause difficulties in therapeutic interventions. The heterogeneity can exist at several levels; for instance, patients with tumors of the same histological type may have tumors that are very different (intertumoral heterogeneity), which could be due to variations in patient-specific factors like their genetic background, differences in the somatic mutation profile or environmental factors. Further, there will be heterogeneity within a tumor (intratumorally), which reflects the instability of tumor cells and the establishment of genetically different subpopulations of tumor cells, either within the primary tumor itself or in metastases or both [140]. Tumors can also be distinct based on frequency of different immune cell types within the TME [141]. Effector T cells are crucial in an immune response against cancer, and tumors can be classified as immunologically hot or cold, depending on the level of T cell infiltration (Figure 3). Hot tumors have high levels of infiltrating T cells, NK cells, TAMs, IFN signaling, PDL-1 expression and other components essential for anti-tumor immune function. Cold tumors on the other hand, also called “T cell excluded”, do not exhibit cellular and gene expression characteristics which are favorable for anti-tumor action, primarily by T cells. The cold tumors are enriched with immunosuppressive cell populations like regulatory T cells, regulatory B cells, MDSCs including M2 macrophages and N2 neutrophils and low PD-L1 expression. Having a hot tumor is associated with improved prognosis in cancer patients, whereas patients with cold tumors are less responsive to conventional therapies and to ICIs [142-145]. ICIs, such as nivolumab and pembrolizumab, are used in some cancer types and have succeeded in clinical trials [146, 147]. However, large number of patients do not respond to ICIs due to lack of PD-L1 expression and infiltration of effector T cells essential for tumor cell killing [148]. Use of CART cells have been successful in some settings, raising the importance of effector T cells in immunotherapy [38, 149]. Since TAMs also have pro-tumor properties and correlate with poor prognosis in several cancer types, targeting TAMs is an attractive approach [150]. Using an antibody to target specifically the macrophage receptor with collagenous structure (MARCO) has been shown to reverse the immunosuppressive effects of TAMs. Silke et al. recently showed that anti-MARCO immunotherapy in melanoma models involved NK cell but not T cells. This therapy also showed a synergistic effect with PD-1 or PD-L1 to

increase tumor killing, adding to existing T cell-based immunotherapies [151]. Research is still ongoing to understand how cold tumors can be switched into hot tumors via T cell priming along with other immune cells, an important mechanism necessary to understand for increased patient survival.

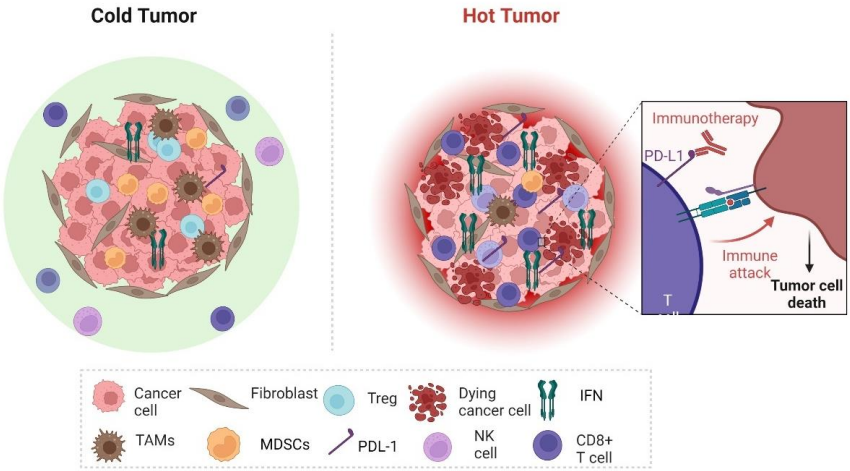

Figure 3. Immune composition of cold tumors vs. hot tumors. Cold tumors are characterized by exclusion of effector T cells, infiltration of immunosuppressive cells including MDSCs, and tumor associated macrophages (TAMs). Hot tumors are characterized by infiltration of effector T cells, and natural killer cells (NK cells), and are responsive to immunotherapy. Created with biorender.com.

1.3.3 Tumor-promoting inflammation

In all tumors, there will be a recruitment of immune cells that could potentially attack the tumor, however, as described above it is now well established that this tumor-associated inflammatory response can enhance tumorigenesis and tumor progression. Thus, tumor-promoting inflammation is considered as one of the hallmarks of cancer, and as reviewed by Hanahan and Weinberg, 2011. It can contribute to several other hallmark capabilities by supplying for instance proangiogenic factors, growth factors, survival factors that limit cell death, and by increasing the genetic instability in the cancer cells via release of mutagenic ROS [43]. Genotoxic treatments including radiotherapy, chemotherapy and surgery commonly used in all cancer patients can induce both local and systemic inflammatory responses due to activation of stress inducing pathways [20]. Usually, this process is beneficial for anti-tumor responses, and immune system activation is also the keystone for current immunotherapies [152]. However, genotoxic treatments can also cause tumor cells to release damage-associated molecular patterns (DAMPs). This could trigger an anti-tumor response or immunosurveillance. It is also evident that DAMPs can act as double-edged sword that could trigger tumor-promoting inflammation and changes in the TME that will aid tumor progression [153]. For example, DAMPs like S100 proteins, especially S100A8/9 and S100A12, are upregulated in multiple tumor types [154]. The expression of S100A8/9 on myeloid cells in colon tumors can cause tumor promotion via activation of mitogen-

activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways [85, 155]. Interestingly, cytokines released in the TME via activation of downstream effectors like STAT, NF-κB and caspases can have dual functions. For example, these cytokines can have either tumor suppressive roles via IFN-γ and IL-12 production or can promote tumor progression via IL-6 and IL-17 secretion [156].

1.4 Innate immune signaling

The innate immune system provides the rapid first line of host defense, classically sparked in response to detection of pathogen associated molecular patterns (PAMPs) or DAMPs released during cellular stress or tissue injury. PAMPs and DAMPs are recognized by various pattern recognition receptors (PPRs) that can be either membrane bound or cytosolic. Among the PAMPs we find bacterial carbohydrates (like lipopolysaccharide or mannose), nucleic acids (bacterial or viral DNA or RNA), bacterial peptides, peptidoglycans, and lipoproteins. The DAMPs include both proteins and non-protein component, as exemplified by the chromatin-associated protein high-mobility group box 1 (HMGB1), free DNA, S100 proteins, nucleotides (like ATP), nucleosides (like adenosine) and uric acid [157]. The membrane-bound PRRs include toll-like receptors (TLRs) and C-type lectin receptors (CLRs), while cytosolic PRRs include retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) cyclic guanosine-monophosphate adenosine-monophosphate synthase (cGAS) and AIM2 (absent in melanoma 2) like receptors (ALRs) [157-161]. After recognition of PAMPs or DAMPs, a variety of downstream responses are triggered; for instance, autophagy, phagocytosis, processing, and activation of pro-forms of cytokines (like pro-IL-1) and production of soluble mediators like cytokines, including interferons (IFNs) [158, 162-164]. Recent advances in understanding the role of innate immune response in cancer, and vaccine design using PAMPs and DAMPs as adjuvants, have gathered great attention [165].

1.4.1 Interferons in innate immune response

Interferons (IFNs) were the first cytokine family to be discovered over 60 years ago by Isaacs and Lindenman in response to viral infection [166]. IFN production has been mostly associated in response to pathogenic infections since then. However, cells that accumulate intracellular damage also report to the immune system by activating the IFN response, inducing inflammation under sterile conditions [167]. Depending upon the unique receptor binding for signal transduction, IFNs are divided into three groups, type I, type II, and type III IFNs, as illustrated in Figure 4 [168, 169]. The type I IFNs, the largest family of IFNs, all bind to the cell surface interferon-alpha/beta receptor (IFNAR), consisting of the two transmembrane subunits IFNAR1 and IFNAR2. Type II IFN (IFN-γ) binds to IFN-γ receptors 1 (IFNGR1) and 2 (IFNGR2) heterodimers. It also induces genes that prime the type I IFN response, and similarly, type I IFN signaling also primes type II IFN signaling [170-174]. Type III IFNs (IFN-

l1/2/3 and 4) share structural features with interleukin-10 (IL-10) cytokine family members, and signal through IL-10 receptor 2 (IL-10R2) and IFN-λ receptor 1 (IFNLR1) heterodimers [168, 175-179] (Figure 4). All three IFN types can initiate various signaling pathways via Janus kinase/signal transducer and activator of transcription (JAK/STAT), which together provide diverse immunological functions [180, 181]. In the canonical, linear kinase-dependent JAK/STAT signaling, binding of IFNs to the specific receptor complexes initiates the phosphorylation of the receptor-associated cytosolic kinases JAK1 and (for type I and III interferons) tyrosine kinase 2 (TYK2). This subsequently leads to phosphorylation of STAT1 and STAT2, which dimerize either as heterodimers (for type I and III IFNs) or as STAT1 homodimers (for type II IFNs). The heterodimers can recruit interferon regulatory factor 9 (IRF9) to form a STAT1-STAT2-IRF9 tri complex (ISGF3), which can translocate to the nucleus and bind to conserved sequences known as IFN-stimulated response elements (ISREs). The activated STAT1 homodimers can after translocation bind to γ-activated sequences (GAS), and binding of these STAT-complexes to either ISREs or GAS will result in induction of interferon-simulated genes (ISGs) (Figure 4) [168, 177, 182, 183].

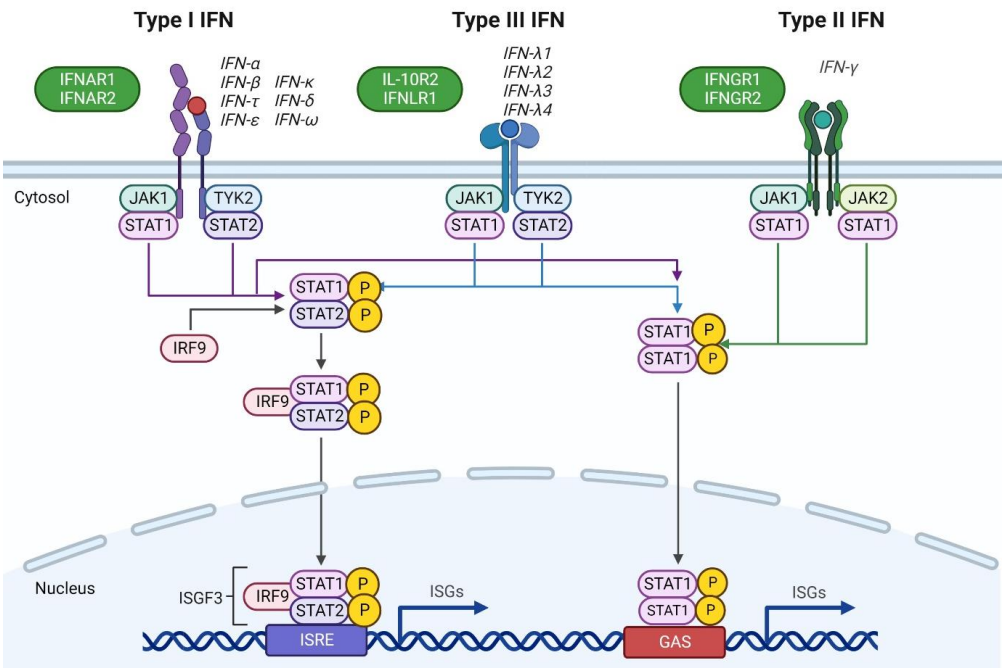

Figure 4. IFN signaling pathways. A schematic view of the canonical, linear signal transduction pathways for the three IFN types. Type I, II and III IFNs signal via different receptors with signal transduction mediated via JAK/STAT for phosphorylation and subsequent STAT activation. STATs, upon activation by IFN-I and IFN-III receptors, can drive expression of interferon-simulated genes (ISGs) with IFN-stimulated response elements (ISRE) or γ-activated sequences (GAS) elements in their promoters, while the IFN-II receptor complex solely

drives the expression of genes with GAS promoter elements. Created with biorendor.com with minor adaptations from [168, 169]

1.4.2 Type I IFN and cGAS-STING signaling

Type I IFNs are the largest class of IFNs and have a vital function in various inflammatory diseases. In addition to anti-pathogenic function, they have a crucial role in cancer prevention, providing anti-tumor immunity [184]. IFN-I can activate immune cells like innate lymphoid cells, T cells, NK cells, and dendritic cells. IFN-I signaling involves both IFN-I production and downstream IFN-I response causing transcription of ISGs. Immune cells, tumor cells, fibroblasts, and epithelial cells within the TME can produce IFN-I. In the TME, IFNs are produced in response to DAMPs; damage response associated within the cells [185-187]. Several studies indicate that IFN-I induction in TME mainly occurs via the cGAS-STING axis.

The cGAS–STING signaling axis, comprising the cyclic guanosine monophosphate–adenosine monophosphate (GMP–AMP) synthase (cGAS; also known as MB21D1) and stimulator of interferon genes (STING) detects cytosolic DNA to activate an innate immune reaction involving a potent IFN-I response. Cytosolic DNA is a highly immune stimulatory and is a strong inducer of IFN-I response [188]. Upon DNA binding, cGAS undergoes a conformational change and produces a second messenger 2’3’cyclic GMP-AMP (cGAMP) from ATP to GTP [189-192]. cGAMP is then sensed by STING, a transmembrane protein that resides on ER [193-196]. Upon cGAMP binding, STING translocates to ER-Golgi intermediate compartment (ERGIC). STING then recruits TANK-binding kinase (TBK1) to initiate downstream signaling where TBK1 phosphorylates itself, STING, and subsequently the transcription factor, interferon regulatory factor 3 (IRF3) [197]. IRF3 undergoes dimerization and enters the nucleus causing transcription of the ISGs [197-199] (Figure 5). In addition, STING can undergo nuclear factor-κB mediated (NF-κB) transcriptional activation of IFN-I response. STING recruits NF-κB, leading to IFN-I production and other cytokines [195, 200, 201].

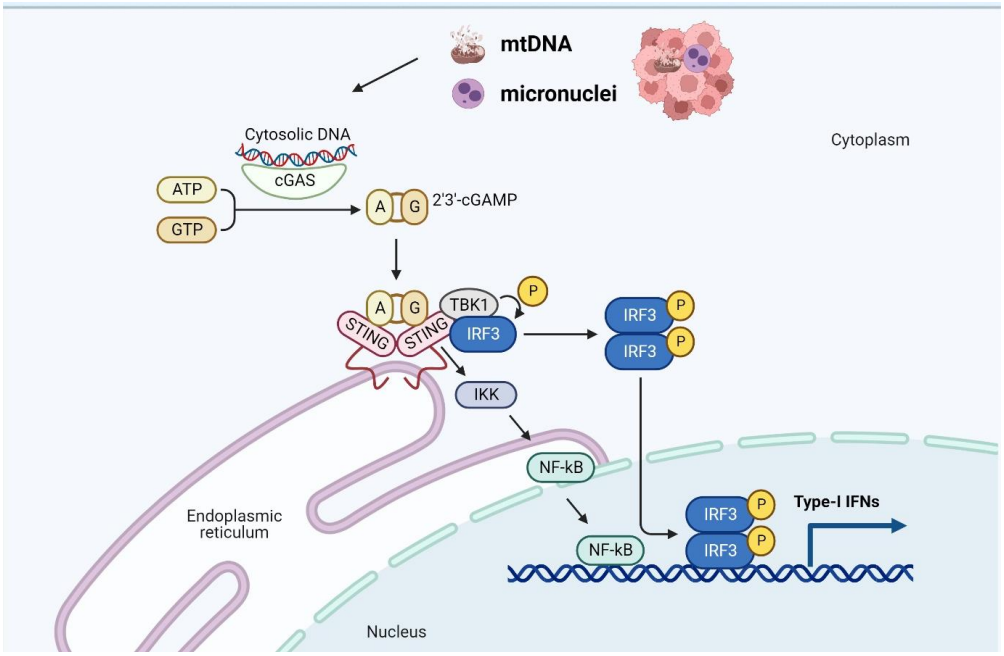

Figure 5. The cGAS-STING signaling pathway. cGAS is activated by binding of self-DNA (mtDNA from damaged mitochondria and micronuclei) present in the cytosol. Activated cGAS uses ATP and GTP to catalyze the formation of second messenger 2'3'cGAMP, which forms a complex with STING, inducing its activation. Activated STING traffics from the endoplasmic reticulum to the Golgi where it recruits and activates TBK1, which in turn phosphorylates IRF3 and cause nuclear translocation of IRF3 and to a lesser extent NF-kB (activated by IKK). This leads to increased transcription of IFN-I genes in the nucleus. Created with biorendor.com with minor adaptations from [202].

1.4.2.1 cGAS-STING activation by self-DNA

Apart from cytosolic DNA sensing from the microbes, cGAS can be activated by the self-DNA emphasizing the importance of cGAS-STING pathway in sterile inflammation, cellular senescence, and cancer [203]. Damage in organelles like the nucleus, mitochondria, endoplasmic reticulum, ribosomes, lysosomes, and cytoskeleton can disrupt organelle homeostasis and activate innate immune sensors, including the cGAS-STING pathway [204].

1.4.2.2 Cytosolic DNA and micronuclei

DNA is usually restricted to the nucleus; however, upon DNA damage, if not properly managed, DNA can leak into the cytosol. In cancer cells, chromosomal material can also be found outside the nucleus. Error during mitosis, double-strand DNA breaks, and telomere erosion can accumulate aberrant DNA structures called micronuclei [205, 206]. For example, genotoxic treatments, including radiotherapy and chemotherapy commonly used in cancer patients, can cause DNA damage causing the formation of micronuclei. Micronuclei form when single chromosomes or fragments of chromosomes like acentric

chromosomes do not segregate with the rest of the chromosomes [207]. As a result, these chromosomes remain excluded from the primary nucleus and become enveloped by their own nuclear membrane [208, 209]. The nuclear envelope in micronuclei appears to be defective and, unlike the nuclear envelope surrounding the primary nucleus, ruptures at micronuclear envelope are never repaired [158]. Micronuclear catastrophe involving nuclear envelope collapse with substantial loss of micronucleus-cytoplasmic compartmentalization can activate the cGAS-STING signaling to induce proinflammatory signaling [210-212]. Nucleases like Three Prime Repair Exonuclease (TREX) are important for the clearance of damaged DNA in the cytosol. TREX1 is reported to inhibit cGAS activation at micronuclei by degrading micronuclear DNA [213]. Accordingly, mutations in TREX1 can cause accumulation of micronuclear DNA and trigger activation of cGAS-STING signaling [214].

Micronuclei frequently form in cancer cells, and increased micronuclei frequency is observed in breast cancer patients compared to healthy controls [210, 215]. Interestingly, some patients treated with focal radiotherapy in combination with immune-checkpoint inhibitors clearly benefit from the presence of ruptured micronuclei and accumulation of cytosolic DNA. This is because the cytosolic DNA sensor cGAS can access chromosomal DNA and initiate innate immunity and inflammatory pathways. Unfortunately, this is not always the case. Several preclinical and clinical reports show that focal radiotherapy can instead increase the occurrence of distant metastasis [216-218]. It has also become clear that micronuclei are not simple consequences of tumors, but instead are main contributors to tumorigenesis [219]. Presence of micronuclei is a sign of damage in a cell, and normally a cell tries to get rid of this damage. The mechanisms underlying the formation of micronuclei are well understood but not much is known about the potential fate of micronuclei and micronucleated cells.

1.4.2.3 Cytoplasmic detection of mitochondrial DNA (mtDNA)

Mitochondria and their DNA replicate autonomously from the nuclear genome. Since mtDNA proofreading does not occur during the replication, the mtDNA is extremely susceptible to errors. During cellular, local environmental stress, infection, and injury, the mitochondrial contents can leak into the cytosol and cause activation of several downstream pathways [220, 221]. Inside the cytosol, mtDNA is sensed by NLRP3 inflammasome, thereby promoting the activation of pro-inflammatory cytokines like Interleukin-1 beta and Interleukin-18. Additionally, Toll-like receptor 9 can also bind mtDNA in the endosome and cause the expression of pro-inflammatory cytokines [222-224]. Importantly, mtDNA sensing by cGAS, causing activation of cGAS STING pathway-mediated IFN-I production has been described in multiple conditions [225]. The mechanism of mtDNA release in cytosol needs proper understood. However, during apoptosis, the formation of BAK-BAX macropores in the outer mitochondrial membrane is known to aid the inner mitochondrial membrane in protruding into the cytosol to initiate the cGAS STING pathway [226]. Studies in transgenic mouse models have shown that depletion of transcription factor A mitochondrial (TFAM) cause moderate mtDNA stress

and lead to release of mtDNA in the cytosol activating the cGAS- STING signaling to increase IFN-I production [227].

1.4.3 IFN-I dysregulation in cancer

In TME, stress conditions like hypoxia and secretion of cytokines like IL-1 can inactivate IFNAR and suppress downstream signaling and induction of ISGs, thereby blocking antitumor responses [228, 229]. Classical treatment strategies, including chemotherapy, radiotherapy, and immunotherapy, depend on IFN-mediated immune signaling [228, 230, 231]. Unfortunately, dysregulation of IFN-I signaling is observed in most tumor types and it is associated with resistance to classical treatment regimens, including immune checkpoint inhibitors (ICIs). For example, loss of IFNAR1 is an important resistance mechanism in cancers due to ubiquitination-mediated degradation of IFNAR1 due to ER stress, integrated stress response, and hypoxia [229, 232]. Loss of IFNAR1 is reported in melanoma patients, and overexpression of IFNAR1 mutant in melanoma mouse models showed delayed melanoma progression and increased responsiveness to PD-1 inhibitors [233]. In breast cancer models, downregulation of interferon regulatory factor (*Irf7*) target genes was associated with increased bone metastasis and reduced survival. At the same time, high expression of *IRF7* regulatory genes in patients correlated with increased metastasis-free survival [234]. Since IFN-I signaling is essential in cancer treatment, understanding the processes involved in regulating this response are crucial. Therefore, crosstalk between IFN-I signaling and cellular recycling processes like autophagy are thought to be important [235].

1.5 Autophagy

Eukaryotic cells continuously renew their components in a balanced interplay between catabolic and anabolic processes to maintain cellular homeostasis [236]. Autophagy, derived from the Greek meaning ‘eating of self’, is a self-degradative process involved in the sequestration and transport of cytosolic proteins or organelles for lysosomal degradation [237]. During autophagy, cytoplasmic substrate is sequestered into a double membrane vesicle known as an autophagosome, which fuses with a lysosome to form an autolysosome for the degradation and recycling of the cytoplasmic contents. Based on different mechanisms for directing the cytoplasmic content into the lysosomes, three different types of autophagy have been described as macro-autophagy, micro-autophagy, and chaperone-mediated autophagy respectively [238-240]. Only macro-autophagy (hereafter referred to as autophagy) will be discussed here.

Breakthrough genetic studies in yeast by Dr. Yoshinori Ohsumi and colleagues identified the core molecular machinery of autophagy [241]. Comparative studies in animals and other single celled organisms revealed that these genes are highly conserved during evolution of all eukaryotes. For this

outstanding work, Ohsumi was awarded the Nobel Prize in physiology or medicine in 2016 [241-243]. There is an exponential growth in autophagy research, and currently more than 42 autophagy-related (Atg) genes have been identified in yeast [244].

1.5.1 The core autophagy machinery and the autophagic process

Autophagy involves multistep complex processes including induction and nucleation, expansion and sealing, fusion, and degradation (Figure 6) [245, 246]. A subgroup of approximately 20 ATG proteins are essential for the autophagosome formation and constitute the autophagy core machinery [247]. This can be classified into six functional modules; (1) the unc-51 like autophagy activating kinase (ULK) complex; (2) the class III phosphatidylinositol 3-kinase (PI3-kinase) complex (VPS34); (3) the ATG9-positive vesicles; (4) a complex of ATG2/Atg18 homologs (WD-repeat protein interacting with phosphoinositides; WIPI proteins); (5) the ATG12 conjugation complex and (6) the ATG8/LC3 conjugation complex (Figure 6) [246, 247].

In addition to nutrient and growth factor deprivation, autophagy is also activated by a variety of stress conditions including endoplasmic reticulum stress, hypoxia, redox stress, mitochondrial damage, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [248]. The nutrient- and growth factor-sensitive kinase mammalian target of rapamycin (mTOR) and the energy-sensitive adenosine monophosphate (AMP)-activated protein kinase (AMPK) are major regulators of autophagy [249]. mTOR participates in two signaling complexes; mTORC1 and mTORC2, where mTORC1 is involved in regulation of autophagy whereas mTORC2 regulates cellular metabolism via phosphorylation of ATG proteins [250]. During nutrient rich conditions, mTORC1 phosphorylates ATG13 and ULK1 in the stable complex consisting of ULK1/2, ATG13, FIP200 and ATG101, leading to inhibition of autophagy. In contrast, in nutrient depleted conditions, the ability of mTORC1 to phosphorylate ATG13 and ULK1 is impaired, and thus the lack of the inhibiting phosphorylation causes increased autophagy. However, AMPK can also phosphorylate ULK1, but at different sites and this induces autophagy under nutrient depletion [251]. The initiation of autophagosome formation starts when the ULK1/2 complex assembles at the isolation membrane. ATG9, a transmembrane protein, mediates membrane addition to the phagophore during the nucleation process [252]. At the membrane nucleation site, phosphatidylinositol 3 phosphate (PtdIns(3)P) is formed by the PI3-kinase complex, coordinated by the interaction between its constituents including Beclin-1 (BECN1), ATG14L, vacuole protein sorting 34 (VPS34) and VPS15, and double FYVE-containing protein 1 (DFCP1). The increased concentration of PtdIns(3)P due to the kinase activity of VPS34 is important for the recruitment of various proteins like WIPI/ATG18, ATG2, ATG9 and DFCP1 at the phagophore formation site [253]. The phagophore expansion is mediated by two ubiquitin-like conjugation systems. The ATG12 conjugation system is initiated when ATG12 and

ATG5 are covalently conjugated via the E1-like enzyme ATG7 and the E2-like enzyme ATG10. The ATG12-ATG5 then binds ATG16L, generating a complex which localizes to the forming autophagosome via ATG16L's binding to FIP200 in the ULK1/2 complex. The ATG12-ATG5-ATG16L complex has E3 ligase activity that can act on the ATG8/LC3 conjugation system. There are many mammalian ATG8 homologues, including microtubule-associated protein light chain 3 (MAP1LC3) A/B/B2/C, and γ-aminobutyric acid (GABA)-receptor associated protein (GABARAP), GABARAPL1 and GABARAPL2 [246, 254, 255]. The ATG8/LC3 is synthesized as a precursor and following cleavage by ATG4 a C-terminal glycine residue is exposed which is conjugated to phosphatidylethanolamine (PE) via the sequential action of ATG7, ATG3 and the ATG12-ATG5-ATG16L complex acting as E1-, E2- and E3-like enzymes, respectively. Since the ATG12-ATG5-ATG16L complex is located at the autophagosomal membrane, the conjugation takes place here and the lipidated product, ATG8/LC3-II, can be incorporated into the phagophore. The cargo for autophagy is recruited to the surface of the phagophore by cargo receptors (Figure 6) [246, 256, 257]. The sealing of the elongated phagophore requires endosomal sorting complex required for transport (ESCRT-III) and VPS4 [258, 259]. The expansion of the phagophore and enclosing of cargo forms the autophagosome, which fuses with the late endosomes/lysosomes to form autolysosomes where the cargo is degraded. The fusion of autophagosome to lysosome is governed by the autophagosomal SNARE (Syntaxin 17, soluble SNAp-29, lysosomal membrane protein Vamp7 and all six HOPS (homotypic fusion and VPS subunits (VPS11, VPS18, VPS16, VPS16A, VPS33A, VPS39 and VPS47)) [230, 260, 261].

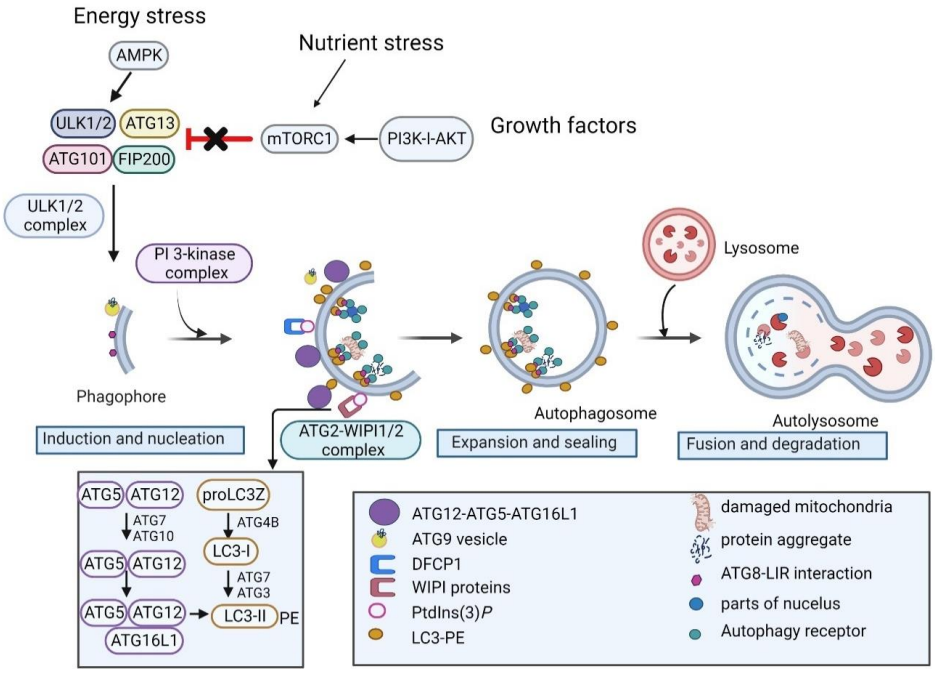

Figure 6. Schematic overview of core autophagy machinery in autophagosome formation. Autophagy is regulated by nutrient and energy stress via mTORC1 and AMPK. During cellular stress, the inactivated mTORC1 dissociates from the ULK1/2 complex (ULK1/2, ATG13, FIP200 and ATG101). Activation of the ULK1/2 complex is required for the induction of autophagy. During nucleation, the ATG proteins and lipids are recruited to the phagophore. The cytosolic cargo is sequestered during the expansion and sealing process via ATG proteins for autophagosome formation. The autophagosome fuses with the lysosome making an autolysosome where the degradation of cargo occurs. Created with Biorender with adaptations from [245, 246, 262].

1.5.2 Selective Autophagy and Sequestosome Like Receptors (SLRs)

Autophagy was long considered as a non-selective process. However, in the last decades it is well accepted that the process can be a highly selective and tightly regulated process that requires cargo recognition and recruitment to the autophagosome [263]. Based on the type of substrate sequestration, different types of selective autophagy have been described; aggrephagy (protein aggregates), mitophagy (mitochondria), pexophagy (peroxisomes), ribophagy (ribosomes), reticulophagy (endoplasmic reticulum), nucleophagy (parts of nucleus), micronucleophagy (micronucleus), lipophagy (lipid droplets), glycophagy (glycogen delivery to lysosome), ferritinophagy (ferritin), lysophagy (lysosome) and xenophagy (intracellular pathogens) [264-272]. Polyubiquitination of targets is fundamental for the recognition and physical binding by the autophagy receptors that selectively link the cargos to the lipidated ATG8s located on the expanding autophagosome membrane (ATG8-PE). Sequestome1 ((SQSTM1), also known as p62) was the first autophagy receptor to be identified which links cargo to LC3B before the whole complex is degraded in the autolysosome [273, 274]. SQSTM1 contains an N-terminal self-interacting Phox and Bem1 (PB1) domain, a ZZ-type zinc finger domain, an LC3

interacting region (LIR) motif and a C-terminal ubiquitin-associated (UBA) domain. The UBA domain of SQSTM1 interacts with polyubiquitinated cargo, SQSTM1 polymerizes via the PB1 domain and makes protein aggregates, followed by cargo connection to the autophagosome via the LIR motif. Hence, SQSTM1 plays a vital role in selective autophagy by recognizing the cytosolic cargo for the lysosomal degradation [275]. With the discoveries of selective autophagy, more than 20 autophagy receptors have been identified in human. These receptors are known as sequestosome-1 (SQSTM1) like receptors (SLRs) since they recognize cargo in a similar manner as SQSTM1 via the UBA domain. Additional receptors identified that recognize ubiquitinated substrates include neighbor of BRCA1 gene 1 protein (NBR1), nuclear dot protein 52 kDa (NDP52) /calcium-binding and coiled-coil domain-containing protein 2 (CALCOCO2), optineurin (OPTN), Tax1-binding protein 1 (TAX1BP1), toll interacting protein (TOLLIP), FK506-binding protein 8 (FKBP8), nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog (NIPSNAP)1/2, nip3-like protein X (NIX), prohibitin-2 (PHB2), autophagy and beclin-1 regulator 1 (AMBRA1), BCL2-Like 13 (BCL2L13) and tripartite motifs (TRIMs) [246, 276]. Like SQSTM1, all these undergo oligomerization, bind to ATG8 family proteins via a LIR interaction and guide the cargo to the autophagosome for later degradation of both the cargo and the receptors in the autolysosome [277].

1.5.3 Context dependent role of autophagy in cancer

Autophagy-based degradation is essential for normal development, differentiation, aging, and cellular remodeling [278]. Dysregulated autophagy has been implicated in neurological diseases such as Alzheimer's, Parkinson's, and Huntington's diseases [279, 280]. In cancer, autophagy is induced by hypoxia, metabolic stress, and therapeutic stress such as chemotherapy and radiation. Autophagy plays a context dependent role in cancer, either as a tumor suppressor or as a promoter [281]. Currently, the understanding of the context dependent role(s) of autophagy during tumor progression is incomplete. However, genetic alterations in autophagy genes have been implicated in cancer and we know that the process is under control of fundamental pathways for cellular regulation. (Figure 7).

1.5.3.1 Autophagy as a tumor suppressor

At the early stages of cancer development, basal autophagy prevents cancer progression by eliminating the oncogenic protein substrates, toxic unfolded proteins, and damaged organelles [262]. The first evidence showing the link between dysregulated autophagy and cancer was observed by Liang CH et al. in 1999, where they show that ATG6/(BECN1) disruption enhanced tumor proliferation and increased frequency of various cancers like lung, liver, and lymphomas [282]. *BECN1* is monoallelically lost in 40% to 75% of human prostate, breast, and ovarian cancers [282, 283]. However, a study by Laddha et al. in 2014 demonstrated that the deletion of *BECN1* in human tumors is accompanied by deletion of the proximal tumor suppressor breast cancer 1 gene (*BRCA-1*), thus making

the importance of *BECN1* unclear [284]. Studies using genetically engineered mouse models demonstrate the complexity, since loss of *Becn1* has been shown to promote p53 activation and reduce tumorigenesis in a Partner and localizer of BRCA2 (*Palb2*)-deficient hereditary breast cancer model, while others demonstrate that heterozygous disruption of *Becn1* increases the frequency of spontaneous malignancies [285-287]. Other autophagy genes like *ATG5* and *ATG7* are identified as tumor suppressors since hepatocytes lacking these genes cause hepatocellular cancer due to oxidative stress and damaged mitochondria [288]. Deficiency in other ATGs like *ATG3, ATG9* and *ATG16L1* are also associated with tumorigenesis [289-292]. In a *Drosophila* model of malignant rat sarcoma (RAS)-driven cancers, the tumor cells show large numbers of damaged mitochondria and increased ROS, indicating metabolic stress, and the cells can stimulate non-autonomous autophagy [293]. It is known that stress signals like ROS can potentially induce autophagy and subsequently eliminate these damage signals in the tumors.

Autophagy cargo receptors play a vital role in tumorigenesis. Autophagy also prevents tumor formation via regulation of ROS production. It is known that autophagy deficiency causes oxidative stress that activates the master regulator of antioxidant defense, Nuclear factor erythroid 2-related factor 2 (NRF2), which also stimulates tumor growth and cancer cell survival [294, 295]. It has been shown that deficiency in SQSTM1 and NRF2 subsequently abolishes the progress of oncogenic RAS-driven non-small-cell lung cancer in mouse models, suggesting NRF2 deficiency might prevent tumorigenesis induced by SQSTM1 accumulation and limited autophagy [296]. Accumulation of SQSTM1 has been detected in various cancer types like prostate cancer, hepatocellular carcinoma, gastrointestinal cancers, breast cancer and lung adenocarcinoma, suggesting that SQSTM1 accumulation positively correlates with cancer progression [297-304]. Increased levels of SQSTM1 could be an indicator of either reduced autophagic flux, since SQSTM1 is degraded itself via autophagy, or it could be a result of the cells increasing the autophagic process by increasing the expression of central proteins, like SQSTM1. However, *in vivo* studies have shown that accumulation of SQSTM1, DNA damage and genomic instability that all have cancer promoting roles are due to inhibition of autophagy, thereby highlighting the role of autophagy in tumor suppression [305-307].

1.5.3.2 Autophagy as a tumor promoter

Although autophagy prevents cancer progression at the early stages, several studies have shown the opposing role of autophagy: as a tumor promoter in advanced cancers [308, 309]. In contrast to normal cells, most cancer cells have high basal autophagy since they experience extreme stress, demand more nutrient supply, are hypoxic, and fundamentally depend on autophagy for survival. During tumor progression, autophagy undergoes an anti-tumorigenic to pro-tumorigenic switch, enabling cancer cells to survive at the later times [310]. Autophagy can be beneficial for tumors and help them survive in a metabolically challenging and harsh tumor microenvironment by mobilizing nutrients to the cancer

cells through increased local recycling. Martinez-Outschoorn UE et al. proposed the autophagic tumor stroma model of cancer cell metabolism or "Battery-Operated Tumor Growth", highlighting that autophagy within the tumor microenvironment can provide fuel for tumor growth and metastasis [311, 312]. Kirsten RAS (KRAS)- and Harvey RAS (H RAS)-driven tumors that are common in many cancer types have been demonstrated to have increased autophagy where autophagy promotes the growth, survival, increased tumorigenesis, subsequently leading to invasion and metastasis [313, 314]. Since many RAS-driven cancers rely on autophagy for their survival, they are termed "autophagy addicted" [314-316]. The *TP53* gene encoding p53 is a major tumor suppressor and commonly mutated gene in many cancers. Autophagy is known to promote mammary tumor growth by suppressing p53 activation [316].

Recent evidence focuses on protective function of autophagy to tumor cells against immune-mediated destruction [317]. Deletion of FIP200 in mammary tumor cells suppresses tumor progression and increases survival in a breast cancer mouse model via increased immune surveillance. The tumors from FIP200 knockout mice have elevated expression of IFN response genes and chemokines like CXCL10 along with increased infiltration of effector T cells in the tumor microenvironment [318]. Baginska et al. show that hypoxia-mediated autophagy in MCF-7 breast cancer cells blocks natural killer (NK) cell-mediated lysis of tumor cells via sequestration of granzyme B and perforin granules inside the autophagosomes. The tumor growth is also reduced in autophagy deficient tumor cells in *in vivo* models of melanoma and breast cancer [319]. In pancreatic ductal adenocarcinoma (PDAC) cells, autophagy promotes degradation of MHC-I molecules thereby reducing their surface expression. NBR1, a selective autophagy receptor, targets MHC-I molecules for selective degradation, a unique mechanism facilitating immune evasion by PDAC cells. Inhibition of autophagy via chloroquine (CQ) and mutant ATG4B increases the surface expression of MHC-I and cytotoxic lymphocytes, thus indicating a possible use of CQ in cancer treatment [320, 321].

These studies indicate the pivotal role of autophagy in promoting tumorigenesis, assisting immune evasion, and are thus pointing out autophagy as a therapeutic target in cancer treatments. Yet, even if it seems evident that autophagy plays an important role in cancer, whether it functions as a tumor promoter or a suppressor is still not fully understood, and the mechanisms for regulation of the process in a complex tumor microenvironment are still unclear [310, 317].

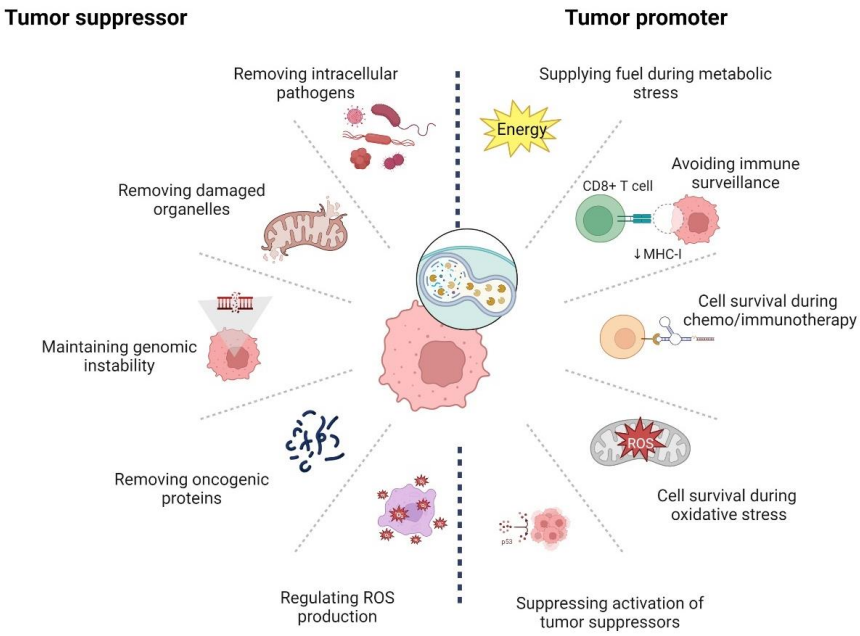

Figure 7. Dual role of autophagy in cancer. Autophagy plays a context dependent role in tumorigenesis by either suppressing or promoting cancer progression. The specific role of autophagy in cancer is individually mentioned under tumor suppressor and tumor promoter. Created with Biorender.com with adaptations from [322].

1.5.4 Autophagy and metabolic reprogramming in cancer cells

Cancer cells experience metabolic challenges and may rely on an elevated uptake of nutrients for their energy needs, growth, proliferation, and survival. Therefore, metabolic reprogramming is one of the hallmarks of cancer [40]. Already in the 1920s, it was acknowledged that cancer cells can reprogram the glucose metabolism from oxidative phosphorylation (OXPHOS) to aerobic glycolysis, known as the Warburg effect [323, 324]. It was thought quite illogical since the cells end up with only two ATP molecules via glycolysis compared to 36 ATP molecule produced by OXPHOS, raising the question: "Do the cancer cells really benefit from this?" Glycolysis is the fastest way for cancer cells to obtain ATP from glucose in an oxygen independent manner, which is beneficial at hypoxic conditions like those that can arise in a fast growing, solid tumor [325, 326]. Also, cancer cells can choose glycolysis to preserve Krebs cycle metabolites for anaerobic process for tumor proliferation. The high glycolytic flux also provides sufficient glycolytic intermediates for fatty acid, lipids, and nucleotide synthesis [327-331].

A role of autophagy in cancer metabolism is highly expected because only cells that adapt their metabolism to the harsh conditions can sustain and contribute to tumor development. Autophagy plays a crucial role in regulating the shift to aerobic glycolysis in cancer cells. Studies have shown that Glucose transporter-1 (GLUT1), necessary for glucose transport, enhances autophagy in breast cancer cells [332]. FIP200 depletion leads to reduced glycolysis in mammary mouse tumor models [318]. In pancreatic tumors, with KRAS mutation, genetic and chemical inhibition of autophagy leads to suppressed proliferation and prolonged survival in mouse and xenograft models [315]. Glutamine, the most abundant amino acid in mammalian cells, is an important metabolic intermediate, along with glucose, and is essential for cancer cells. The tumor cells rely on glutamine to restore the TCA cycle intermediates and maintain ATP production [333]. During long-term amino acid starvation, glutamine that is converted to glutamate contributes to an autophagy-dependent mTORC1 reactivation, important for further growth and proliferation [334]. Autophagy is also linked to lipid metabolism in $KRAS^{V12}$-driven non-small cell lung cancer (NSCLC) mouse models, where deletion of *Atg7* lead to development of predominantly benign tumors with accumulation of dysfunctional mitochondria and neutral lipids [335]. In a pancreatic cancer model, Yang et al. demonstrated that autophagy can promote tumor growth via both cell autonomous and non-autonomous mechanisms [336]. However, recent studies highlight the non-autonomous aspect of autophagy regulating the cell metabolism outside of the tumor cells [337-339]. Researchers have also shown that cancer cells just grow by stealing nutrients from the neighboring cells. A recent study by Katheder et al. using a *Drosophila melanogaster* malignant tumor model shows that transformed tumor cells benefit themselves by inducing non-autonomous autophagy in the neighboring epithelial cells and distal tissues, suggesting the importance of considering interactions in TME while studying the role of autophagy in cancer. They show that reduced tumor growth in autophagy deficient animals is reactivated when they are transplanted into autophagy competent hosts [293, 340]. It is also evident that autophagy in the host mediates nutrient mobilization via organ wasting that is used for tumor growth [341]. Laura Poillet-Perez et al. showed that conditional depletion of the autophagy genes *Atg5* and *Atg7* in mice with tumor allografts significantly suppresses tumor growth. They reported that the lack of autophagy is related to limited levels of the semi essential amino acid arginine in circulation, and this is partly restored by supplementation of dietary arginine [342]. Although we know that autophagy has an important role in altering metabolism in cancer, a deeper understanding on how autophagy affects metabolism inside the tumors may lead to better therapeutic approaches.

1.5.4.1 Arginine biosynthesis and uptake

Arginine is a semi-essential amino acid important for protein synthesis and cell growth, wound healing, ammonia disposal, hormone biosynthesis and the immune system [343]. The major sources for L-arginine in the body are via dietary intake, *de novo* production from citrulline by the intestinal-renal axis and from degradation of proteins. In the biosynthesis of arginine, argininosuccinate synthase 1

(ASS1) and argininosuccinate lyase (ASL) are central enzymes, with ASS1 being the rate-limiting enzyme. ASS1 converts the urea cycle component L-citrulline and aspartic acid to argininosuccinate, which is then converted by ASL to L-arginine and fumaric acid [344]. L-arginine is also the precursor of L-citrulline and nitric oxide via nitric oxide synthase (NOS) [345, 346]. Additionally, ARG1 enzyme also utilizes arginine, converting it to ornithine, which is a precursor of polyamines (Figure 8). Polyamines are essential for proper cell physiology, protein synthesis and function, and protection from oxidative damage, inflammation, and wound healing [347, 348]. Besides endogenous synthesis and dietary supplementation, arginine availability also relies on the action of arginine transporters located in the mitochondrial and plasma membranes [346]. Several cationic amino acid transporters (CATs) are involved in the uptake of L-arginine; CAT-1, also known as solute carrier family 7 A1 (SLC7A1), CAT-2A (SLC7A2A), CAT-2B (SLC7A2B) and CAT-3 (SLC7A3) in different cell types [349]. For example, CAT-1 mainly mediates the arginine transport in human T cells whereas CAT-2 has this function in myeloid cells [350, 351].

1.5.4.2 Arginine dependence in cancer cells

Downregulation of urea cycle components in cancer cells, directing metabolites away from arginine synthesis, is frequently occurring due to metabolic reprogramming [125]. Further, in many cancer cells, ASS1 abundance is either reduced or the cells are completely unable to synthesize arginine *de novo* [352]. The combination of metabolic reprogramming, deficient synthesis, and increased needs due to high growth rate, causes dietary arginine to become essential for the survival of many cancer cells. This condition is known as arginine auxotrophy, an under-recognized metabolic vulnerability in cancer [353, 354]. Many tumors including melanoma, hepatocellular carcinoma (HCC), breast, and prostate carcinoma are deficient in ASS1 [355-357]. In ASS1 deficient cancer cells, Cheng et al. showed that arginine starvation damages the mitochondria, resulting in accumulation of excess ROS, which causes genome instability [358]. This could lead to a novel form of cell death induced by arginine starvation called chromatin-autophagy or chromatophagy [359]. Arginine-free diet also significantly retards tumor growth in orthotopically xenografted breast cancer cells, presenting dependence on extrinsic arginine as a possible target for therapies [358]. Several clinical trials have been performed using agents that will reduce arginine availability, such as pegylated versions of ARG1 or arginine deiminase (ADI) (ADI-PEG20), thus targeting arginine auxotrophic tumors. Phase I/II clinical studies using ADI-PEG20 and recombinant human Arginase (rhArg) have shown increased median progression-free survival and overall survival in patients with acute myeloid lymphoma, HCC and melanoma [360]. However, the phase III clinical trial of ADI-PEG20 in HCC failed with no difference in the median progression-free survival in control and treatment groups [361]. It is also evident that ARG1 expression positively correlates with poor prognosis in HCC. The high ARG1 activity seems to be important for increased expression of Vimentin, N-cadherin, and β-catenin, essential for EMT process suggesting oncogenic

function of ARG1 [362]. Therefore, the efficacy of therapeutic strategies involving depleting arginine levels may not be of general benefit for cancer patients, and for some even unfavorable.

1.5.4.3 Arginine levels influence immune responses

Arginine has many biological functions, including being an important regulator of immune responses [363]. Detrimental effects of arginine starvation on human T cells were already described in 1968 and it is well appreciated now that arginine is essential for antitumor T cell responses [364]. During tumor progression, there is a large recruitment and expansion of MDSCs that have immunosuppressive effects and thus help tumors avoid immune surveillance [97]. The inhibitory effect MDSC have on T cell responses is mediated partly by their ability to make ARG1, causing depletion of arginine, which the T cells need for their activity [365]. Arginine deficiency also leads to reduced IL-2 production in human T cells and reduced expression of CD25 and CD69 activation markers [366]. In accordance with this immune dampening effect of arginine deprivation, several studies have shown that cancers with high degree of MDSC infiltration are associated with poor prognosis. Fletcher et al. showed that arginine depletion using a pegylated form ARG1 increases tumor growth, blunts antitumor T cell response and increased activation of MDSCs, suggesting a need for co-targeting MDSC in these settings [97]. This approach could be beneficial in addressing problems associated with resistance in immunotherapy, where MDSCs are involved, and additional treatment to manipulate the effects of MDSCs on arginine levels and T cell function could be added to the treatment regime. This is because the immune therapy might be effective only for a limited time, and then recruitment of MDSCs can dampen the T cell response, subsequently causing development of resistance to therapy and increased tumor growth. Using drugs that counteract the effect of MDSCs, such as arginase inhibitors, might be a better approach in treating therapy resistant tumors. So, manipulating the arginine availability may be used in a therapeutic manner but it is still necessary to provide more detailed information about the interplay between various cancer cells and the TME, including the immune cells, to ensure that the therapeutic strategy results in reduced cancer growth and not the opposite.

1.5.4.4 Arginine sensing and autophagy

As mentioned above, arginine is an amino acid which can influence on a variety of physiological processes. One of the most central directors of cellular growth and metabolism is mTOR, and among the many regulators of mTOR activity we find amino acids. Activation of mTORC1 involves a complex interplay between many different proteins, and the first step where mTORC1 is recruited to the surface of lysosomes involves Ras-related GTP binding (Rag) GTPases and occurs when the Rag proteins are activated by sufficient amino acid levels. At the lysosomes, mTORC1 can be directly activated by ras homolog enriched in brain (RHEB), another small GTPase. For some amino acids there are other sensor systems upstream of the Rag GTPases than can cause activation of mTORC1, and for arginine this

includes the amino acid transporter solute carrier family 38 members 9 (SLC38A9), which is located in the lysosomal membrane, and also the cytosolic arginine sensors for mTORC1 subunit 1 (CASTOR1) and CASTOR2 [367-372]. Upon arginine depletion, mTORC1 will dissociate from the lysosomal surface and become inactivated. While active mTORC1 suppresses catabolism via inhibitory phosphorylation of the ULK1-complex, the inactivation of mTORC1 following arginine deprivation will lead to reduced inhibitory phosphorylation, and consequently this will enable initiation of autophagy to replenish the low arginine levels. Several reviews have highlighted the mechanism coupling autophagy and immunity [162, 163, 373, 374]. It is intriguing to further explore how arginine starvation in the tumors can control the signaling leading to immunosuppressive TME.

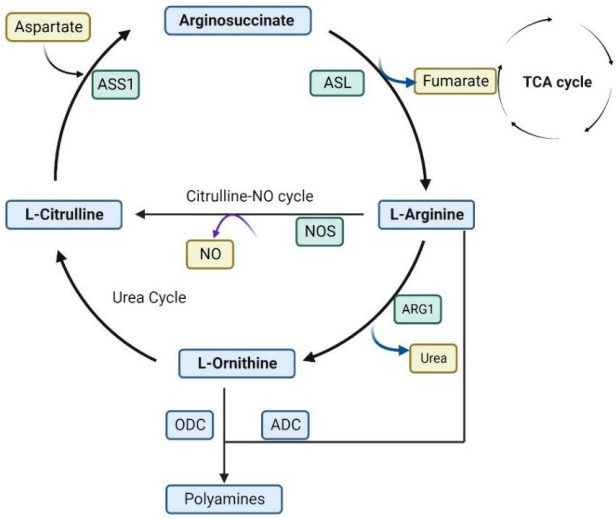

Figure 8: Arginine-related pathways in metabolism. L-arginine can be formed from L-citrulline by the enzymes argininosuccinate synthase 1 (ASS1) and argininosuccinate lyase (ASL) and recycled into L-citrulline and nitric oxide via nitric oxide synthase (NOS) or transformed to L-ornithine and urea via ARG1. Created with Biorender.com with minor modifications from [363].

1.5.5 Interplay between autophagy and immune responses

There is a comprehensive crosstalk between autophagy and innate immune responses [163, 235, 375]. Several studies have reported interplay between autophagy proteins in direct regulation of IFN-I signaling [376-379]. In response to bacterial and viral infections, autophagy receptors such as SQSTM1, NDP52, TAX1BP1 and TRIM23 can promote the degradation of RIG–like receptors, mitochondrial antiviral-signaling protein (MAVS) and TIR-domain-containing adapter-inducing interferon-β (TRIF), causing reduction in IFN-I signaling [376, 380, 381]. In mice deficient of ATG16L1, autophagy represses IFN-I in response to intestinal microbiota [382]. A pro-tumorigenic role of autophagy is also reported in *in vivo* models, where FIP200 conditional knock out mice have elevated IFN expression

along with increased infiltration of effector T cells, highlighting that autophagy proteins can be used as potential targets in cancer therapy [318].

The molecular link between autophagy and inflammation is also reported in a study by Jena et al. where they identify IRGM as a negative master regulator of IFN-I response in autoimmune diseases. IRGM mediates SQSTM1-dependent autophagic degradation of cGAS, RIG-I, and TLR3 thereby controlling IFN-I pathways [383]. Autophagy can also suppress protein complexes that initiate IFN-I production. For example, the cGAS-STING axis consists of several proteins that trigger IFN-I. ATG9A, the only multispanning membrane transport protein that generates autophagosomes, negatively regulates trafficking of the ER-associated STING and inhibits type I IFN activation [384, 385]. The ATG9A-knockout MEFs show increased phosphorylation of IRF3 and have increased transcription of IFN-I related genes like CXCL10 and IFN-B upon dsDNA stimulation [386]. cGAS and STING are also targeted by the autophagy machinery via SQSTM1 for subsequent degradation in the lysosome [387, 388]. SQSTM1 can recognize ubiquitinated cGAS and direct for autolysosomal degradation, thereby inhibiting the cGAS-STING signaling [388]. TBK1 is an essential component in the cGAS-STING pathway that causes phosphorylation of IRF3 and activation of IFN-I expression [200, 389]. Along with this, TBK1 is also crucial for autophagy where TBK1 is involved in multiple steps of autophagy. TBK1 is involved in autophagosome formation, and it phosphorylates as well as interacts with autophagy receptors [163, 390-393]. Prabakaran et al. 2018 show that SQSTM1 causes feedback inhibition of IFN-I by repressing STING-TBK1 activation. They show that the cGAS-STING pathway, upon recognition of cytosolic dsDNA, activates TBK1 to induce IFN-I expression. TBK1 also phosphorylates SQSTM1 which interacts with the ubiquitinated STING to promote autophagic degradation, thus mitigating IFN-I production [387]. Similarly, blocking the trafficking mediated STING degradation using autophagy inhibitor BafilomycinA1 has been shown to elevate the cGAS-STING activity in mouse embryonic fibroblasts [394]. In addition, it is well appreciated that autophagy can also mediate the degradation of IFN receptors [395-397].

The regulation between autophagy and IFN-I is a bilateral process. Autophagy as described above can regulate IFN-I response and IFNs can also mediate autophagy to eliminate damage responses in the cells [375]. Genome wide screen study by Orvedahl et al. showed that many genes involved in IFN-alpha signaling are essential for viral autophagy [398]. Several studies have shown that autophagy is induced by IFN-I via JAK/STAT pathway in numerous cancer cell lines [399-402]. For example, in chronic myeloid leukemia cells, JAK/STAT activation is essential for IFN-I induced expression of the autophagy-related protein BECN1 [399]. In glioma cells, IFN-β induces autophagy via the PI3K/Akt/mTOR and ERK 1/2 pathways [401]. In hepatoma cells, treatment with IFNα-2a has been shown to block autophagic degradation and accumulation of LC3 puncta and decrease phosphorylation of Akt/mTOR [403]. Similarly, interferon stimulated gene (ISG) products can regulate autophagy to deploy their innate immune response [404, 405]. A recent study demonstrates that IFN-β regulates

autophagy via microRNA *mir1* expression, where *mir1* lowers the levels of RAB GTPase protein TBC1D15, thereby inducing autophagy in HeLa cells [406]. Although, the mutual role of autophagy and IFN-I in response to infection and other stimuli is known, very little is known about how the TME alters autophagy and regulates IFN-I signaling in cancer. Therefore, improved understanding of the interplay between autophagy and the innate immune response in tumorigenesis can add unique target regimens to boost antitumor immunity and immunotherapy.

1.6 Cancer cachexia

As elaborated above, intratumor events greatly influence cancer development and patient prognosis. However, cancer patients may experience severe complications that extend far beyond the physical margins of solid tumors. Cachexia is derived from the Greek word, kakos and hexis, simply meaning "bad condition". This systemic condition affects up to 80% of advanced cancer patients and results in a life-threatening degradation of muscle tissue with or without loss of adipose mass [407, 408]. Cachexia can ultimately reach a severe condition where patients cannot even fulfil their basic daily needs due to reduced muscle strength that may lead to cardiac arrhythmias, respiratory syndromes and other problems resulting in premature death [408]. There is no strict correlation between cancer type, tumor size and cachexia. In addition, patients with similar diagnosis that undergo the same treatment approach might have different cachectic phenotypes underscoring the complexity of the disease [409, 410]. Cancer cachexia is also associated with reduced tolerance to anti-cancer therapies, so in addition to severely reducing the quality of life, cachexia as such is accountable for about 20% of all cancer deaths [411-413]. "Understanding and reversing cachexia and declining performance status in cancer patients" was considered one of the eight grand challenges in cancer research by The National Cancer Institute and Cancer Research UK in 2021 [414]. This emphasizes that both the cancer research community and people affected by cancer, consider cachexia a major unmet medical need.

1.6.1 Mechanisms of muscle loss in cancer cachexia

Cachexia is a systemic condition, involving multiple organs. Yet, muscle atrophy (of both heart and skeletal muscle) is a central limiting factor for survival and functionality for cachectic patients [415]. Therefore, understanding the mechanisms that lead to muscle atrophy is likely crucial for development of targeted treatment. Skeletal muscle is a major storage of amino acids, and proteolysis of muscle proteins provide energy to metabolically active organs [416, 417]. Under normal conditions, these processes are maintained under muscle homeostasis. However, during physiological response to starvation and other catabolic states accompanying cancer, such as inflammation and cachexia, the homeostasis is altered and shifts towards skeletal muscle wasting. This shift involves inhibition of muscle protein synthesis and allows degradation of muscle proteins. Patients with cachexia show hyper-metabolism, hyper-catabolism, and hypo-anabolism simultaneously, causing impaired energy metabolism and muscle atrophy [418].

1.6.2 Potential mediators of muscle loss in cancer cachexia

The putative underlying mechanisms that drive the metabolic abnormality in cachectic muscles are greatly studied, and various systemic mediators have been suggested. Since the only successful approach to treat cachexia is curative cancer therapy, cancer cells must be key initiators of cancer cachexia. Therefore, many factors that are released from the tumor and distributed systemically have been suggested as mediators of muscle loss. For example, tumor-derived factors such as TGF-β family members myostatin and Activin A may directly affect muscle signaling cascades, and their elevated levels have implications for muscle loss in cancer patients [419-422]. Also, increased presence of circulatory inflammatory cytokines like interleukin (IL) IL-1, IL-2, IL-6, IL-11, TNF-α [407, 408, 423-425] is associated with cachexia and these factors have been suggested as possible mediators of muscle loss. Studies have shown the interplay between potential mediators in cachexia. For example, inhibition of activin signaling causes reduced IL-6 secretion in *in vitro* and *in vivo* cachexia models [426, 427]. However, clinical interventions that target such single factors have generally shown disappointing results in reversing cachexia, suggesting that the underlying mechanisms causing cachexia are more complex and not yet fully understood. Possibly, the interplay between distinct inflammatory and other mediators released from cancer cells can cause the patient to undergo muscle atrophy. Or alternatively, the atrophy-inducing factors are not derived directly from the cancer cells, but rather from other non-cancerous cells whose presence and activities are altered in cancer patients. Given the massive implications tumor development has on immunity, immune cells may possibly have a greater impact on cachexia development, than currently acknowledged. Our current knowledge regarding the role of immune cells in cancer cachexia is addressed below.

1.6.3 Immune cells in cachexia

The skeletal muscle microenvironment is a mixture of myofibers, satellite cells, fibroblasts, endothelial cells, and immune cells [428]. It is known that muscle regeneration during growth, injury, exercise, and hypertrophy involves a strong interaction between multiple cell types inside skeletal muscle environment [429, 430]. There is also a keen interest in understanding how cancer alters skeletal muscle microenvironment and how tumor-derived factors can drive the skeletal muscle catabolism. Despite limited studies linking the function of immune cells and cachexia, recent studies suggest the involvement of numerous immune cell types in cachexia. Immune cells account for 2-6% of skeletal muscle cells that maintain muscle homeostasis [431, 432]. In skeletal muscle wasting in cachexia, a dynamic role of specific immune cells like neutrophils, monocytes, monocyte-derived macrophages, MDSCs and T cells, has been reported [428, 433]. The specific roles are discussed hereunder:

1.6.3.1 Monocytes and macrophages:

Myogenic cells, mesenchymal progenitors and macrophages are important cells needed for muscle regeneration indicating their potential role in skeletal muscle wasting [434]. Several muscle regeneration defects were observed in a C26 model of cancer cachexia along with reduced numbers of

myogenic cells, mesenchymal progenitors, and macrophages [435]. In a study in of pancreatic ductal adenocarcinoma (PDAC)-associated cachexia by Burfeind et al. they showed that microglia (resident macrophages of the central nervous system) accumulate in the hypothalamus in brain [436]. Depletion of microglia by CSF1-R antagonists increased cachexia, anorexia, fatigue, and muscle catabolism suggesting a protective function of microglia [437]. In addition to this, increased microglial cells in the hypothalamus positively correlated with increased IL-1b and Arg1 secretion both *in vivo* and *ex vivo*, indicating the defensive function of macrophages. A similar protective function of macrophages was also observed in transgenic mouse model of hepatocellular carcinoma-associated cachexia. The myeloid cell specific *Hif1a* knock out mouse, having defective myeloid cell activation, exhibited increased adipose tissue degradation and reduced macrophage infiltration in the adipose tissue [438]. It is known that macrophage function in the adipose tissue dependent on their polarization [439]. In contrast to the protective role of macrophages, another study showed that macrophages boosted pancreatic tumor cell induced myotube atrophy. The frequency of proinflammatory $CD163^{+}$M2 macrophages negatively correlated with muscle fiber density in patients with pancreatic cancer, suggesting an important role of M2 macrophages in muscle wasting. Also, myotubes generated from the C2C12 cell lines cocultured with M2 macrophages exposed to conditioned media from cultured tumor cells showed significant reduction in myosin heavy chain (a core myofibrillar protein) and increase in proteins involved in ubiquitin mediated degradation [440].

1.6.3.2 Neutrophils

Studies showing a role of neutrophils in cachexia emerged with observations of increased neutrophil influx in response to ischemia/reperfusion-induced skeletal muscle damage [441-443]. Increased neutrophil infiltration is associated with delayed skeletal muscle repair, and increased neutrophil accumulation blunts the signs of skeletal muscle wasting in this model [444]. However, studies in cancer cachexia models have shown contradictory roles of neutrophils. In C26 cachexia mouse models, decreased numbers of neutrophils and a reduction in CXCL3 (a chemokine essential for neutrophil recruitment) were reported along with reduced macrophage populations [435]. However, in mouse models of PDAC, increased neutrophil infiltration expressing brain specific chemotactic receptor CCR2, were observed in the central nervous system. Blocking CCR2, both genetically and via chemical inhibitors, led to a reduction in infiltrating neutrophils as well as diminished cachexia in this model [445]. In addition, a high neutrophil to lymphocyte ratio in advanced cancer patients have shown to correlate with cachexia [446, 447]. More studies are needed and are ongoing to understand the role of neutrophils within the circulation and the TME.

1.6.3.3 MDSCs

MDSCs having immunosuppressive functions are frequently observed in tumors from gastric and pancreatic cancer patients, as well as in several mouse models of cancer associated cachexia: 4T1 breast cancer, C26 colon adenocarcinoma and Lewis lung carcinoma (LLC) [77, 448-450]. The levels of MDSCs in bone marrow and spleen also positively correlated with adipose tissue loss along with loss in total body weight indicating the potential role of MDSCs in cachexia [448, 449].

1.6.3.4 T cells:

The role of adaptive immune cells, especially T cells, remains elusive in cachexia. Like neutrophils, not much is understood about function of T cells in cancer-induced skeletal muscle wasting. In gastrointestinal cancer patients with signs of muscle impairment, the presence of effector $CD8^+$T cells were associated with elevated muscle mass whereas the regulatory T cells and memory T cells showed negative correlation with the muscle mass, implying a protective function of $CD8^+$T cells in cachexia [451, 452]. It is shown that the muscle microenvironment with reduced levels of cytokines like IL-12, IFNβ and IFNγ, establish an environment where $CD8^+$T cells can flourish. It is however unclear how these muscle environments form and how they interact with muscle atrophy. One could speculate that these $CD8^+$T cells may consume muscle-derived amino acids for their growth and cytokine production [453].

With the current available studies, it is obvious that immune cells can have both anti- and pro-cachectic effects. Additional research is warranted to fill the significant gaps in exploring the role of specific immune cells, including how the local and infiltrating immune cells affect the tissue metabolism in cachexia.

1.6.4 Autophagy and muscle loss in cancer cachexia

Regardless of which atrophy-inducing signal(s) are involved, several metabolic pathways seem to be affected in cachectic muscles. Skeletal muscle accounts for almost 40% of the overall body mass and is the largest tissue in the human body [454]. Although both anabolic and catabolic pathways may be affected in cachexia [455, 456], the focus herein will be on catabolic pathways that may facilitate muscle loss. The degradation of muscle mass is mostly regulated by the ubiquitin proteasome system and autophagy, as well as caspases and calpains depending upon the stimulus [457-461]. Emerging studies have shown accelerated autophagy process in cachexia. Increased levels of autophagy markers like BECN1 and LC3B-II were observed in muscle samples from cachectic patients and in several mouse models of cachexia [457, 462, 463]. Since cachexia involves both skeletal and heart muscle wasting, studying the involvement of autophagy has gained particular interest in these tissues. In fact, muscles of both heart and skeleton showed upregulation of autophagy marker LC3 and SQSTM1 in rat cachexia

models. Treatment with appetite stimulant megestrol acetate reduced SQSTM1 protein as well as autophagy flux in the gastrocnemius and heart of the tumor-bearing rats and improved survival [464]. In muscles of C26 tumor bearing mice, autophagy proteins BECN1, SQSTM1 and LC3B-II levels were higher than in the control mice, indicating increased autophagy activity in cachectic muscles [462]. However, an increase in autophagy markers does not directly reflect increased autophagy if the animals are not treated with autophagic inhibitors.

Genetic studies showing autophagic activity *in vivo* that affects tumor growth or even organ wasting are limited. A study by Penna et al. showed BECN1 knock down slightly reduced muscle wasting in tibialis anterior muscle in C26 bearing mice but did not cause morphological changes [465]. A role of autophagy in systemic muscle wasting in cachexia was recently reported [245]. Khezri et al. reported an important role of autophagy (using *Atg13* knockouts) in promoting organ wasting and nutrient mobilization for tumor growth in malignant tumor model in *Drosophila melanogaster* [341]. Beyond muscle wasting via autophagy, mitochondrial impairment is also associated with onset of cachexia [465-467]. In LLC tumor bearing mice, mitochondrial degeneration and dysregulation occurred prior to cachexia development [468]. This indicates that the mitochondria are damaged and of poor quality in cachectic muscles. These studies pinpoint to a potential role of mitophagy in cachexia [469, 470]. However, studies involving selective autophagy in cachectic muscles are limited and will therefore be essential for a deeper understanding of how selective autophagy plays a role in cachexia development.

1.7 Models used in study

In this PhD research project, three different models were used (Figure 9). The mouse 4T1 breast cancer model is a syngeneic model of metastatic breast cancer that was established by Dexter et al. already in 1978. Originally, four cell lines were isolated from one, spontaneously arising mammary tumor of a BALB/cfC3H mouse [471] and since then, various cell lines and variants of the original cell lines have been developed. Here we used two of these cell lines; 67NR and 66cl4 [471, 472]. When injected back into the mammary fat pad of BALB/c mice, both form primary tumors, but they have different metastatic propensity; 67NR does not metastasize at all, while 66cl4 metastasizes to the lungs (Figure 9). Use of this immunocompetent model allows us to study the interplay between cancer cells and the full specter of immune cells and thus identify features associated with metastatic tumor development. In addition to the 4T1 model, two human breast cancer cell lines that are characterized as non-invasive (MDAMB453) and invasive (MDAMB231) were also used. The MDAMB cell lines both originate from metastases, yet the MDAMB453 is classified as non-invasive based on its expression of matrix metallopeptidase 14 (MMP14) [473, 474], and is considered as tumorigenic only in semi-solid medium (ATCC). For the cachexia model, we used a human cardiomyocyte cell line (AC16, Sigma Aldrich, Cat.no. SCC109) and a mouse myoblast cell line that can be differentiated into myotubes (C2C12, ATCC, nat.no. CRL-1772).

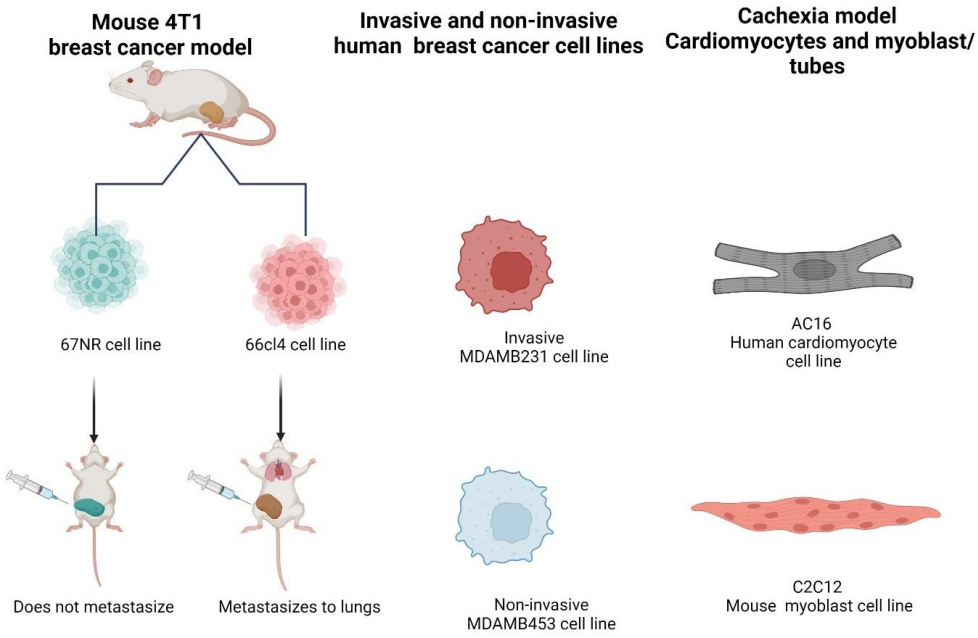

Figure 9. Mouse model and cell lines used in the study. Two cell lines from the mouse 4T1 breast cancer model, two human breast cancer cell lines with invasive and non-invasive propensity, one human cardiomyocyte and one mouse myoblast cell line were used in this study.

2 Aims of Study

Tumorigenesis involves a complex interplay between the transformed cancer cells and local immune cells. This interplay can be either pro-tumorigenic or anti-tumorigenic, depending on the context. Although our understanding of anti-cancer immune responses is increasing, it is still uncertain how immuno-suppressive TMEs, that facilitate tumorigenesis, are shaped and how some cancer cells survive, metastasize, and orchestrate systemic effects in their hosts. The aim of this thesis was to provide more knowledge about the interplay between cancer cells and the immune system that facilitates tumorigenesis and promotes cancer-related muscle loss. More specifically we aimed to:

- Identify biological processes that facilitate immune suppression in the TME and are associated with metastatic tumor development.
- Determine whether autophagy can regulate tumor immune-suppressive processes in metastatic and invasive tumors.
- Explore whether immune responses that are associated with tumorigenesis may have systemic effects on muscle wasting in cancer cachexia.

3 Summary of papers

Paper I:

Opposite and dynamic regulation of the Interferon response in metastatic and non-metastatic breast cancer

The synergy between cancer cells and stromal cells has a strong impact in tumor development and metastasis. In this study, we studied the distinct gene expression patterns altered in cancer cells when they are grown in culture compared to when they form a primary tumor in the 4T1 mammary carcinoma model. Using transcriptomics performed in the metastatic (66cl4) and non-metastatic (67NR) cells and their primary tumors, we found IFN-I response as a potentially altered biological process. We identified a gene signature of 11 IFN-I related genes that were "oppositely expressed" in cells in culture and tumors of the metastatic and non-metastatic cells. While this IFN-I gene signature was highly expressed in 66cl4 cells *in vitro* it was dampened in 66cl4 tumors, and while it had a low expression in 67NR cells *in vitro* it was increased in the 67NR tumors. The potential dampening of the IFN-I response in the 66cl4 tumors were also validated using proteomics and immunoblotting. The clinical relevance of our findings from the 4T1 model (a triple negative breast cancer (TNBC) model) is supported by the observation that lower mRNA levels of these 11 IFN-I gene signatures correlates with reduced relapse-free and overall survival exclusively in TNBC patients. We also identified mechanisms involved in the constitutive activation of IFN-I in the metastatic cancer cells. In these cells, when grown *in vitro*, the IFN-I response was activated via the cGAS-STING pathway due to elevated levels of cytosolic DNA, including micronuclei and mtDNA. Our study adds to increasing knowledge on the role of IFN-I response in cancer and highlights the importance of identifying the negative regulators of cGAS-STING in patients with aggressive breast cancer. These regulators may represent future therapeutic targets.

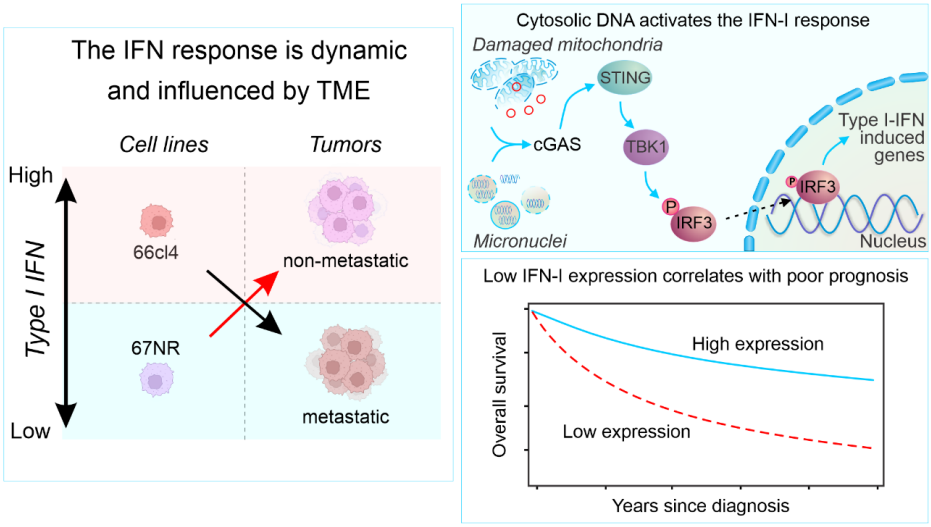

Figure 10. Summarizing illustration of paper I. Using open approach transcriptomics and proteomics we found that the IFN response is dynamic and is oppositely expressed in the metastatic (66cl4) and non-metastatic cells (67NR) in culture, and their respective primary tumors. The cytosolic DNA (damaged mitochondria and micronuclei) in the metastatic and invasive breast cancer cells can activate the IFN-I signaling and cause induction of IFN-I response. Lower IFN-I expression is associated with the reduced overall and relapse free survival in triple negative breast cancer patients.

Summary of paper II

Local arginine restriction dampens IFN-I response via autophagy in breast cancer

The anti-tumor innate immune reactions are often quenched in solid tumors, especially in the patients with an aggressive tumor phenotype. The fundamental interplay between cancer cells and infiltrating immune cells is important for the tumor tissue homeostasis. In this study, we aimed to make significant contributions to our understanding of how a suppressed immunity is established and maintained in some solid tumors and find novel ways to reprogram such a tumor supportive microenvironment. We recently identified that the arginine level in 66cl4 tumors can be depleted by the presence of myeloid cells that produce ARG1. Arginine restriction in the murine and human breast cancer cells led to significant reduction in the IFN-I response, indicating that arginine depletion could be a potential contributor to dampened IFN-I response in aggressive tumors. Amino acid starvation is a strong inducer of autophagy and autophagy has been shown to regulate IFN-responses following pathogenic infections. Still, very little is known about the role of autophagy that is not limited to pathogens, and if autophagy also regulates the DAMPs associated to the cytosolic components. We here found that arginine restriction induced selective autophagy receptors and the autophagy process. Autophagy negatively regulated the IFN-I response in the breast cancer cells by acting on upstream intracellular mediators of the cGAS-STING axis, like cGAS and micronuclei. Our data support that targeting ARG1 or the autophagy machinery in the cancer cells may reactivate inflammatory signaling in the tumor microenvironment and aid in treatment of aggressive tumors.

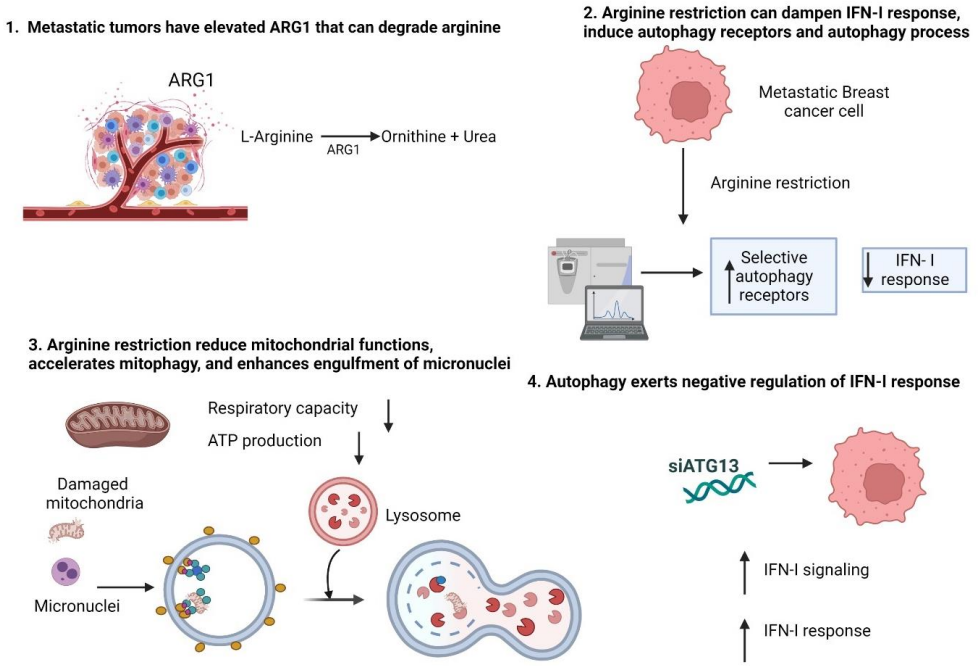

Figure 11. Summarizing illustration of paper II. The metastatic tumors contain higher ARG1 protein that have a potential to dampen local arginine availability in the tumor. Restricting arginine in metastatic cancer cells dampens the IFN-I response, induces several autophagy receptors, and accelerates the autophagy process. Arginine restriction reduces the mitochondrial function causing reduced respiratory capacity, and ATP production, accelerates mitophagy. Micronuclei also attracts autophagy machinery upon arginine restriction. Silencing a core autophagy gene *ATG13* by siRNA exerts a negative effect in both IFN-I signaling and IFN-I response in the metastatic breast cancer cell.

Summary of article paper III

A role of Arginase1- expressing myeloid cells in cachexia

Cachexia is a severe muscle loss condition that remains a global burden due to lack of specific treatments to reverse it. The muscle microenvironment, like TME, is a complex tissue with arrays of cell types, extracellular matrix, and immune components. In this study, we extended our knowledge from paper I and II to investigate if the myeloid cells that express ARG1 can infiltrate the muscle environment and be involved in the muscle degradation of cancer cachexia. Analyzing ARG1 protein in plasma derived from gastrointestinal and pancreatic cancer patients with and without muscle loss indicated significantly higher ARG1 protein level in the patients with weight loss compared to control and weight stable patients. Patients with gastric cancer often develop cachexia, and metanalyses in publicly available databases demonstrated a negative correlation between *ARG1* expression and overall survival in gastric cancer patients. When analyzing the transcriptomics of muscle biopsies from three cachexia mouse models (4T1, LLC and C26), revealed upregulation of biological processes involved in myeloid, neutrophil or granulocyte migration in cachectic muscles compared to healthy muscles. We therefore expected that the myeloid cells expressing ARG1 can infiltrate the muscle tissue and reduce the local arginine availability. In line with reports linking autophagy in skeletal muscle wasting and mitochondrial dysfunction in cachexia, we demonstrated that arginine restriction induced selective autophagy reporters, activated the autophagy machinery, and reduced mitochondrial functions in skeletal muscle cells and myoblasts. This study introduces a novel mechanism for muscle atrophy and point out the importance in exploring the role of ARG1-harbouring myeloid cells in cachexia pathogenesis.

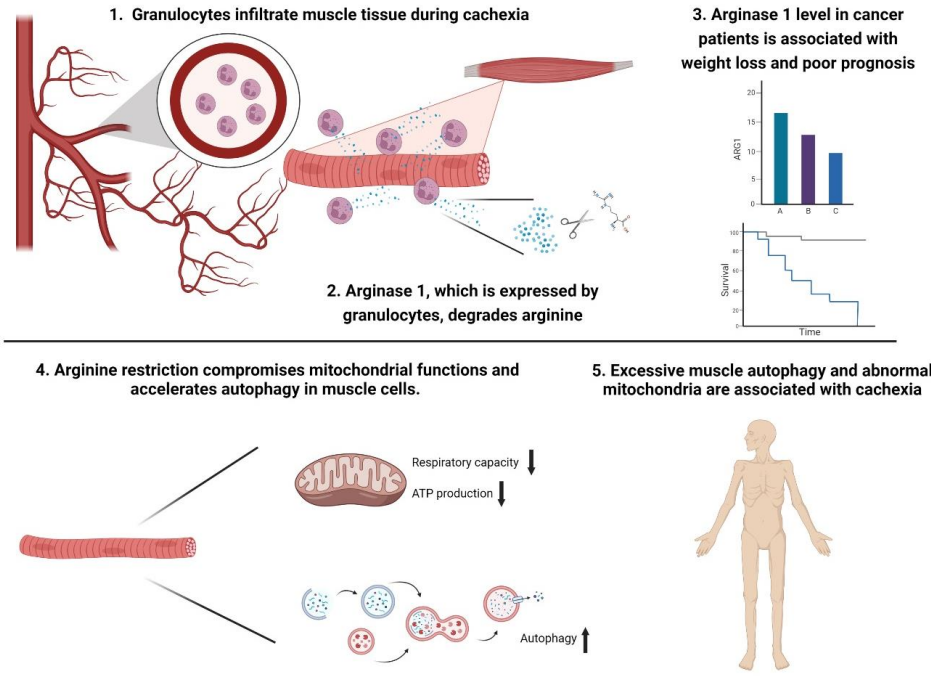

Figure 12. Summarizing illustration of paper III. Studies using cachexia mouse models indicate granulocyte population infiltrate the muscle tissue. Arginase-1 expressed by granulocytes have a potential to degrade arginine and cause local arginine availability in the muscle. Increased Arginase-1 correlates with weight loss in cancer patients and associates with reduced survival in gastric and lung cancer patients. Arginine restriction impairs mitochondrial function and induces autophagy in the muscle cells, an important characteristic observed in cachexia.

4 Discussion

With around 2.3 million new cases and 685 000 deaths in 2020, breast cancer accounts for most diagnosed cancer and represents a global health burden [475]. The cancer stage and the tumor subtypes determine the patient survival [476, 477]. To opt in an appropriate treatment strategy, proper understanding of tumor type is critically important. As mentioned in the introduction, tumors can be immunologically hot or cold based on the presence of immune cells with either pro or anti-tumor function [142-145]. In accordance with this, IFN expression is also used to classify these tumors; cold tumors have less IFN expression than the hot tumors. Unfortunately, IFN-I signaling is suppressed in most aggressive tumor types and is associated with resistance to classical treatment regimens [229, 232] Thus, immunologically "cold" tumors correlate with poor prognosis. This corresponds to our findings that lower mRNA expression of IFN-I related genes, correlates with a reduced relapse free and overall survival in triple-negative breast cancer patients, underlining the importance of IFN-I response in aggressive tumors. Although different forms of immune therapy have revolutionized cancer treatment, only a small fraction of the patients are responsive. Further progress in this field likely depends on a need for a deeper understanding of the mechanisms that initiate and maintain immune suppression in immunologically cold tumors. The complex heterogeneity in breast cancer, interplay between the cancer cells, immune cells, and stroma in the TME are key factors in establishing an immunosuppressive TME [478, 479].

In this thesis, we focus on the distinct dampening of IFN-I response in tumors that are associated with metastatic propensity (**paper I**) and find that dampening of IFN-I response in these metastatic tumors is regulated through the immune-suppressive innate immune cells tuning the local arginine availability, thereby inducing selective autophagy (**paper II**). Moreover, we find that the presence of these immune suppressive immune cells potentially has systemic effect in cancer associated cachexia (**paper III**). This PhD project extends on previous findings on selective autophagy from the research group [273, 480-482] and explores how TME can be controlled by autophagy and regulated through immune suppressive innate immune cells. Together, this work contributes to exploring a novel concept of cancer immunity.

In search of understanding the mechanism underlying the metastatic tumor development, we utilized the immunocompetent mouse 4T1 breast cancer model. In **paper I**, we show that IFN-I response is a key differentially regulated pathway among metastatic and non-metastatic cell lines and their primary tumors. In a related work in the group (unpublished data), we have found that metastatic tumors in this model also have increased presence of ARG1-expressing myeloid cells. Based on this observation and the findings in paper I, we hypothesized that ARG1-expressing immune suppressive cells infiltrate in the metastatic tumors and induce autophagy, with dampened IFN-I response consequently. Although the role of autophagy in regulating IFN-I response during viral infections is understood, the mechanisms

for suppression of IFN-I response in solid tumors are still unclear [376, 380, 381, 387, 388]. In **paper II**, we found that ARG1 protein are present in the metastasis competent primary tumors and cause local arginine deficiency. We further identified that arginine restriction in breast cancer cells with metastatic ability accelerates the autophagy process and induction of selective autophagy receptors. Concurrently, arginine restriction also potently dampened the expression of IFN-I response related proteins. These findings indicate a novel role of arginine deprivation by downregulating IFN-I response and contribute to immune escape of aggressive cancer. Our results in the invasive human breast cancer cell lines support this notion. In **paper III**, we present experimental data that suggest that the expansion of these characteristics myeloid cells with high expression of *Arg1* may also occur in cancer cachexia (**paper III**). We report an association among ARG1 expression, weight loss and survival in cancer patients, implying a role of ARG1-producing cells in cachexia. In line with this possibility, we find that arginine restriction in muscle cells, enhanced expression of autophagy-related proteins and stimulate the autophagy process, as observed in the breast cancer cells. With these findings that are linked together in a unique way, we have examined why some tumors appear as "never healing wounds" with suppressed immunity.

4.1 Choosing "an appropriate" model

Metastasis is a primary cause of (breast) cancer related mortality. It is therefore crucial to understand the molecular pathways that are altered during metastatic tumor development. Identifying these mechanisms facilitate the expansion of new treatment targets that help in personalized treatment. The IFN-I signature is an example of how our studies in the 4T1 breast cancer model can be extended to a clinical significance. Therefore, choosing an appropriate model is important to perform studies that allow understanding of tumor-host interactions that are clinically relevant. The first study in the thesis involves comparison of the cells in culture and primary tumors formed by the non-metastatic 67NR cell line and the metastatic 66cl4 cell line from the 4T1 breast cancer mouse model. The use of this model allowed to perform bulk transcriptome analysis of the cancer cells under controlled conditions in culture and their respective primary tumors, further validated by proteomics in 67NR and 66cl4 primary tumors.

To obtain an understanding of the tumor-host interactions that occur during cancer development and metastasis, it is crucial to use models with a complete immune system. We chose the 4T1 model because it is immunocompetent and represents a range of metastatic abilities, allowing us to study metastasis as it may occur when tumor-host interactions are operable. In addition, this is an orthotopic model making it more "disease relevant" in assessment of breast tumor growth that grows inside a breast tissue. To translate our findings from this murine model to humans, we first validated in invasive and noninvasive human breast cancer cell lines, before examining the clinical relevance by using publicly available databases, that provides data from thousands of patients. Nevertheless, all disease models, including this model, is restricted by certain limitations. For example, the lower transfection efficiency in the cells

in the model (especially 66cl4) led to difficulty in performing experiments involving transient transfection. We observed substantial heterogeneity between subclones generated from these cancer cells. This heterogeneity made it very difficult to conclude on putative phenotypic changes after different types of genetic manipulation. In addition, even though human and mice have similar risk for cancer development, huge differences in phenotype, karyotype and tumor origin are observed between human and mouse cancers. The inflammatory responses can also be totally different between mice and human tumors [483, 484]. Therefore, validation of data obtained in mouse models with human models are important.

4.2 Amino acid metabolism: a key player in innate immune response

Amino acid levels can control transcription, protein translation and organelle biogenesis since amino acid availability can be sensed by regulatory kinase like mTOR [485]. Recent reviews have focused on the metabolic regulation of various immune cells, role of mTOR signaling and autophagy in the immune system [374, 486]. Amino acid auxotrophy, a need for external supplementation of amino acid may have evolved as an immunoregulatory control mechanism [125, 487]. This is because amino acids not only provide building blocks and energy to fuel cells but also function as modulators of signaling pathways. Reprogramming energy metabolism, an established hallmark of cancer, is an important event in tumorigenesis and therefore have been extensively studied. Only cancer cells that withstand metabolically harsh conditions can survive and metastasize [40]. In tumor metabolism, where the cancer cells constantly demand high nutrient supply, even specific amino acid levels can have an important regulatory role. However, it is difficult to interpret the exact consequences of amino acid starvation within the TME, for cancer cells and the host. For example, tumors containing myeloid cells with suppressive function produce enzymes like arginases (ARG1 and ARG2), reduce arginine levels, and increased ARG1/ARG2 activity correlates with poor clinical outcomes [125, 363]. This could be because certain immune cells, specifically T cells are inhibited upon arginine deficiency, beneficial for the cancer cells but bad for the host. In such circumstances, amino acid supplementation therapy could have beneficial effects for the host. At the same time, in cancer cells with higher metabolic needs, supplying amino acids might help them to efficiently survive, leaving with poor consequences for the host. In our model, we find that reduced arginine levels have a positive function for cancer cells, as it results in dampened IFN-I response, and thus facilitates immune escape. Yet, it is also conceivable that the cancer cells experiencing this restriction face metabolic challenges like reduction in mitochondrial functions. Nonetheless, they still manage to survive, possibly since arginine is largely recycled in the cells because of increased autophagy activity. In these conditions, therapeutic interventions that lead to increased arginine levels can have both better and poor outcomes.

In **paper II**, we found that the primary tumors that can metastasize contain increased levels of ARG1 protein potentially produced by myeloid cells with an immune suppressive function. We also have

observations that the population of immature myeloid cells that are positive for ARG1 have functionally active ARG1, as reflected by an increased arginine/ornithine ratio (unpublished data). Arginine is a semi essential amino acid but becomes essential in the cells where ASS1 is silenced. Cells that lack ASS1 expression are unable to synthesize their own arginine from citrulline and aspartate [344]. The transcriptomics performed in murine breast cancer cells 66cl4 and 67NR showed very low *Ass1* mRNA [488]. Using data from The Cancer Genome Atlas (TCGA), Cheng et al. showed *ASS1* gene expression was downregulated across multiple cancer types including breast cancer. They also characterized the human breast cancer cell line MDAMB231 as *ASS1* negative [358]. To confirm this, we investigated the CCLE database and noticed that MDAMB231 cells indeed express very low levels of *ASS1* mRNA [489]. Thus, the minimal *ASS1* expression in such cancer cells likely make arginine to function as an essential amino acid in these cells, where they depend on external arginine supplementation, a mechanism called arginine autotrophy. However, we do not know if cells with minimal *ASS1* expression are truly arginine auxotrophs and are still sensitive to arginine restriction, or whether the limited ASS1 expression is sufficient to exert some arginine-generating function. We also do not know if *ASS1* expression can be activated in *ASS1*-negative cells under different circumstances, such as in arginine-deprived conditions. This probably will depend on how they are silenced. We tried to mimic the *in vivo* tumor conditions by depleting the arginine levels in breast cancer (**Paper II**) and muscle cells (**Paper III**) *in vitro* and looked for potential pathways affected. We found that arginine restriction had a significant effect both mitochondrial functions, autophagy (**Paper II and III**) and IFN-I expression (**Paper II**) in these cells. This shows that, at least in our experimental settings, a putative ASS1 upregulation in response to arginine-deprivation is not sufficient to block the effects on these processes.

The cell culture conditions differ from the TME in many ways. In our study in breast cancer and muscle cells, we used varying concentrations of arginine ranging from the normal concentration in this culture medium; 400µM or 700µM (depending on cell line) as control, and 40 µM, 16µM, 4 µM and 0µM, respectively, for various degree of arginine restriction. In humans, it is reported that the normal arginine concentration in blood is in the range of 21-137µM [490, 491]. In fact, we do not know the local concentration of arginine in specific tissues or in tumors and if the concentration varies by blood supply and distance to the closest blood vessel. The control concentration selected here is possibly different than the actual concentration present *in vivo*. However, we do not know how rapid arginine is used up by the cells during culturing. Moreover, complete arginine depletion may not be physiologically relevant in tumors or any tissue. Instead, there may be limited arginine levels. But these limited arginine conditions may persist over long periods of time. In our experiments, we have tested for responses in short time frames, meaning up to 48 hours. In growing tumors, the cancer cells grow and develop in close interplay with its TME over weeks, months, and years. Even if the concentrations and time frames are difficult to compare, we found that the results obtained upon complete arginine restriction (0µM)

and limited arginine restriction (4μM) were similar. This shows that complete arginine depletion is not necessary to observe the phenotype.

4.3 Arginine restriction as a signal for autophagy induction

Intracellular arginine levels are known to be sensed by the amino acid transporter SLC38A9, in the lysosome and CASTOR1 [368, 370]. In absence of arginine, CASTOR1 interacts with GAP Activity Towards Rags complex 1 (GATOR1) which inhibits mTORC1 possibly enabling activation of autophagy [492]. We therefore expected elevated autophagy upon arginine restriction. We started with an open question; do we find signs of any change in autophagy receptors? Indeed, we found that arginine restriction in the murine metastatic 66cl4 cells caused a striking induction in *Sqstm1* mRNA and a slight, but significant, increase in *Tax1bp1* (**Paper II**). The *Sqstm1* mRNA was also slightly induced after lysine restriction and after complete amino acid restriction. However, compared to the responses to arginine restriction, the increase in *Sqstm1* was minimal after lysine or complete amino acid restriction. Only amino acid degrading enzymes like IDO and ARG1 exclusively have emerged as key players in the regulation of tumor-induced immune tolerance [493]. Therefore, in cancers or normal tissues, lysine and complete amino acid restriction is not of physiological relevance. It is however quite striking that we observed a higher induction in *Sqstm1* transcript with arginine restriction compared to complete amino acid restriction where arginine is deprived along with other amino acids in the medium. The absence of arginine is sensed via a sensor system that is specific for arginine opposite to what happens upon absence of all amino acids. It could very likely be that several other mechanisms are involved when the cells experience complete amino acid restriction. However, one could also expect arginine sensors are also activated upon complete amino acid restriction which needs further investigation.

Along with increased transcripts, we also identified SQSTM1 as an early protein to be induced upon arginine starvation. This was opposite of what might be expected, that when cells are starved for semi-essential amino acids like arginine, the cells would normally degrade the proteins to overcome the nutrient stress. Intriguingly, SQSTM1 induction after arginine restriction imply that SQSTM1 is a priority protein to be made to mediate the autophagy process. With prolonged incubation in arginine free medium, other autophagy receptors and proteins were elevated highlighting the role of autophagy upon arginine restriction. The induction of autophagy in breast cancer cells in **paper II** were in line with autophagy induced by arginine restriction in the muscle cells in **paper III**. In muscle cells, both the autophagy process and autophagy receptors like, SQSTM1, TAX1BP1, starch binding domain-containing protein 1 (STBD1) and OPTN were induced upon arginine restriction. Although we find an increase in arginine uptake channels in muscle cells upon arginine restriction, suggesting arginine-restriction in this tissue, we do not know if the arginine levels in the muscle cells are sensed in the same way as in cancer cells.

4.4 Crosstalk between immune suppressive cell, autophagy, and innate immune response

Several recent publications have defined mechanisms that link autophagy and immunity [376-379, 485]. In this context, it is interesting that immune cells control specific amino acid levels and establish an immunosuppressive TME. In the TME, suppression of T cell function by limiting arginine via ARG1 and NOS2 is a primary mechanism of MDSC-mediated suppression [90]. Cells that accumulate intracellular damage due to mutations, downregulated apoptosis or intracellular pathogens, report this to the immune system through activation of the IFN response [494]. In **Paper I**, we extended on understanding the mechanism of cGAS-STING mediated IFN-I regulation in cancer instead of an infection mediated response.

Cancer cells need to escape immune surveillance for their own benefit. This may be particularly challenging for cancer cells accumulating DNA and damaged proteins and organelles in their cytosol. In cancer cells displaying elevated IFN-I response, we focused on putative upstream activators of cGAS-STING pathway and how they are regulated. We (**paper I**) and others have shown that damaged mitochondria and micronuclei are responsible for activation of cGAS-STING axis [210, 227]. The role of autophagy in regulating the IFN-I response is extensively studied in response to pathogens [376, 380, 381, 387, 388]. Interestingly, Gui et al. showed that induction of autophagy via STING is a primordial function of the cGAS-STING pathway. They report that that autophagy induction occurs at earliest stage and is evolutionary conserved which is distinct from IFN induction. They also showed that cGAMP induced autophagy hence is important for clearance of cytosolic DNA and DNA viruses [495]. We also presume that this function of autophagy has evolved prior to the development of advanced, adaptive immune systems. The role of autophagy in removing intracellular pathogens is well appreciated [376, 380, 381, 387, 388, 496-498]. In this context, it is logical to consider autophagy as a central innate immune response since it is the fundamental system for clearing intracellular pathogens. Autophagy mediated protective innate immune system is functional in all cell types and can be anticipated that certain immune cells may have a crucial role in stimulating autophagy in the infected cells, potentially inducing a local nutrient depletion. In such a scenario it can be envisioned a role of autophagy could also be involved in clearing the endogenous DAMPs in the cancer cells. In agreement with this, we found increased autophagy correlated with the dampened cGAS-STING dependent IFN-I response in metastatic cancer cell lines (**paper II)**. We also observed that cytosolic DNA containing micronuclei attracted the autophagy machinery as seen by presence of LC3+ vesicles and SQSTM1 puncta. Our observation supports earlier findings from Santiago et al. demonstrating that autophagy leads to the degradation of the micronuclei via LC3 and SQSTM1 [499]. In another study performed in ASS1 deficient prostate cancer cells, arginine depletion using arginine deiminase induced autophagy in two distinct phases. The conventional autophagy in the early 48 h treatment protected the cells from dying whereas prolonged incubation led to excessive autophagy and giant autophagosome formation

causing chromatin autophagy "chromatophagy" in dying cells [500]. In our experiments, we observed reduced numbers of micronuclei until 48 h arginine starvation where conventional autophagy could be involved in clearance of the micronuclei. However, we do not know what happens upon prolonged arginine restriction since the cells do not tolerate arginine restriction for longer time. It would be interesting to establish if autophagy also differs by the size of the micronuclei and if smaller micronuclei are cleared up more easily than the large micronuclei. Still, we do not know if micronuclei are completely turned over by autophagy. For this, one could generate stable cell lines expressing fluorescently tagged LC3 to monitor the autophagy activity at micronuclei and perform live cell imaging. To monitor and identify areas of micronucleus degradation, histone2B tagged with mKeima, could be used to determine if arginine restriction causes the degradation of micronuclei. It would also be interesting to study if autophagy regulates the upstream signaling proteins in cGAS-STING axis inside a solid tumor. To do so, one could study the turnover of upstream proteins like cGAS, markers of DNA damage, damaged mitochondria if they are altered upon autophagic activity inside the tumor. Unfortunately studying these processes *in vivo* comes with many limitations and challenges that needs to be addressed.

Using metastatic breast cancer cell lines, we have tried to mimic the conditions that might not completely reflect what exactly happens in the tumor tissue. It would be therefore important to measure autophagy activity in the tumor to determine if ARG1 expressing tumors have higher autophagic activity. This could be done by measuring the autophagy activity *in vivo* by using fluorescent proteins like GFP-mCherry fused to autophagy markers; SQSTM1 or LC3 in mouse models. Even though better strategies have been developed, technical limitations still exist during flux measurement in animal models. This is basically because variation exists, and autophagy is induced in different animals at different time points [501]. Therefore, this should be optimized depending upon the specific tumors to be tested. SQSTM1 is known to exert negative control in IFN-I regulation where SQSTM1 knockout induced IFN-I production in mouse embryonic fibroblasts [387]. However, we did not find involvement of SQSTM1 in the negative regulation of the IFN-I signaling in the human breast cancer cells. This could be explained by the inability of siRNAs to completely abolish the SQSTM1 in the cells, where the remaining SQSTM1 protein could exert sufficient function. In addition, other autophagy receptors might exert regulation and compensate when SQSTM1 is downregulated/lost. The shRNA SQSTM1 mediated knockdown studies in murine 66cl4 cells, were inconclusive because of heterogeneity between the different clones (data not shown). Clonal selection and generation of pool from a single clone would be better to perform knockdown/knock out studies in the difficult cells. In fact, it could also be true that SQSTM1 does not exert immunoregulatory function in all models. Study by Zach et al. showed that SQSTM1 knock out mice accelerated the osteoclastogenic process and lead to Paget's disease of bone like phenotype [502]. However, no autoimmune disease upon SQSTM1 knock down was addressed by the authors, suggesting that autophagy might not have immune regulatory function.

In our study, we indeed found that ATG13, an important protein in the core autophagy process, negatively regulated IFN-I production as well as signaling under both arginine rich and restricted condition supporting the role of autophagy in IFN-I regulation in cancer.

4.5 MDSCS: An emerging player in cancer Cachexia

Our findings from paper I and II, indicate that cancer is associated with a specific increase in immune cell population. We think that these immune cells also play a crucial role in cancer cachexia. These specific immune cells migrate from the bone marrow and are released in the bloodstream. With this ability, these cells can enter the muscles and reshape the muscle microenvironment. We also know that the arginine homeostasis that these immune cells affect can increase autophagy activity in cancer cells. A systemic increase in autophagy is also seen in cachexia [427]. In paper III, we therefore asked whether these immune cells also enter the muscle tissue of cancer patients who develop cachexia. Recent studies have suggested the involvement of arrays of immune cells including neutrophils, macrophages, MDSCs in cachexia patients and animal models [433]. Despite increasing reports about the role of immune cell involvement in maintaining muscle homeostasis, our knowledge regarding their function in cancer cachexia is limited. **In paper III**, datamining of the RNA sequencing performed in three cachexia mouse models (4T1, C26 and LLC) [503, 504], we found an increase in markers of neutrophil and MDSCs in cachectic muscles attributing an expansion of these cells in cachexia. ARG1 expression is characteristics of myeloid cells [125]. We therefore checked if ARG1 expression had any correlation with pathogenesis in patients with cancer cachexia. Our results from the ARG1 in plasma showed elevated levels of ARG1 in cancer patients with weight loss compared to the healthy control and weight stable cancer patients suggesting infiltration of ARG1 positive cells in cancer cachexia.

Despite extensive increase in several MDSC and neutrophil markers, we do not find elevated *Arg1* mRNA expression in cachectic muscles from any of the three cachexia mouse models studied here. Since ARG protein is formed early during neutrophil development and that we are therefore unsure how well the mRNA levels reflect the actual protein level in the muscle. As discussed in **paper III**, further studies are needed to perform proper assays like measurement of specific arginine levels in muscle or more specifically assessment of ARG1 activity in identifying the potentiality of MDSCs in muscle cells. Nevertheless, this is also not straight forward. Even so, if we expect increase in ARG protein, a protein extract from the muscle will probably be dominated by the proteins that arise from the muscle cells which might make detection even more difficult. In this case, performing immune staining to detect ARG protein in muscle would be a better approach. Quantifying arginine and ornithine could be also beneficial to identify the ARG activity. In fact, studies have been carried out that have analyzed arginine and ornithine levels in cachectic muscles. However, it must be remembered that what has been analyzed is a sum of extracellular and intracellular muscle content. It is also known that arginine levels in some cellular compartments can be very high and are probably buffered by autophagy. A measurement of the

amino acid level in the extracellular muscle environment therefore will be accurate that represents arginine availability in the tissue. Computed tomography-based approaches has been successful in identifying organ volume changes during cachexia in *Drosophila melanogaster* models [505]. Still, determining specific amino acid levels in extra cellular space in animal models have not been identified. This should be focused on further studies.

Linking the findings from **paper II** and **III**, we hypothesized that the circulating immune cells containing ARG1 with arginine degrading activity in the local muscle environment could also alter local arginine levels in the muscle. In cachexia, as described by extensive loss of lean muscle mass, the role of autophagy in cancer induced muscle wasting has been extensively studied. For example, skeletal muscle samples from cachectic cancer patients and cachexia mouse models have shown increased levels of autophagy markers like Beclin-1, LC3B-II, SQSTM1 etc. [457, 462, 463]. Our research group and others have well acknowledged the involvement of autophagy in degradation of muscle content in cachexia [426, 427, 465]. Together with our findings from **paper II,** this led us to ask if arginine restriction also induce autophagy of muscle cells. As in breast cancer cells, arginine restriction increased the expression of autophagy-related proteins and induced the autophagy process in muscle cells. In line with studies showing reduced mitochondrial functions and ATP production in cachectic muscles we found reduced mitochondrial function in muscle cells that are depleted for arginine [506]. In addition, we observed reduction of inner mitochondrial membrane protein suggesting that damaged dysfunctional mitochondrial are degraded via mitophagy. However, it is necessary to perform mitophagy flux assays to confirm this notion. One could generate reporter muscle cells with LDHB-mKeima and mt-mKeima as performed in **paper II**. Our results suggests that expansion in the number of ARG1 expressing immune cells may be causally involved in elevated catabolism and reduced mitochondrial function in muscle during cancer cachexia. To further confirm the potential effect of ARG1, studies using myeloid-specific knock out of ARG1 mouse model would be beneficial. With this, we could learn if ARG1 knock out mice can confer resistant to cachexia development. In parallel, the commercially available ARG1 inhibitors could also be used to see if ARG1 inhibition regulates cachexia progression *in vivo*.

4.6 The future of Arginase and autophagy inhibitors

To develop future therapeutic strategies, we need to uncover the mechanisms that can reactivate the inflammatory signaling in the TME and turn the immunologically “cold” tumor to “hot”. IFN based therapies using recombinant type I IFNs and IFN inducers like TLR agonists, STING agonists, chemotherapeutic and targeted drugs have shown promising results in clinical trials [507-509]. Nevertheless, these strategies still have limitations, either associated with severe side effects or increased production of immunosuppressive cells. Studies have also shown that combining IFN-I and immune checkpoint-based therapies are not suitable for all cancer patients [508]. From our findings in **paper I, II and III** and unpublished data from the group, we argue that increasing arginine levels would

be beneficial in both breast cancer patients and cachexia. It is known that many cancer patients undergo immune suppression and have lower plasma arginine levels [127, 510, 511]. Increased ARG expression and activity is observed in many cancers, reporting its role in tumorigenesis [116-118]. In accordance with this, our observations demonstrate that infiltrating ARG1-positive myeloid cells were abundant in metastatic-competent tumors and higher ARG1 levels correlated with a large cohort of human breast cancer (unpublished data). This indicates that ARG1 could be used as a potential prognostic marker to predict patient outcomes.

In paper I, we identified that IFN-I is dampened in the tumors with metastatic ability and lower IFN expression correlates with poor patient outcome in TNBC breast cancer patients having aggressive tumors. Our findings presented in **paper II** with autophagy mediated dampening of IFN-I response in the cancer cells also highlight the benefits of raised arginine levels in the tumors. The presence of myeloid cells in cachexia mouse models and higher ARG1 levels in plasma of cachectic patients add to the prognostic value of ARG1 in systemic disease like cachexia. We therefore suggest targeting both ARG1 and autophagy to establish an anti-tumor immune response and counteract muscle wasting as an attractive approach for future treatment.

Both arginase and autophagy inhibitors have been used separately and have demonstrated beneficial effects [116, 512, 513]. In accordance with this, studies using mouse models where ARG1 is pharmaceutically targeted using nor-NOHA or CB-1158 have shown to reduce tumor growth in mice [511, 514]. Study by Steggerda et al. additionally showed blocking MDSC-mediated ARG1 with CB-1158 increased tumor and plasma arginine levels in syngeneic mouse models. In addition, it reversed the ARG1-mediated suppression of T cell proliferation, increased inflammatory cytokines and increased IFN responsive transcripts [511]. This was in full agreement with "turning an immunologically cold tumor to hot" by using ARG1 inhibitor. At the same time, targeting arginase systemically can be challenging due to importance of the urea cycle in maintaining the proper cellular functioning and wound healing properties. Despite this, arginase inhibition is shown to be well tolerated in several animal models [514, 515]. Moreover, arginase inhibitors in clinical trials have shown beneficial effects [516]. However, the arginase inhibitors in clinical trials are mostly in phase I and II, but phase III trials as monotherapy have not reached intended goals [361].

Like ARG1 inhibitors, recent studies have also shown the importance of autophagy in switching the cold TME to hot, hence increasing the response to immunotherapy [517, 518]. The US Food and Drug administration (FDA) accepted autophagy inhibitors chloroquine (CQ) and hydroxychloroquine (HCQ) have shown promising effect in some cancer patients [517]. Several Phase I/II trials are determining the safety and efficacy of CQ and HCQ as both monotherapy and a combination therapy. CQ and HCQ when combined with chemotherapeutic drugs and radiation enhanced the efficacy of tumor cell killing in patients with solid tumors [517, 519]. A recent study showed the beneficial effect of the combination

therapy both *in vivo* and *in vitro* using HCQ and 2-Deoxyglucose (2-DG), a glucose analogue that inhibits glycolysis [520, 521]. This potential combination therapy has also shown reduced tumor size and metastasis in liver and lungs in both the 4T1 mouse model and CMT-7364 canine model [519]. However, CQ and HCQ showed minimal effect as monotherapy in breast and pancreatic cancer patients [522, 523] but the long-term use in pre- and post-operative cancer patients showed better clinical outcomes [522, 524-526]. Since both arginase and autophagy inhibitors alone have not gained expected outcomes, we suggest combination therapies using ARG1 and autophagy inhibitors could aid to reprogram an aggressive tumor microenvironment. However, it is essential to note the timing of treatment to avoid severe side effects because of context dependent role of autophagy in cancer. For example, early administration of autophagy inhibitor can induce apoptosis whereas the late inhibition can activate a pro survival mechanisms thereby enabling cancer cells to rescue cell death [527]. Thus, future strategies to combine arginase and autophagy inhibitors demands improved mechanistic understanding.

Arginine supplementation therapy has also shown to have beneficial effect for cancer patients. In a study involving 96 breast cancer patients, arginine supplementation led to increased pathological response compared to the healthy controls indicating arginine supplementation might have an anti-tumor effect in cancer patients [528]. However, arginine supplementation also comes with some side effects. The oral arginine supplementation led to several gastrointestinal disorder and excess arginine metabolism by the intestinal mucosa [529-531]. Therefore, the use of ARG1 inhibitors has potential in maintaining arginine levels in patient without having gastrointestinal disorders compared to dietary supplementation. In addition, citrulline supplementation could be a better option because of its higher stability and is known to be more efficient at increasing arginine availability in mice [532]. But gastrointestinal and other side effects should be taken in account if there is any. Even if arginine or citrulline supplement were to be given, it is unknown whether this leads to an increased arginine level in tissues that are enriched with ARG-positive immune cells, which continuously degrade arginine.

In cachexia, the underlying mechanisms are incompletely understood, and no treatment regimen has been shown to successfully reverse cachexia, and no cachexia drugs are approved by the FDA. Oral treatment regimens like Anamorelin, a gherlin receptor agonist, that improves appetite were approved for certain cancers in Japan [533]. Anamorelin was not approved in Europe since it showed a marginal effect on lean body mass but no proven effect on strengthening the grip of patients and improved quality of life [534]. Cachexia therefore is still an unmet medical need, where no strategies targeting autophagy or immune cells for reduction of muscle mass are clinically tested. In fact, iron supplement in cachexia mouse models and patients have shown to prevent muscle loss, suggesting important mechanism to avoid cancer induced muscle atrophy [535]. It is therefore tempting to speculate that ARG and

autophagy inhibitors alone or in combination can achieve clinical efficacy in cancer patients with cachexia.

5 Future Perspectives

As a tumor progresses different cell types, including immune cells, infiltrate the tumor. Large clinical studies have shown that both the number and type of infiltrating immune cells have therapeutic and prognostic implications [536, 537]. Many studies have focused on interaction between the primary tumor and its microenvironment [538, 539]. The role of infiltrating immune cells, especially neutrophils in tumor progression, remains largely uncharacterized. Nevertheless, cancer associated neutrophilia and increased numbers of neutrophils in tumors have been associated with poor prognosis in many cancers, suggesting their potential function [446, 540]. A preclinical study has shown that neutrophils bodyguard circulating tumors cells (CTCs) in the bloodstream and expand their metastatic potential. The CTCs associated with neutrophils were highly proliferative and efficient in metastasis formation. RNA sequencing analysis in CTCs associated neutrophils from patients or orthologous mouse, showed elevated expression of *ARG1*, *CXCR2* and several chemokines [446]. In line with this, unpublished data from our group show that metastatic cancer cells display elevated expression of CXCL3. CXCL3 is a chemoattractant for neutrophils that signals through the CXCR2 receptor, which subsequently drive mature neutrophils into circulation [541]. This pinpoints the unique capacity of the metastatic cancer cells is not only restricted to recruiting neutrophils but also utilizing neutrophils to efficiently establish metastasis. It has been shown that the neutrophils escort the CTCs into the distant organs where the MDSCs or neutrophils can establish a premetastatic niche [542]. Hence, it would be interesting to study what are the potential phenotypes of these recruited neutrophils and how they can aid in metastasis in addition to being established in the tumors. The presence of neutrophils and MDSCs in tumors have been known for years, but their functional role in tumor progression is still incompletely understood. Functional studies of these cells are limited by their fragility and by their tendency to be activated during sample preparation. Together, this might lead to misleading results not exactly mimicking the real situation inside a solid tumor or other areas in the body. In addition, after mature neutrophils leave the bone marrow, they have a short half-life and contain very low mRNA levels [541, 543]. This makes such cells challenging to characterize through gene expression-based studies. Therefore, new methods for sensitive proteome characterization will improve our understanding of the interplay between cancer cells and neutrophils. Identifying the putative roles of MDSCs and neutrophils in metastasis progression could also lead to development of new cancer therapies.

The results presented in this thesis indicate an interplay between cancer cells, infiltrating immune cells and autophagy in the TME. Recently, it was published that more than 60% of all breast cancers have a microbiome of intracellular bacteria that is richer and more diverse compared to normal breast tissues. This very surprising finding that may represent a new dogma for our understanding of peripheral immunity, but also indicate dampened immune reactions in the tumor [544]. It is likely that the infiltration and activity of the immune cells are a consequence of the acquired features of the cancer cells. Based on our recent observations, increased infiltration of ARG1 containing cells in the metastatic

tumors alter autophagy in the cancer cells which subsequently leads to suppression of local immunity. Our study identifies a unique ability of the infiltrating immune cells to tune local arginine availability, induce autophagy and negatively regulate the innate immune response. As discussed in **paper II**, SQSTM1 stands out as an important autophagy receptor to be affected upon arginine restriction. However, SQSTM1 was not crucial for regulating the IFN-I response in the metastatic cancer cells. Understanding the detailed mechanism involved in increased *Sqstm1* transcription upon arginine restriction would help to identify its potential role in regulating immune reactions inside a tumor, if any. A recent study in Zebrafish model show that certain bacteria may use neutrophils as a proliferative niche, and neutrophils can use Sqstm1 to target for bacteria's subsequent degradation [545]. Identifying if neutrophils and SQSTM1 are interrelated in solid tumors would also be interesting.

ASS1 silencing is a unique phenotype observed in many cancer cells [546]. The cells used in this study had minimal to negligible ASS1 expression, making them dependent on external arginine, and were sensitive to arginine restriction. It would therefore be interesting to overexpress ASS1 in these cells to see if this reduces the response to arginine restriction. Interestingly, a recent study in hepatocellular carcinoma (HCC), demonstrated that ASS1 overexpression increased sensitivity in HCC spheroid cells to chemotherapeutic agents [547]. However, we would expect that the immune system would get overactivated and might have extreme consequences upon ASS1 overexpression.

6 Conclusion

Even with progress in the diagnosis and treatment of breast cancer, there is still little or no cure for metastatic cancers. This demands a new and more personalized therapy to improve survival and reduce side effects. Recent technological advances have finally opened for detailed characterization of the infiltrating immune cells and studies of how cancer cells control their presence and phenotype. Studying the TME closely is therefore needed to better understand cancer progression. It is also crucial to understand how patients with similar tumors, respond differently to treatment. The results of this project have added to our understanding of the mechanisms underlying aggressiveness in breast cancer to reduce over treatment of those with favorable prognosis and to indicate new therapeutic strategies for those with poor prognosis.

The three studies presented in this PhD thesis are well connected, suggesting a novel mechanism where the myeloid cells with immune suppressive function can infiltrate the tumor and muscle microenvironment and regulate arginine availability in the tumor and muscle. Targeting these cellular mechanisms may improve innate immune response in the aggressive breast cancer patients as well as reversing muscle atrophy in systemic cachexia. Our results add to the increasing knowledge of the interplay between immune cells, innate immune response, autophagy, and cachexia. Altogether, this work also adds to the understanding of mechanisms underlying tumor aggressiveness as well as provide new markers for targeting aggressive cancers and cancer cachexia.

7 References

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Paper I

Lamsal *et al.*
Cell Communication and Signaling (2023) 21:50
https://doi.org/10.1186/s12964-023-01062-y

Cell Communication and Signaling

RESEARCH Open Access

Opposite and dynamic regulation of the interferon response in metastatic and non-metastatic breast cancer

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Abstract

Background To our current understanding, solid tumors depend on suppressed local immune reactions, often elicited by the interaction between tumor cells and tumor microenvironment (TME) components. Despite an improved understanding of anti-cancer immune responses in the TME, it is still unclear how immuno-suppressive TME are formed and how some cancer cells survive and metastasize.

Methods To identify the major adaptations that cancer cells undergo during tumor development and progression, we compared the transcriptome and proteome from metastatic 66cl4 and non-metastatic 67NR cell lines in culture versus their corresponding mouse mammary primary tumors. Using confocal microscopy, RT-qPCR, flow cytometry and western blotting, we studied the signaling pathway and the mechanisms involved. In addition, we used public gene expression data from human breast cancer biopsies to evaluate the correlation between gene expression and clinical outcomes in patients.

Results We found that type I interferon (IFN-I) response was a key differentially regulated pathway between metastatic and non-metastatic cell lines and tumors. The IFN-I response was active in metastatic cancer cells in culture and markedly dampened when these cells formed primary tumors. Interestingly, the opposite was observed in non-metastatic cancer cells and tumors. Consistent with an active IFN-I response in culture, the metastatic cancer cells displayed elevated levels of cytosolic DNA from both mitochondria and ruptured micronuclei with concomitant activation of cGAS-STING signaling. Interestingly, decreased IFN-I-related gene expression in breast cancer biopsies correlated with an unfavourable prognosis in patients.

Conclusion Our findings show that IFN-I response is dampened in the tumors with the metastatic ability and lower IFN-I expression predicts poor prognosis in triple-negative and HER2 enriched breast cancer patients. This study highlights the possibility of reactivating the IFN-I response as a potential therapeutic strategy in breast cancer.

Keywords 4T1 model, 66cl4, 67NR, IFN-I, Metastasis

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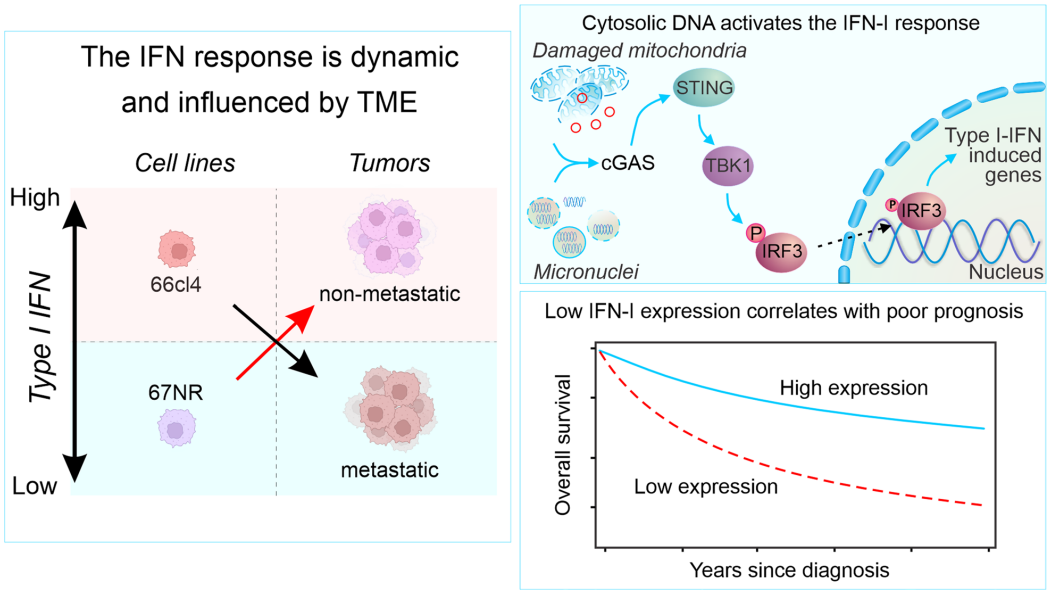
Graphical abstract



Background

The interaction between cancer cells and the tumor microenvironment (TME) profoundly impacts tumor development by influencing processes that lead to either tumor eradication or tumor progression and metastasis [1–4]. In a solid tumor, the transformed cancer cells co-evolve with the TME, which includes fibroblasts, endothelial cells and infiltrating immune cells, blood vessels, signaling molecules, secreted factors, and extracellular matrix [5]. Immune cells are essential components of the TME since a proper antitumor immune response will destroy the transformed cancer cells, while a protumor immune response may support tumor growth and metastasis. Cancer cells can avoid immune recognition by actively suppressing antitumor immune responses by releasing anti-inflammatory cytokines, recruiting immunosuppressive immune cells, and shaping the TME towards a more permissive state [6–14].

Interferons (IFNs) have a crucial role in the immune response against infections, intracellular pathogens, and cancer cells. These proteins are released by infected or transformed cells and activate the immune response that promotes cytokine production, natural killer cell functions, and antigen presentation [15, 16]. Type I IFNs (IFN-I), the largest class of IFNs, have a pivotal role in cancer prevention, inducing anti-tumor immunity [17]. Downregulation of IFN-I response prevents $CD8^+$T cell-mediated recognition and elimination of tumor cells. For instance, loss of the type I interferon receptor chain (IFNAR1) in colorectal cancer models led to aggressive cancer growth, while the activation of IFN-I response increases the $CD8^+$T cell effector function and tumor regression [10, 17, 18]. In breast cancer models, downregulation of interferon regulatory factor (Irf7) target genes was associated with increased bone metastasis and reduced survival in this model. On the other hand, high expression of Irf7 regulatory genes correlated with increased metastasis-free survival in more than 800 patients studied [19].

IFN-I expression can be induced by activating the cGAS-STING pathway, which induced tumor regression in breast, colon cancer and melanoma mouse models when STING agonists were administered [20–24]. Moreover, STING agonists are currently used in clinical trials in combination with chemotherapy or Programmed Cell Death Ligand 1 (PDL1) antibodies highlighting the importance of IFN in cancer treatment [25]. However, a better understanding of the mechanism that controls IFN-I responses and its relationship

in the TME components is needed to extend the success of this combined therapy.

Despite our improved understanding of anticancer immune responses in the TME, it is still unclear how immuno-suppressive TME are formed and how some cancer cells survive and metastasize [26]. We aimed to identify specific adaptations of metastatic cancer cells that enable them to grow in the TME, avoiding immune response and facilitating tumor progression and metastasis. We used cell lines derived from the well-established metastatic mammary carcinoma model 4T1 with different metastatic capacities. We used 66cl4 cells that metastasize to the lungs after injection into the mammary fat pad of mice, while 67NR cells do not metastasize [27]. Using the unbiased approach of transcriptomics and proteomics to compare metastatic and non-metastatic cancer cells grown in culture versus their corresponding tumors, we showed that the IFN-I response differed. Specifically, we found a significant dampening in the IFN-I response in the metastatic tumors compared to the cells in the culture. In contrast, an increase in the IFN response was observed in the non-metastatic tumor. In addition, we found that elevated IFN-I response in metastatic cancer cells was related to the high cytosolic DNA levels and activation of their sensor system. Our results suggest that factors in the TME enable metastatic tumors to silence their IFN-I response, thus avoiding the antitumor immune response. Hence, a better understanding of the mechanism used by metastatic tumors to dampen the local IFN-I signaling could lead to novel targeted therapies to reactivate local immune reactions and boost responses to conventional therapies.

Methods

Cell lines and cell culture

67NR and 66cl4 cells, obtained from Barbara Ann Karmanos Cancer Institute, and MDAMB453 and MDAMB231 cells, kindly provided by Dr. Kaisa Lehti were cultivated as described in Additional file 1: Methods.

Transcriptome analysis

RNA from 66cl4 and 67NR cells, as well as from 66cl4 and 67NRprimary mammary tumors from BALB/cJ mice were isolated and sequenced as described before [28] and analyzed as in Additional file 1: Methods.

Mice experiments

Eight- to twelve-week-old female BALB/cJ mice were obtained from Janvier Labs, France. The tumors were initiated and resected and processed as in Additional file 1: Methods.

Mass spectrometry analysis

Proteins were isolated from 66cl4 and 67NR mammary breast tumors from mice by homogenization in lysis buffer and analyzed by LC–MS/MS as described in Additional file 1: Methods. The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [29] partner repository with the dataset identifier PXD037288.

Quantitative real-time PCR

Total RNA from cells in culture was extracted using RNeasy Mini Kit (Qiagen). RNA concentration and purity were measured by Nanodrop. cDNA synthesis followed by qPCR was performed as described in Additional file 1: Methods.

Immunoblotting

Cells were harvested in 8 M urea lysis buffer (8 M urea, 0.5% (v/v) Triton X-100, 100 mM DTT, 1 × Complete® protease inhibitor (Roche) and 2 × phosphatase inhibitor cocktail II and III (Sigma)). When indicated, the cells were pretreated with the cGAS inhibitor (Invivogen, # inh-ru521) or the TBKI inhibitors MRT67307 and BX795 (Sigma, #HY-13018 and # HY-10514). Frozen tumor tissues were thawed in urea lysis buffer and homogenized as described under sample preparation and MS analyses in Supplementary Methods. Protein concentration was measured and subjected to western blot as in Supplementary Methods.

ELISA

67NR and 66cl4 cells were cultured in full growth medium for three days until they reached 80–90% confluency. Conditioned medium (CM) was collected, centrifuged, and filtered through a 0.22 µm filter. CXCL10 levels were determined using Mouse CXCL10/IP-10/CRG-2 DuoSet ELISA (R&D systems, #DY466) according to the manufacturer's protocols. The data was analyzed using Microplate Manager 6 (Bio-Rad).

Immunofluorescence

Cells were grown on high precision cover glass until desired confluency, fixed, permeabilized, stained with antibodies specific for cGAS and Lamin A and imaged as specified in Additional file 1: Methods.

Analysis of mitochondrial membrane potential and reactive oxygen species.

Mitochondrial membrane potential (MMP) and production of reactive oxygen species (ROS) were assessed as described in Additional file 1: Methods.

Detection of total and cytosolic mtDNA

Total and cytosolic DNA were isolated from 66cl4 and 67NR cells and subjected to qPCR as described in Additional file 1: Methods.

Use of public databases

Kaplan–Meier plotter [30], Broad Institute Cancer Cell Line Encyclopedia (CCLE), (https://portals.broadinstitute.org/ccle) [31] and cBioPortal [32] were used as described in Additional file 1: Methods.

Statistics

Statistical analyses were performed in GraphPad Prism 9. Values are expressed as mean ± standard deviation (SD) or standard error of the mean (SEM) if not otherwise stated. Details about statistical analyses are specified in the figure legends. p-value < 0.05 was considered statistically significant and is labeled with *, $p < 0.01$ is labeled with **, $p < 0.001$ is labeled with *** and $p < 0.0001$ is labeled with ****.

Results

IFN-I-associated gene expression is suppressed in metastatic tumors

To identify transcriptome dynamics that occur during metastatic tumor development, we compared the RNA-sequencing profile of cell cultures and tumors formed by the metastatic 66cl4 cells. This analysis identified 1859 genes that were differentially expressed between 66cl4 cells grown in culture versus their corresponding primary tumors (log2 Fold Change (FC) > ± 1.5; adjusted p-value < 0.05). Of these, 1537 genes were significantly higher expressed, whilst 322 genes were significantly lower expressed in the 66cl4 tumors (Fig. 1A). To understand the biological processes (BP) linked to the differentially expressed genes, we performed gene ontology (GO) enrichment analysis. This showed that the highly expressed genes in the 66cl4 tumors were associated with inflammation and cell chemotaxis (Additional file 1: Fig. S1A) and that the lower expressed genes were associated with viral defense and IFN-I response (Fig. 1B).

To investigate if we could extend these observations to a non-metastatic tumor, we compared the transcriptomes of the non-metastatic 67NR cell line grown in culture versus its primary tumor. This analysis identified 1084 genes differentially expressed between the 67NR samples (log2FC > 1.5; adjusted p-value < 0.05). Of these, 938 genes were significantly elevated, whilst 146 were reduced in the tumors (Additional file 1: FigS1B). GO analysis for BP revealed that the high-expressed genes were also involved in inflammation and leukocyte migration (Additional file 1: Fig.S1C), while the low-expressed genes were related to RNA metabolism (Additional file 1: Fig.S1D). Together, the results obtained from the 66cl4 and 67NR analyses showed that genes with a lower expression are involved with different BP in the metastatic and non-metastatic tumors, raising the possibility that these signaling pathways are associated with the different metastatic ability of the tumors.

To further understand the dynamic changes in gene expression between cell lines and tumors, we compared the differentially expressed genes in 66cl4 and 67NR, both when grown in culture and when forming primary tumors (Additional file 1: Fig.S2). Compared to the 67NR cells, 411 genes were highly expressed in 66cl4 cells (log2FC > 1.5). Strikingly, 11 genes stood out as being significantly elevated in 66cl4 cells in culture but also among the significantly downregulated genes in the 66cl4 tumors. GO analysis for BP of these 11 genes revealed that they were involved in IFN response, especially in IFN-I signaling (Fig. 1C). Interestingly, these 11 transcripts were amongst those showing low expression in 67NR cells in culture but higher expression in the 67NR tumors (Fig. 1D).

To confirm that the expression of these 16 genes was reduced in metastatic cancer cells following tumor formation, we performed RNA sequencing from isolated non-immune cell-enriched (CD45-negative) and immune cell-enriched (CD45-positive) from 66cl4 and 67NR tumors. This analysis showed a significantly lower expression of these 11 genes in 66cl4 isolated from CD45-negative population enriched with cancer cells compared to (CD45-negative) population from 67NR (Fig. 1E, Additional file 1: Fig.S3A). The reduction in expression of 11 genes was also observed in the CD45-positive population from 66cl4 tumor (Additional file 1: Fig S3B). This suggests that metastatic and non-metastatic cells utilize different strategies to successfully form a tumor, and that alterations in IFN-I signaling stand out as strikingly different.

Type I IFN-associated proteins are lower in the metastatic tumor

To investigate whether the transcriptional differences associated with the IFN-I response in metastatic and non-metastatic tumors correlate with protein levels, we analyzed the proteomes of the 66cl4 (n = 6) and 67NR (n = 5) primary tumors. Principal component analysis of the 5906 detected proteins showed a high degree of similarity between the biological replicates of each tumor (Additional file 1: Fig. S4A). In addition, the tumor samples were separated by tumor type (67NR vs 66cl4) based on the relative abundance of individual

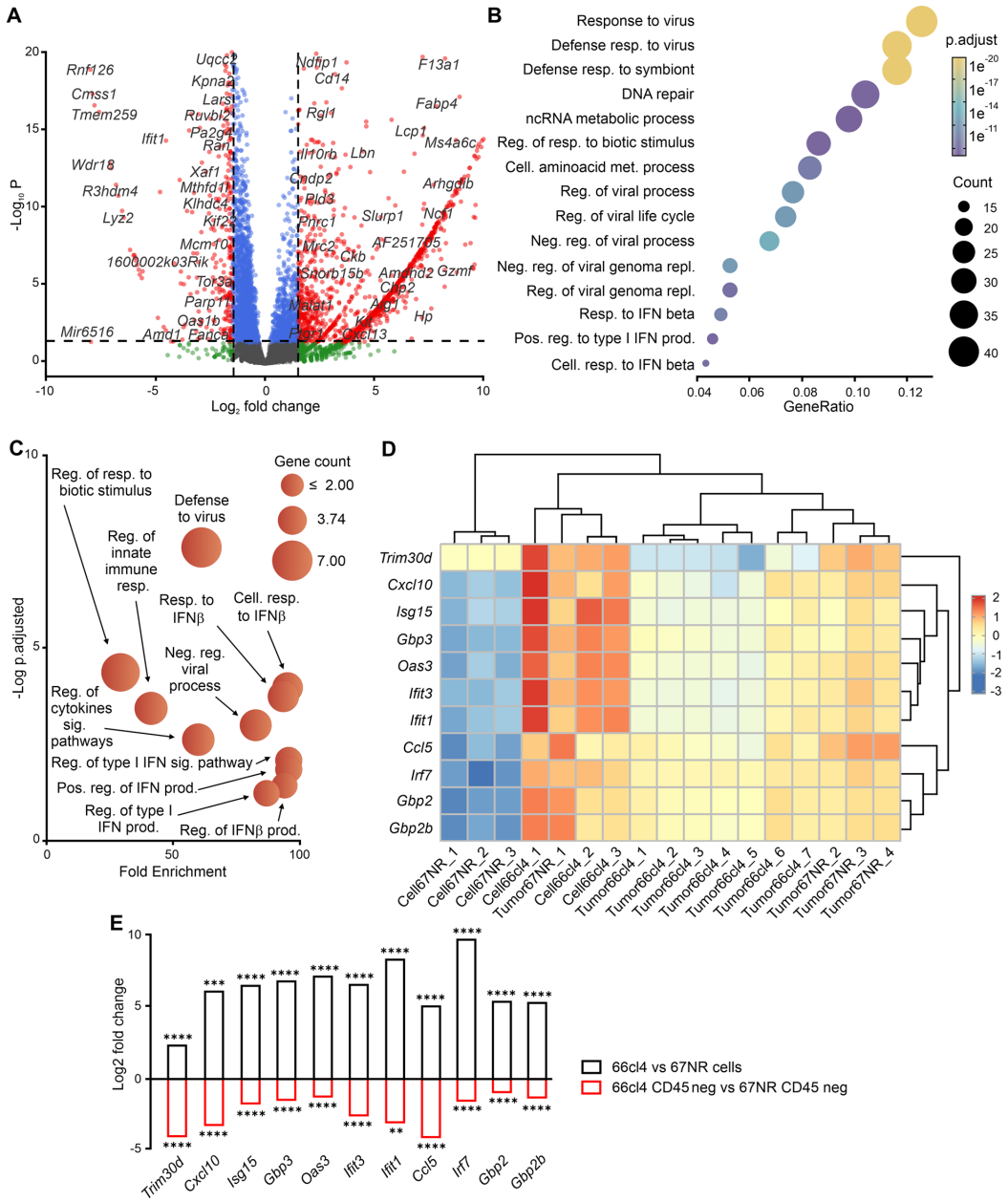


Fig. 1 IFN-I-related gene expression is suppressed in metastatic cancer cells. **A** Volcano plots depicting differentially expressed genes from 66cl4 primary tumors vs 66cl4 cells. Red points represent genes with Log2foldchange within the cut-off (± 1.5) and adjusted *p*-value < 0.05. **B** Gene Ontology (GO) analysis of the most enriched biological processes (BP) associated with the genes with a reduced expression in 66cl4 tumor versus 66cl4 cell line. **C** Gene Ontology analysis of the most enriched biological processes of the 11 genes highly expressed in 66cl4 versus 67NR cells but low expressed in 66cl4 versus 67NR tumors. **D** Heatmap showing transcript per million (after log10 transformation) of the 11 genes that are oppositely expressed in cells in culture and in primary tumors of 67NR and 66cl4. **E** Significantly upregulated IFN-I genes in 66cl4 cells vs. 67NR cells (N = 3) and downregulated genes in 66cl4 (CD45-) vs 67NR (CD45-) population (N = 5) sorted from the primary tumors of 66cl4 and 67NR

proteins, indicating differential protein levels between them (Additional file 1: Fig.S4A). Compared with 67NR tumors, 66cl4 tumors displayed elevated levels of 387 proteins (log2FC > 1.5) and lower levels of 328 proteins (log2FC < − 1.5) (Additional file 1: Fig. S3B). To identify the main biological processes associated with the identified proteins, we performed GO (BP) enrichment analyses of the tumor proteomes. Consistent with the RNA sequencing-based data, primary tumors formed by 66cl4 expressed higher levels of proteins related to leukocyte migration and chemotaxis (Fig. 2A). In contrast, proteins related to adaptive immune response and cytotoxicity were markedly reduced in 66cl4 tumors versus 67NR tumors (Fig. 2B). We also noticed significantly lower levels of interferon beta-associated proteins in metastatic 66cl4 tumors versus 67NR tumors (Fig. 2B), suggesting that reduced transcription of the interferon-related genes in metastatic tumors correlates with a significant decrease in IFN-I associated proteins.

We further examined the proteins associated with the response to interferon beta and other interferon-related proteins selected based on the literature [33]. We found low levels of 14 IFN-related proteins among the less abundant proteins (log2FC < − 1.5) in 66cl4 tumors (Fig. 2C). These were compared with the 11 'oppositely' expressed genes that were identified earlier (high in 66cl4 vs. 67NR cells in culture but low in 66cl4 vs 67NR in tumors), and six proteins were detected by proteomics. Amongst these, three proteins corresponded to transcripts that were dampened in 66cl4 tumors (Fig. 1D):

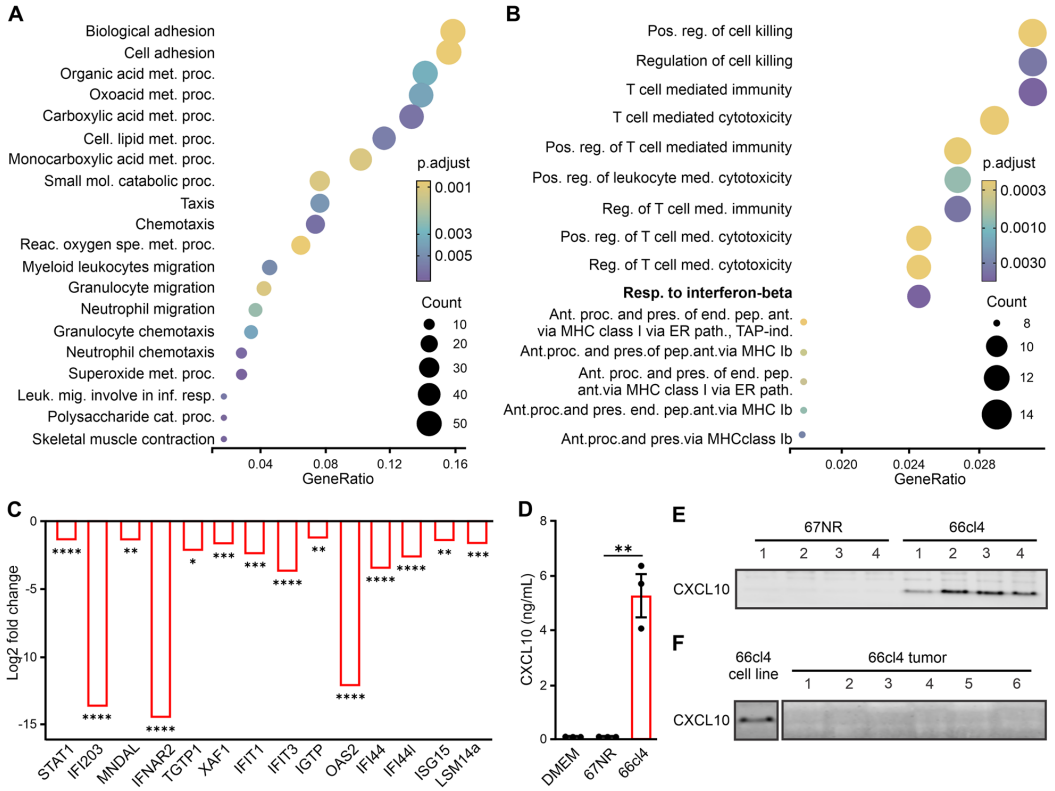


Fig. 2 IFN-I proteins are dampened in metastatic tumors. **A** GO (BP) functional enrichment analyses of proteins with elevated levels in 66cl4 tumors (N = 6) relative to 67NR tumors (N = 5), from MS analysis (log2FC ≥ 1.5, *p*-value < 0.05). **B** GO (BP) functional enrichment analyses of proteins with a reduced expression in 66cl4 tumors (N = 6) relative to 67NR tumors. **C** Significantly downregulated IFN-I proteins in 66cl4 tumors. **D** CXCL10 levels in conditioned medium from 67NR and 66cl4 cells determined by ELISA. Bars represent means ± SEM (N = 3). E) CXCL10 immunoblot of protein extracts from 67NR and 66cl4 cell lines (N = 4). F) CXCL10 immunoblot of protein extracts from 66cl4 cell line (N = 1) and 66cl4 tumors (N = 6). Statistical significance was determined using Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$P < 0.001$ and ****$p < 0.0001$)

IFIT3, IFIT1 and ISG15. Two proteins, OAS3 and GBP2, had significant lower expression in 66cl4 tumors versus 67NR tumors, however, these were outside the cutoff of log2FC < − 1.5. Only one protein, GBP2B, did not correlate with its detected mRNA levels. Among the five undetected proteins in MS (CXCL10, TRIM30d, IRF7, GBP3 and CCL5), we chose CXCL10, CCL5 and IRF7 for further validation [34, 35]. This analysis showed that both mRNA and protein levels of CXCL10, CCl5 and IRF7 protein levels were elevated in metastatic cancer cells in culture and reduced in 66cl4-derived primary tumors (Fig. 2D–F and Additional file 1: Fig.S4C–I). Together, the transcriptomic and proteomic analyses of the cancer cells in culture and primary tumors indicate the dampening of the IFN-I response as the most evident adaptation during tumor formation in the metastatic model.

Cytosolic nuclear DNA is elevated in the murine metastatic and aggressive human cancer cells

The data presented above is consistent with a constitutive IFN-I response in metastatic 66cl4 cells in culture that is downregulated in tumors formed by these cells. Thus, the response is dynamically regulated. We next wondered how the IFN-I response can be constitutively activated in sterile cell culture conditions. IFN-I response is induced by cytosolic DNA [36]. Often cancer cells contain cytosolic micronuclei produced by chromosomal mis-segregation events during mitosis [37]. Immunostaining of DNA and confocal microscopy revealed a similar number of micronuclei in the metastatic and non-metastatic cells (Fig. 3A, B). However, the activation of the IFN-I signaling depends on cGAS physically binding to DNA, a condition that is met at a subset of micronuclei characterized

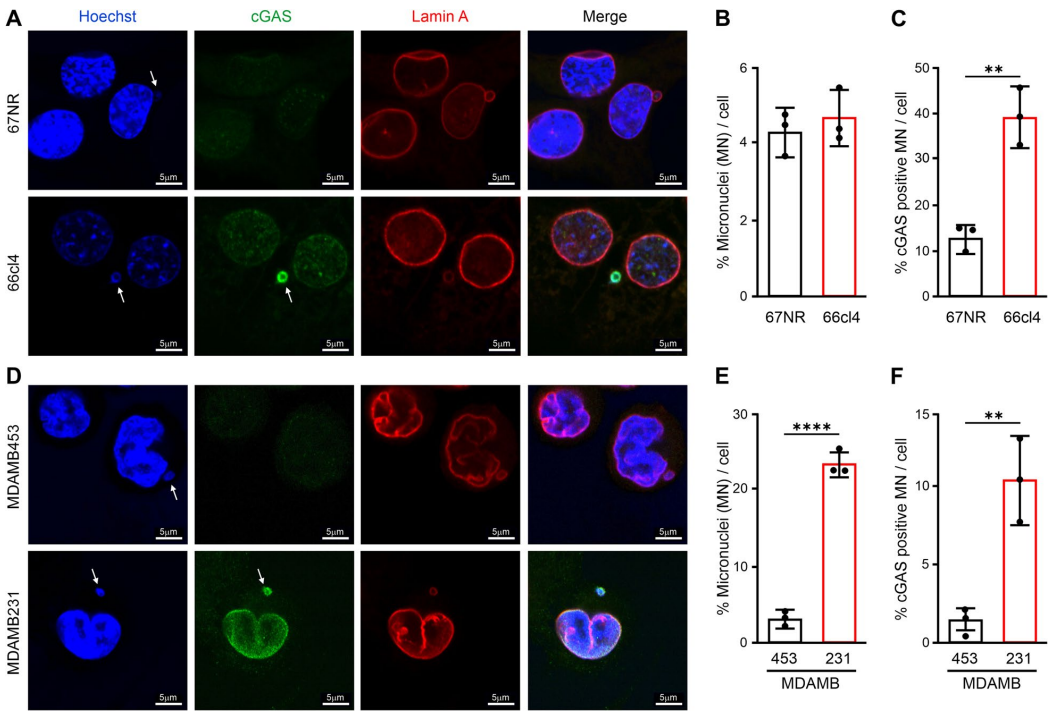


Fig. 3 Metastatic and invasive cancer cells display elevated levels of cGAS-positive micronuclei. **A** Representative immunofluorescence staining of micronuclei in 67NR and 66cl4 cells with cGAS (Green) and Lamin A (red) antibodies. Micronuclei and cGAS-positive micronuclei are highlighted by white arrows. DNA was stained with Hoechst (Blue). Scale bar: 5 µm. **B**–**C** Percentage of micronuclei per cell (**B**) and cGAS-positive micronuclei per cell (**C**) calculated from three independent experiments (N = > 1000 cells per experiment, from three independent experiments). **D**–**F** Representative immunofluorescence staining and quantitation of micronuclei and cGAS-positive micronuclei in MDAMB453 and MDAMB231 cells (N = > 1000 cells per experiment, from three independent experiments). Scale bar: 5 µm. Bars represent mean ± SEM (**$p < 0.01$, ****$p < 0.0001$; Student's t-test)

by a ruptured nuclear envelope [37, 38]. Strikingly, the fraction of cGAS-positive micronuclei was considerably higher in 66cl4 cells than in 67NR (Fig. 3C). Together, transcriptome, proteome and signaling analyses suggest that cGAS binds to DNA in the cytosol of 66cl4 cells in culture and cause a constitutive IFN-I response in these metastatic cancer cells that is not detected in the non-metastatic cells. As 66cl4 and 67NR are mouse-derived breast cancer cell lines, we next postulated whether similar differences in IFN-I expression could be identified in human breast cancer cell lines. We then analyzed the expression of two well-known interferon-induced genes, IFIT3 and IFI44, using mRNA expression data from the Cancer Cell Line Encyclopedia. Interestingly, the expression of these two transcripts showed a strong association with each other (Additional file 1: Fig.S5A). Still, their expression varied among the different cancer cells, including the 60 breast cancer cell lines in the database (Additional file 1: Fig. S5B). For instance, IFIT3 and IFI44 mRNA were highly expressed in the invasive human breast cancer cell line MDAMB231 [39, 40], while their expression was low in the non-invasive MDAMB453 (Additional file 1: Fig. S5B). These findings were confirmed by transcript and protein quantification of IFIT3 in cell extracts of MDAMB231 and MDAMB453 cells (Additional file 1: Fig. S5C–E). In line with the observations seen in mouse breast cancer cell lines, a higher number of micronuclei and cGAS-positive micronuclei were observed in the cytosol of the invasive MDAMB231 compared to non-invasive MDAMB453 human cancer cells (Fig. 3D–F). Together, our data suggest that the IFN-I response could be associated with constitutive activation of the DNA sensor cGAS by recognising nuclear DNA in the cytosol of metastatic and aggressive cancer cells.

Mitochondrial DNA in the cytosol triggers IFN-I response in metastatic cancer cells

The IFN-I response can be activated by cytosolic DNA from other sources, such as mitochondria. We therefore measured the levels of mitochondrial DNA (mtDNA) from intact cells and the cytosolic fractions of 66cl4 and 67NR cells. The mtDNA was measured via qPCR using different mtDNA primers (COX1, Dloop1 and Dloop2). Total mtDNA levels were lower in the 66cl4 cells than 67NR (Fig. 4A and Additional file 1: Fig.S6A). However, in the cytosolic fraction, significantly higher levels of mtDNA were detected in 66cl4 cells (Fig. 4B and Additional file 1: Fig.S6B).

Mitochondrial stress can trigger the release of mtDNA into the cytosol. We, therefore, asked if mitochondria function differently between the metastatic and non-metastatic cells. We performed flow cytometry analysis of the mitochondria mass (mitotracker green; MTG), membrane potential (tetramethylrhodamine ethyl ester perchlorate; TMRE) and mitochondrial ROS production (MitoSox) using fluorescent probes. Despite the mitochondrial mass being similar between the cancer cells, the membrane potential was higher in 66cl4 compared with 67NR cells (Fig. 4C–D). Indeed, the TMRE/MTG ratio, was significantly higher in 66cl4 cells, indicating hyperpolarized mitochondria (Fig. 4E). To further understand if hyperpolarized mitochondria is associated with mtROS production in 66cl4 cells, we quantified mtROS levels. This showed a higher number of 66cl4 cells were positive for mtROS compared to 67NR cells at the basal state (Fig. 4F–H). Furthermore, when the cells were treated with an electron transport chain inhibitor (rotenone), 67NR cells showed a six-fold increase in mtROS levels, while 66cl4 cells showed less than a two-fold increase (Additional file 1: FigS6C). These results suggest that mitochondria in the metastatic 66cl4 cells work at maximum capacity.

Altogether, these data indicate that metastatic cells have poor mitochondrial quality leading to mitochondrial stress and mtDNA release in the cytosol.

The cGAS-STING pathway regulates IFN-I response in metastatic cancer cells

Our results show that the IFN-I response is constitutively active in the metastatic cancer cells in culture, while the IFN-I response is dampened when these metastatic cancer cells form a tumor. A better understanding of the mechanisms that activate this response is important to comprehend how this response could be reactivated in tumors as a therapeutic strategy. Cytosolic DNA is sensed by the cGAS-STING pathway, which is triggered in response to foreign or self-DNA and can activate the IFN response [41]. Transcriptome analysis [28] and qPCR validation showed that the metastatic 66cl4 cells had higher *Sting* mRNA than 67NR cells (Additional file 1: Fig.S7A). In line with this, STING protein level was also higher in 66cl4 (Additional file 1: Fig.S7B). Upon activation, STING recruits and phosphorylates TBK1 and IRF3 to induce the production of type I IFNs [42]. Chemical inhibition of cGAS or TBK1 led to significantly reduced production of the IRF3 target CXCL10 in 66cl4 cells (Fig. 5A–D), indicating that the cGAS-STING-TBK1 pathway is important for IFN-I expression in the metastatic mouse breast cancer cells. Also, in invasive human breast cancer cells MDAMB231, the protein level of STING and phosphorylated TBK1 was higher than in the non-invasive MDAMB453 cells (Additional file 1: Fig.S7C-D). Together, these results indicate that elevated IFN-I

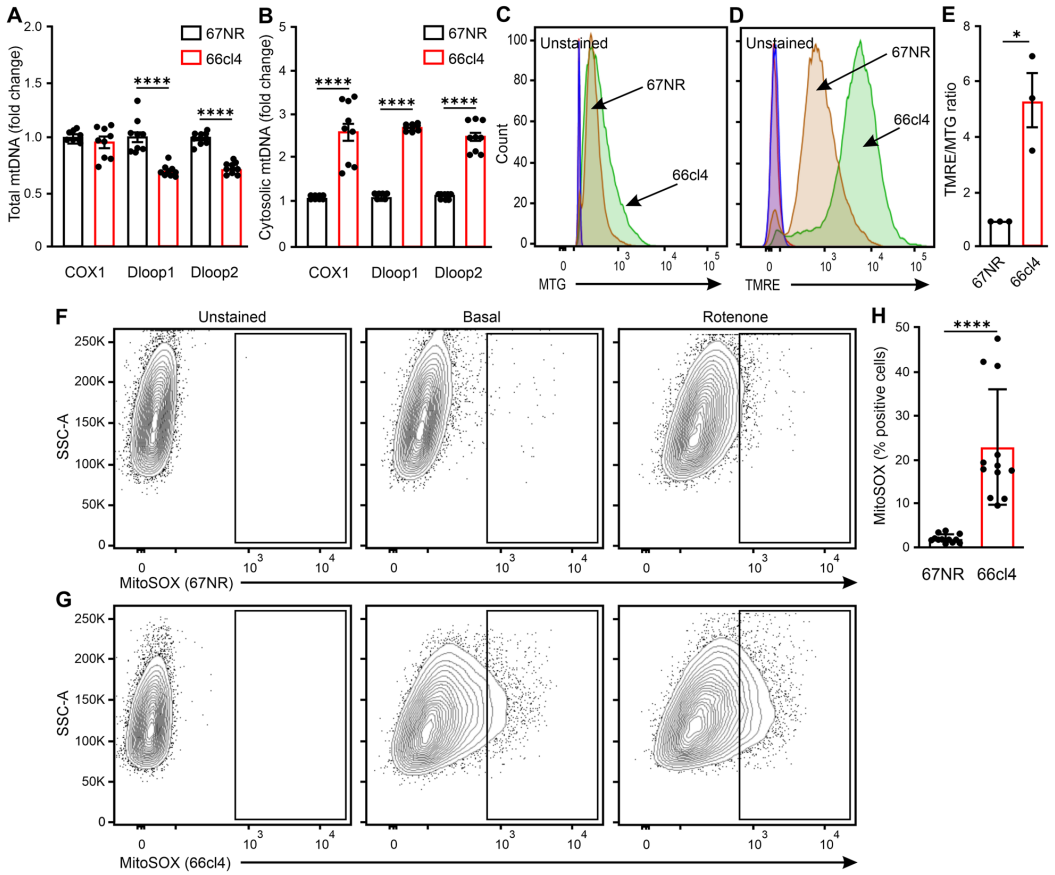


Fig. 4 Mitochondrial DNA release into the cytosol in metastatic cell lines associates with mitochondrial stress. **A** Relative amount of total (**A**) and cytosolic (**B**) mitochondrial DNA (mtDNA) in 67NR and 66cl4 cells, normalized to 18s. Bars represent mean ± SEM (N = 3, each in triplicate, one sample t-test, ****$p < 0.0001$). **C**–**D** Representative histograms of MTG (**C**) and TMRE (**D**) in 67NR and 66cl4 cells. **E** TMRE/MTG ratio in 67NR and 66cl4 cells. Bars represented mean ± SEM (N = 3, one sample t-test, *$p < 0.05$). **F**–**G** Representative histograms showing MitoSox positive populations in 67NR (**F**) and 66cl4 cells (**G**). Rotenone was used as a positive control. Black box represents the gating strategy to identify positive cells. **H** Bars represent mean ± SEM of MitoSOX positive cells in 67NR and 66cl4 cells (N = 4, t-test, and ****$p < 0.0001$)

response in both mouse and human breast cancer cell cultures is associated with activation of the cGAS-STING pathway. The markedly dampened IFN-I in tumors formed from these invasive/metastatic cell lines suggest that the signaling in this pathway is disrupted in vivo to allow such cancer cells to form a metastatic tumor.

Lower IFN-I expression correlates with a poor prognosis in breast cancer patients

To examine if an active IFN-I response has a prognostic relevance in breast cancer patients, we performed a meta-analysis using the Kaplan–Meier plotter database [30] of gene expression in breast cancer biopsies.

We analysed whether the oppositely expressed transcripts encoding IFN-I-related genes correlated with prognosis monitored as relapse-free survival and overall survival. For this, we used data from aggressive triple negative breast cancer (TNBC) and HER2 enriched breast cancer patients, which were compared with the ER positive patient group [44–47]. TNBC and HER2 enriched subtypes are both characterized by the lack of estrogen and progesterone receptor expression, although only HER2 enriched subtype express human epidermal

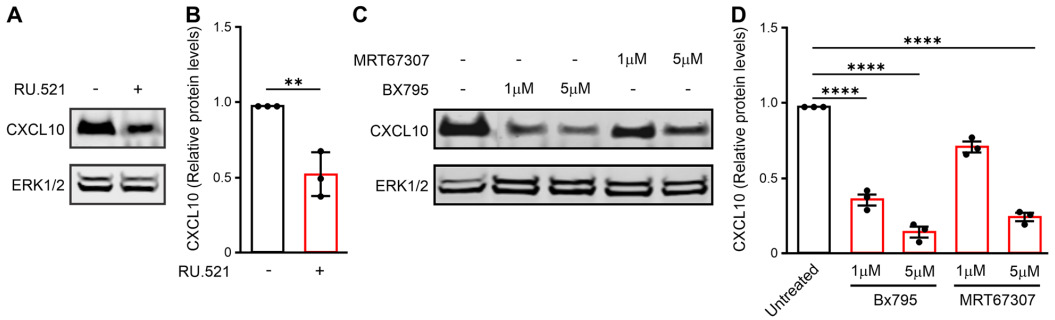


Fig. 5 IFN-I expression in invasive cancer cells is dependent on cGAS-TBK1 signaling. **A** Representative CXCL10 and ERK1/2 immunoblot of protein extracts of 66cl4 cell line treated with and without cGAS inhibitor (RU.521, 6 μM, 24 h). **B** Quantification of A. Bars represented mean ± SEM relative to ERK1/2 (N = 3, one sample t-test, $^{**}p < 0.01$). **C** Representative CXCL10 immunoblot of protein extracts of 66cl4 cell lines treated with and without TBK1 inhibitors (BX795: 1 μM, 5 μM and MRT67307: 1 μM, 5 μM, 6 h). **D** Quantification of C. Bars represented mean ± SEM relative to ERK1/2 (N = 3, ANOVA, Dunnett's multiple comparisons test, $^{****}p < 0.0001$)

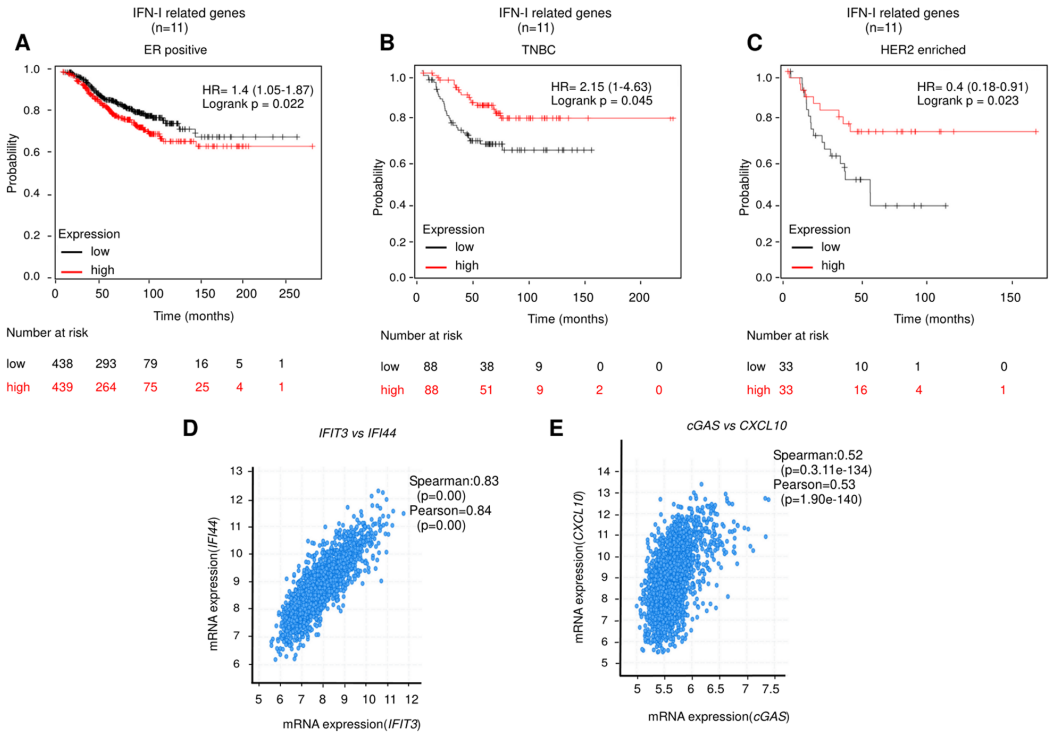


Fig. 6 Low IFN-I expression correlates with poor relapse-free survival in breast cancer patients. **A**–**C** Analysis of relationships between gene expression and relapse free survival (RFS) in breast cancer patients using the online tool KM plotter. High and low expression were defined as above and below median. Relationship between mean expression of IFN-I related genes (n = 11) in ER positive (**A**), TNBC (**B**) and HER2 enriched subtypes (**C**). HR, hazard ratio. **D** Dot plot showing a positive correlation between mRNA expression of *IFIT3* and *IFI44* in METABRIC cohort (N = 1904). **E** Dot plot showing a positive correlation between mRNA expression of *cGAS* and *CXCL10* in METABRIC cohort

growth factor 2 (HER2). In addition, TNBC and HER2 enriched are more aggressive subtypes compared to the ER positive [44, 48–51].

This analysis showed that lower expression of IFN-I related genes correlate with a reduced relapse-free survival in TNBC as well as HER2 positive patients but not in ER positive cancer patients (Fig. 6A–C). In addition, when we evaluated the overall survival, we found that lower levels of IFN-I related genes in tumor biopsies were unfavorable markers exclusively for TNBC patients (Additional file 1: Fig.S8A–C). We extended these results using the public dataset from the "Molecular taxonomy of breast cancer international consortium" (METABRIC) cohort [43], which facilitated analysis of mRNA expression data from 1904 patients. Here, we observed a strong correlation between several IFN-I genes. For instance, *IFIT3* correlated with *IFI44* (Fig. 6D), *CXCL10*, *STAT1*, *IFIT1*, *IFI44L*, *ISG15*, *IRF9* and several cytotoxic T cell markers including *CD8A*, *CD8B*, *CD28* (Additional file 2: Table S1). In addition, *cGAS* expression correlated significantly with the expression of several IFN-I induced genes (Fig. 6E and Additional file 3: Table S2). Together these results suggest that lower levels of IFN-I related genes in more aggressive cancer subtypes are associated with unfavourable prognosis.

Discussion

Although all cancer cells can form a tumor, metastatic cancer cells must endure unique adaptations that help them to undergo immune escape and spread to distant sites [52]. To identify these unique adaptations, we compared metastatic and non-metastatic breast cancer cells when grown in culture, and in the tumors these cells induce when injected in immune-competent mice. We found that the IFN-I response was oppositely regulated in metastatic and non-metastatic cells versus their corresponding tumors. While the IFN-I response was active in the metastatic cell lines when grown in culture, it was markedly dampened in the primary tumor. Interestingly, we also found that the IFN-I response was active in human breast cancer cells in culture, which is associated with an invasive phenotype. Further, the IFN-I response was not active in the non-metastatic cell lines, yet it was induced in the primary tumors. This adaptation may be fundamental to avoid the anti-tumor immune response and metastasis. It is well appreciated that dampened IFN-I signaling aids tumor progression [53]. IFN response can be activated in both cancer cells and immune cells inside the tumor; however, the function is different [54]. In cancer cells, the IFN-I response acts as an alarm system, alerting the immune system to kill the transformed cancer cells. In immune cells, IFN-I response can act as an effector system that contributes to eliminating cancer cells via T cell priming and effector cytokine production [54, 55].The selection of cancer cells in a growing tumor likely involves acquirement of mechanisms that downregulate anti-tumor immunity, including IFN signaling but how cancer cells turn off IFN-I response in the tumors remains incompletely understood. In any case, these findings further underscore potential therapeutic strategies involving reactivation of the IFN-I response to trigger anti-tumor immune reactions. The IFN-I response has long been a key contributor to effective antiviral responses. The induction of IFN-I signaling is essential for the immune system to eliminate cells infected with viruses and intracellular bacterial infections. During infection, the presence of foreign DNA in the cytosol leads to activation of cGAS and subsequent induction of the IFN-I response [53, 55, 56]. Here, we identified elevated levels of cytosolic DNA from micronuclei and mitochondria in metastatic cancer cells in sterile culture conditions. In murine metastatic and invasive human breast cancer cell lines, cytoplasmic DNA foci were often associated with cGAS. This suggests that micronuclei are ruptured and sensed by cGAS. Our data is supported by previous reports showing that disruption of the micronuclear envelope exposes self-DNA to the cytosol, followed by recruitment of cGAS and activation of cGAS-STING signaling [38, 57]. Our observation of mtDNA leakage is supported by our previous findings that show dysfunctional mitochondrial in metastatic cancer cells, characterized by high mtROS production, hyperpolarized mitochondria with higher proton leak, and lower respiratory capacity [58]. We therefore speculate that elevated levels of mitochondrial components and hyperpolarized mitochondria could be a compensatory mechanism to increase ATP production, but also result in mitochondrial damage and mtDNA release in the cytosol. Here we show that the IFN-I response is constitutively active in breast cancer cells with metastatic ability grown in culture, while an effective dampening of this response occurs when they grow as tumors. Likely, this downregulation is either due to stimulated removal of DNA from the cytosol or downregulation of the cGAS-STING signaling pathway in the cancer cells within the tumor. While IFN-I inducers are well-known, negative regulation of IFN-I signaling is poorly understood [59]. Nevertheless, it is well established that cytosolic DNA is degraded by autophagy [60–63]. Even if dampening of the IFN-I response involves elevated autophagy in the cancer cells of a tumor, it remains unknown which factors of the TME cause this effect. It is tempting to speculate that local nutrient restriction could stimulate autophagy in cancer cells of solid tumors. However, the

ability to accurately quantify autophagic flux in biopsies is currently limited.

In human tumor biopsies, elevated IFN-I signaling correlates with induced T cell responses against tumor specific antigens [64, 65] and it may represent a mechanism that limits tumor development. In line with this, our METABRIC analysis of 1904 breast cancer patients showed that the expression of several interferon-induced genes correlates with several T cell markers.

Using KM plotter, we observed that reduced expression of the 11 oppositely expressed IFN-related genes predict poor prognosis in both TNBC and HER2 enriched aggressive breast cancer subtypes but not in ER positive subtypes. In the TME, IFN signaling is commonly induced by tumor-associated antigens or due to immunogenic cell death in response to chemotherapy and radiotherapy [66]. In TNBC and HER2-enriched breast cancers patients that undergo trastuzumab monotherapy or in combination with chemotherapy, high tumor-infiltrating lymphocytes were associated with a better prognosis in patients. In contrast, no association was observed in ER-positive patients [67–70]. This could be because $CD8^+$ T cells can produce and respond to IFNs mediating antitumor responses [71]. While systemic IFN-I based therapies have been shown to increase the efficacy of the checkpoint inhibitors in TNBC, no predictive values were obtained in the ER-positive [68, 72] patients. This leads to an open question of whether inducing IFN-based immune therapy is beneficial to ER positive patients, which still needs to be addressed. Here, our results highlight the clinical significance of an elevated IFN-I response, supporting the therapeutic potential of increasing IFN-I response in patients where these responses are suppressed. One limitation of this type of data analysis, is the lack of evidence about which cells, immune or non-immune, contributes to the IFN-I response in the patient biopsies. Likely, reduction of IFN response could be either due to the downregulation of immune components that induce IFN-I signaling or due to the stimulated removal of the endogenous DAMPs in the cancer cells in the tumor as mentioned earlier in the discussion [73–75].

Currently, more than 370 (recruiting or active) clinical trials aiming to target IFN-I signaling in cancer patients are ongoing (www.clinicaltrials.gov). However, IFN-associated toxicity has been a significant obstacle for this strategy to be translated to the clinic. Recently, other approaches to activate IFN-I response have been explored including cGAMP-based nanoparticles. cGAMP, is a second messenger that is synthesized in response to cytosolic double stranded-DNA. These nanoparticles enhance the cytosolic delivery of cGAMP and trigger formation of an immune competant TME with enriched T cell infiltration [76]. Alternatively, tumors that are non-responsive to immune checkpoint inhibitors could be transformed into the immune competent tumors by using STING agonist-mediated T-cell priming and infiltration [12, 77–81]. Since STING agonists can redesign the TME to promote stronger antitumor T cell responses [22, 78], they are good candidates for combination with established immunotherapies. However, despite several completed and ongoing Phase II studies detecting signs of clinical activity for STING agonists, no Phase III studies have been registered yet. Even with the best-characterized STING agonist DMXAA, most of the trials with mono and combination therapy have failed due to low efficacy and toxicity issues [77]. These findings highlight the importance of identifying better therapeutic combinations and improving understanding of the underlaying mechanisms controlling this signaling in a complex tumor.

Conclusion

In this study, we utilized an immunocompetent mouse model of breast cancer to demonstrate that IFN-I signaling represents an important mechanism supporting tumor progression. Further research is needed to uncover the full repertoire of mechanisms that control this immunological switch and find novel strategies to efficiently target aggressive tumors, reduce the risk of metastasis and improve the survival of breast cancer patients.

Abbreviations

Cell	Cellular
Met	Metabolic
Resp	Response
ncRNA	Noncoding RNA
med	Mediated
sig	Signalling
prod	Production
resp	Response
ext	External
Adapt	Adaptive
imm	Immune
som	Somatic
recomb	Recombination
rec	Receptors
Superfam	Superfamily
Ant	Antigen
proc	Process/processing
pres	Presentation
pep	Peptide
Rib Prot	Ribonucleoprotein
Spli	Splicing
Transesterif	Transesterification
React	Reaction
Ade	Adenosine
Sub	Subunit
Org	Organization
Nucelocytopl	Nucleocytoplasmic
End	Endogenous pathway
Mol	Molecule
Reac	Reactive

Spe	Species
Leuk	Leukocyte
Mig	Migration
inf	Inflammatory
cat	Catabolic
cGAS	Cyclic GMP-AMP synthase
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
STING	Stimulator of interferon genes
CD45	Cluster of differentiation 45
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
ISG15	Interferon-stimulated gene 15
IRF3	Interferon regulatory factor 3
IRF7	Interferon regulatory factor 7
XAF1	XIAP-associated factor 1
IGTP	Interferon gamma induced GTPase
STAT1	Signal transducer and activator of transcription 1
OAS3	2′-5′-Oligoadenylate synthetase 3
GBP2	Guanylate binding protein 2
CXCL10	C-X-C motif chemokine ligand 10
Trim30d	Tripartite motif-containing 30D
IFF7	Interferon regulatory factor 7
H2-T22	Histocompatibility 2, T region locus 22
GBP3	Guanylate binding protein 3
CCL5	C–C motif chemokine ligand 5
H2-Q4	Histocompatibility 2, Q region locus 4
IFI44	Interferon induced protein 44
COX1	Cytochrome c oxidase subunit 1
COX2	Cytochrome c oxidase subunit II
Dloop1	Displacement loop1
Dloop2	Displacement loop1
TBK1	TANK binding kinase 1

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12964-023-01062-y.

Additional file 1. Supplementary figures S1 to S8 and supplementary methods.

Additional file 2. Correlation between IFN-I induced genes expression.

Additional file 3. Correlation between cGAS and IFN-I induced genes expression.

Acknowledgements
We would like to thank Ulrike Neckmann and Camilla Wolowczyk for providing access/inputs for the transcriptome data. Professor Kaisa Lehti and Dr. Andrew Single for reading and providing valuable insights for the manuscript, Proteomics and Modomics Experimental Core Facility (PROMEC), NTNU for proteomics, Membrane dynamics in tumorigenesis group (OUH) for access to imaging facilities.

Author contributions
AL, SBA, MV, IJ, ABA and FR performed experiments and analyzed data. NJK analyzed part of the proteomics data. MSG and GB designed and supervised the study. AL, SBA, MV, IJ, KP, MSG and GB interpreted data. AL, SBA, KP prepared the first draft. AL, MSG, and GB prepared the final manuscript. MV provided valuable insights for the final manuscript. All authors contributed to the article and approved the submitted version.

Funding
Open access funding provided by Norwegian University of Science and Technology. This work was supported by grants from the Norwegian Cancer Society (project number #6846671 and #419654), Research Council of Norway (Centres of Excellence funding program, # 223255/F50), Central Norway Regional Health Authority (project number #90181900), PhD-grant to AL from NTNU, Faculty of Natural Sciences. Additional funding from Southern and Eastern Norwegian Health Authority (#2018043, MV), Tromsø Research Foundation and Trond Mohn Foundation (180°N project, MSG).

Availability of data and materials
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [24] partner repository with the dataset identifier PXD037288. All other data and materials mentioned in this article can be requested by email.

Declarations

Competing interest
The authors declare that they have no competing interests.

Ethics approval and consent to participate
The mice studies were approved by the National Animal Research Authorities and carried out according to the European Convention for the Protection of Vertebrates used for Scientific Purposes (FOTS ID 17895 and FOTS: 26021). All experiments were performed according to approved guidelines and regulations.

Consent for publication
Not applicable.

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Received: 27 October 2022 Accepted: 30 January 2023
Published online: 07 March 2023

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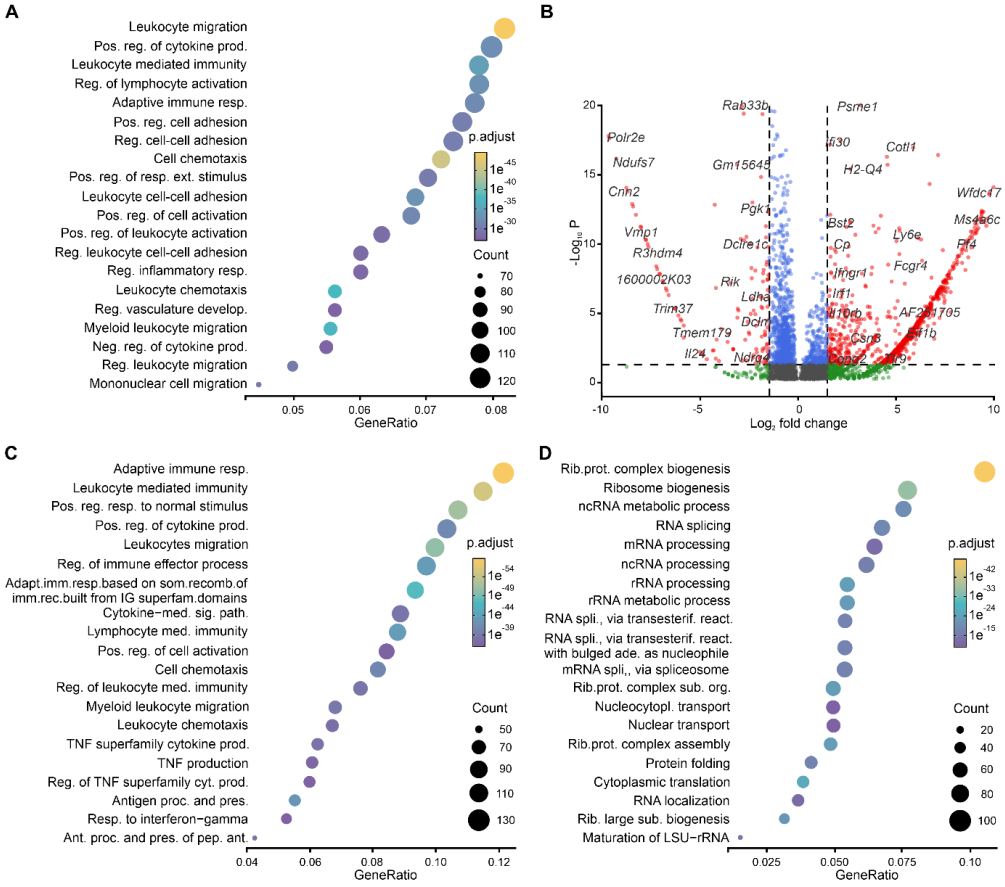

Fig. S1: Gene ontology analysis of RNA seq. data from metastatic and non-metastatic cell lines and primary tumors reveals dysregulation of multiple biological processes

A) Gene ontology (GO) analysis for biological processes (BP) of genes with increased expression in 66cl4 tumors versus 66cl4 cell line. **B)** Volcano plots depicting differentially expressed genes from 66cl4 tumors vs 66cl4 cells. Red points represent genes with Log2foldchange(log2FC) within the cut off (±1.5) and adjusted *p*-value <0.05. **C)** GO (BP) functional enrichment analyses of genes highly expressed in 67NR tumor relative to 67NR cells. **D)** Gene ontology analysis for biological processes of genes with low expression in 67NR tumor vs 67NR cells.

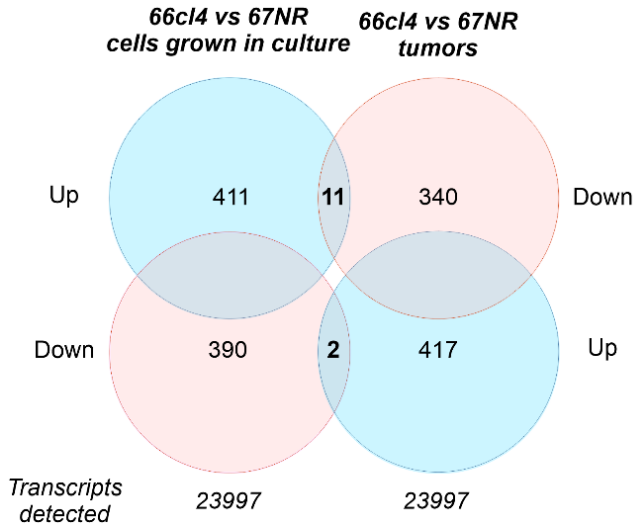

Fig. S2: Strategy for comparison of gene expression in metastatic cell and non-metastatic cells in culture and in primary tumors

Venn diagram showing differentially expressed genes. Gene expression in the 66cl4 vs 67NR cells in culture and 66cl4 tumors vs 67NR tumors were compared. With a cutoff of log2FC ±1.5 and *p*.adj: <0.05; 411 genes had higher expression and 390 had lower expression in 66cl4 compared to 67NR cells grown in culture. With similar cutoff in the primary tumors formed by 66cl4 and 67NR cells, 340 genes were low expressed, and 417 genes were highly expressed in 66cl4 tumors compared to 67NR tumors. Among the 390 low expressed genes in 66cl4 cells, 2 genes were highly expressed in the 66cl4 tumor. Among 411 genes that were highly expressed in 66cl4 cells, 11 were low expressed in the 66cl4 tumors compared to 67NR tumors. These 11 genes were highly expressed in 66cl4 cells (vs. 67NR cells) while in the 66cl4 tumors they were low expressed (vs 67NR tumors); **"oppositely expressed"**.

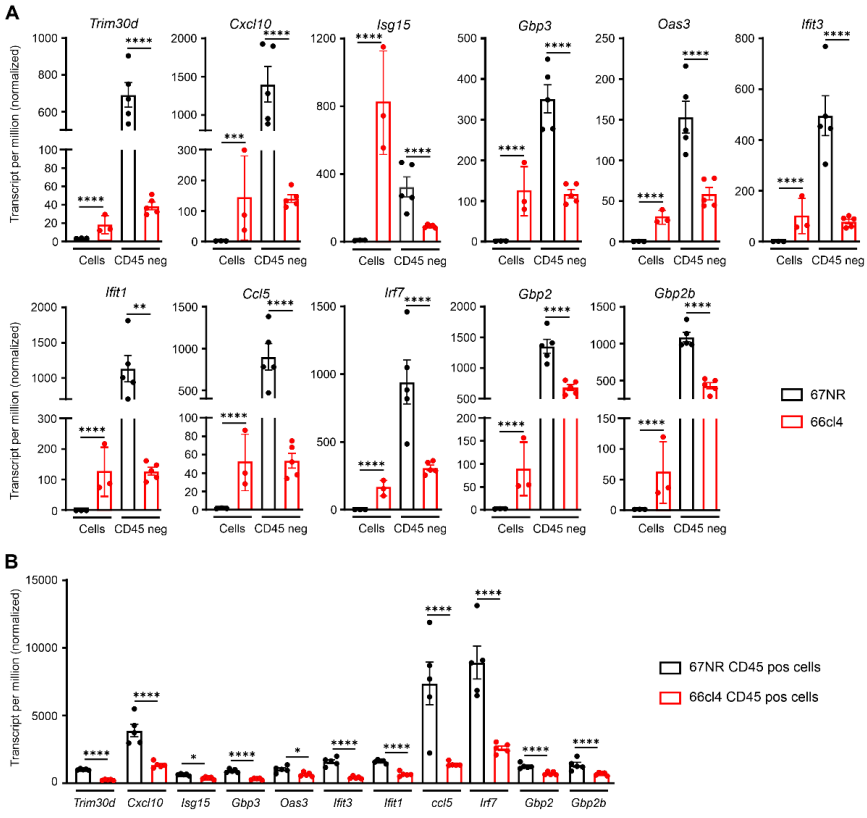

Fig. S3: IFN-I-related gene expression is suppressed in metastatic tumors.

A) Significantly upregulated IFN-I genes in 66cl4 cells vs. 67NR cells (N=3) and downregulated genes in 66cl4 (CD45-) vs 67NR (CD45-) population (N=5) sorted from the primary tumors of 66cl4 and 67NR. **B)** Significantly downregulated genes in 66cl4 (CD45+) vs 67NR (CD45+) population (N=5) sorted from the primary tumors of 66cl4 and 67NR. Bars represent means ± SEM and each data point represents a single animal; (*p <0.05, ** p <0.01, ***p <0.001 and **** p <0.0001). Average expression levels of the selected genes as transcripts per million (TPMs). Bars represent means ± SEM and each data point represents a single animal. Statistical significance was determined using Mann Whitney t-test, *p< 0.05; ** p<0.005; *** p<0.0005 vs 67NR tumor.

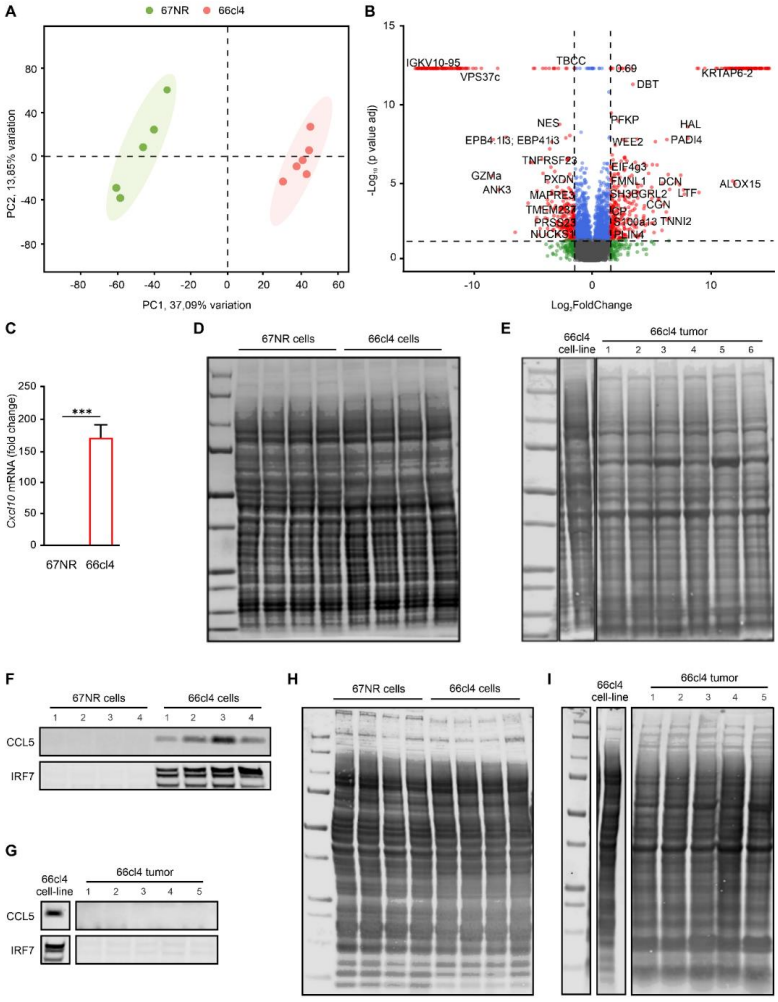

Fig. S4: IFN-I proteins are constitutively expressed in metastatic cancer cells in culture but dampened when they form primary tumors

A) Principal Component Analysis of the proteins from 67NR (N=5) and 66cl4 (N=6) primary tumor lysates. **B)** Volcano plots depicting differentially expressed proteins in 66cl4 tumor (N=6) vs 67NR tumor (N= 5). **C)** *Cxcl10* mRNA levels in 67NR and 66cl4 cell lines in culture (N=3). *Actb* was used as a housekeeping gene and the data was normalized to 67NR. Bars represent means ± SEM (**$p < 0.01$, One sample t-test, N=3). **D)** Total protein stain of western blot membrane shown in Fig.2E. **E)** Total protein stain of western blot membrane shown in Fig. 2F. F) CCL5 and IRF7 immunoblot of protein extracts from 67NR and 66cl4 cell lines (N=4). G) CCL5 and IRF7 immunoblot of protein extracts from 66cl4 cell line (N=1) and 66cl4 tumors (N=5). **H-I)** Total protein stain of western blot membrane shown in F and G respectively.

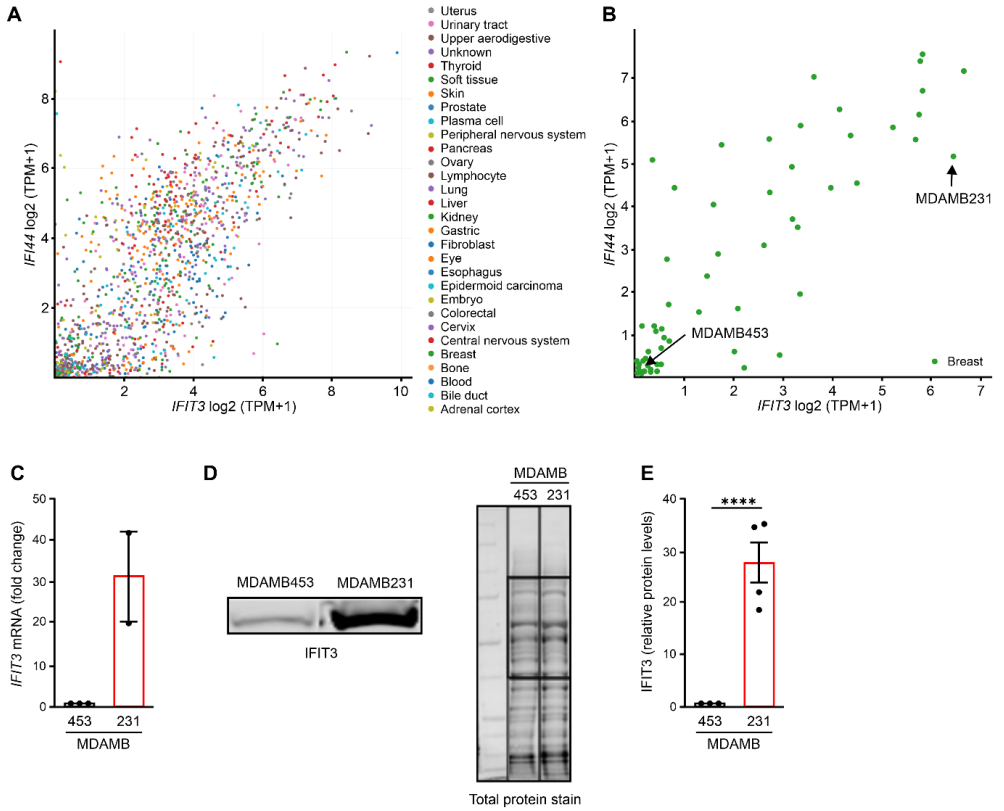

Fig. S5: IFN-I expression correlates with the invasive phenotype in human breast cancer cell lines

A) *IFIT3* and *IFI44* mRNA expression in all cancer cell lines using the Expression 22Q2 Public database in Cancer Cell Line Encyclopedia, CCLE. Each dot represents a cell line and color code is based in their lineage. **B)** *IFIT3* and *IFI44* mRNA expression in breast cancer cell lines. **C)** *IFIT3* mRNA expression levels in MDAMB453 and MDAMB231 cell lines (N=2). *RNA18SN5* was used as a housekeeping gene, and the data are presented relative to the MDAMB453 cells. **D)** Representative IFIT3 immunoblot of protein extracts of the MDAMB453 and MDAMB231 cell lines (N=3-4). Total protein staining was used as loading control, and the square indicates the quantified area. **E)** Quantification of IFIT3 protein level. Bars represent mean± SEM relative to MDAMB453 (N=4, ****p<0.0001, One sample t-test).

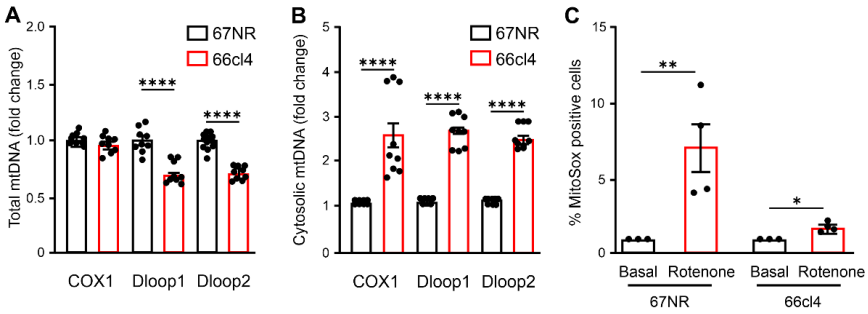

Fig. S6: Metastatic cancer cells release mtDNA into the cytosol

Relative amount of total **(A)** and cytosolic **(B)** mitochondrial DNA (mtDNA) in 67NR and 66cl4 cells, normalized to Tert. Bars represent mean± SEM (N=3, each in triplicate, t-test, $**p < 0.0001$, $****p < 0.0001$). **C)** MitoSOX positive cells (%) in 67NR and 66cl4 cell in presence or absence of rotenone. Bars represent mean± SEM (N=4, one sample t-test, $**p < 0.01$, $*p < 0.05$).

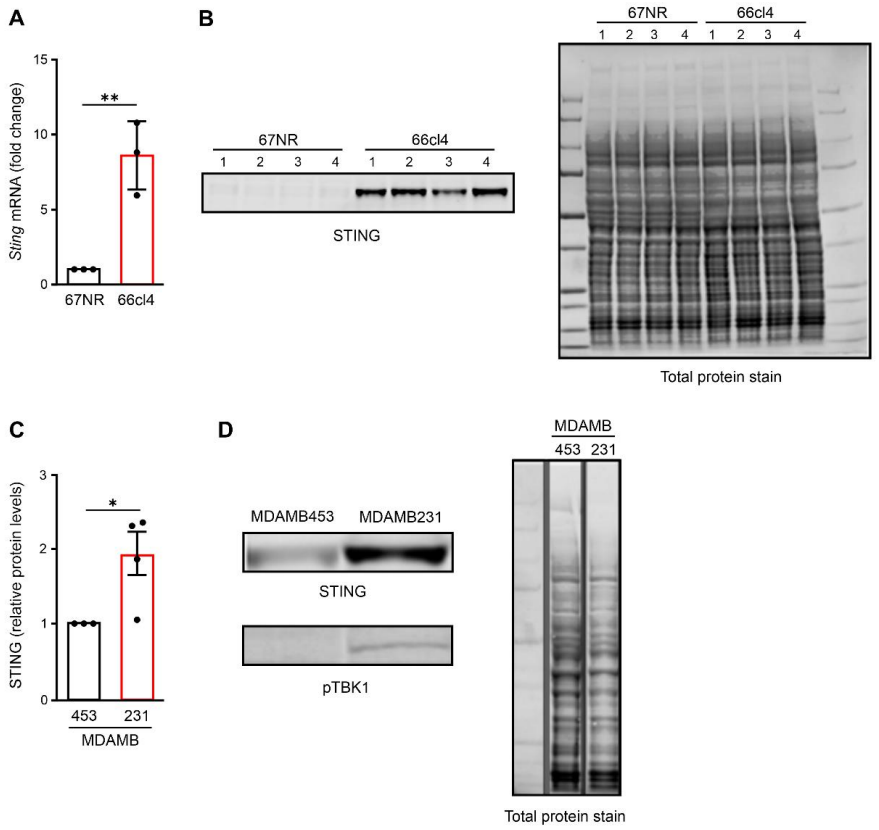

Fig. S7 High levels of STING and pTBK1 are associated with metastatic ability in breast cancer cell lines.

A) *Sting* mRNA expression in 67NR and 66cl4 cell lines (N= 3). *Actb* was used for normalization and fold change was calculated relative to 67NR. Bars represent mean ± SEM (N=3, one sample t-test, ***p* <0.01). **B)** STING immunoblot from 67NR and 66cl4 cell lines lysate. Total protein staining was used as loading control. **C)** STING protein levels in MDAMB453 and MDAMB231 cell lines. Bars represented mean ± SEM relative to total protein staining (N≥3, one sample t-test, * *p* <0.05). **D)** Representative STING and pTBK1(Ser172) immunoblot (N= 5) from MDAMB453 and MDAMB231 cell lines. Total protein staining was used as loading control.

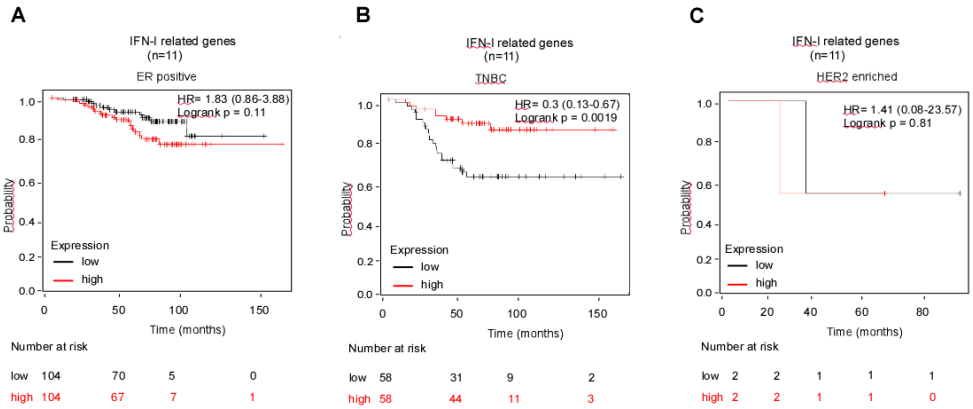

Fig S8: Low IFN-I expression correlates with poor overall survival in breast cancer patients.

A-C) Analysis of relationships between gene expression and overall survival (OS) in breast cancer patients using the online tool KM plotter. High and low expression were defined as above and below median. Relationship between mean expression of IFN-I related genes (n=11) in ER positive **(A)**, TNBC **(B)** and HER2 enriched **(C)** subtypes. HR, hazard ratio.

Supplementary Methods

Cell lines and Cell culture

67NR and 66cl4 cells from the 4T1 model were obtained from Barbara Ann Karmanos Cancer Institute, Detroit, MI, USA. In addition to the 4T1 model, two different human breast cancer cell lines that are characterized as non-invasive (MDAMB453) and invasive (MDAMB231) were also used. The MDAMB cell lines both originate from metastases, yet the MDAMB453 is classified as non-invasive based on their Matrix Metallopeptidase 14 (MMP14) expression (1, 2) and is considered as tumorigenic only in semi-solid medium (ATCC). MDAMB453 and MDAMB231 were kindly provided by Dr. Kaisa Lehti. All cell lines were cultured in DMEM (Lonza, BioWhittaker, #BE12- 604F) supplemented with 10% fetal calf serum (Thermo Fischer Scientific,Gibco #10272-106), 2 mM L-Glutamine (Lonza Group, Cat #De-17-605E), and 50 U/ml 50 U/ mL penicillin-streptomycin (Thermo Fischer Scientific, Gibco, #15070-063). Cells were incubated at 37°C with 5% CO2.

Transcriptome analysis

As described previously, RNA was isolated from three passages of 67NR and 66cl4 in culture, four and seven primary tumors of 67NR and 66cl4, respectively. Detailed information about sample preparation and data analysis can be found in (3). The transcriptome data obtained by sequencing mRNA isolated from cells and primary breast tumors of 67NR and 66cl4 is accessible from NCBI (SUB6422687). The data from(3) was reanalyzed into further detail using RStudio version 4.1.2. Genes that had a total count less than 5 were filtered out using filterfun function in RStudio. Differential expression was then determined with DESeq2 using default settings(4). The presence of differentially expressed genes (DEGs) was determined by comparisons of groups: 66cl4 tumors vs 66cl4 cells ,67NR tumors vs 67NR cells, 66cl4 cells vs 67NR cells and 66cl4 tumor vs 67NR tumor using a significance threshold of $p.adj < 0.05$. The cut off Log2FoldChange of either (±1.5), or (± 1) and $p.adj < 0.05$ was used to define low expressed and high expressed genes. Volcano plots were drawn using the EnhancedVolcano R package (v 1.0.1). Heatmaps of expression data were plotted after log10 transformation and after z scoring the expression values using pheatmap package (v1.0.12). The *hclust* function in pheatmap package was used to performed hierarchical clustering.To evaluate common biological functions of results of DEGs, functional enrichment analyses of all significantly highly expressed genes and all significantly low expressed genes were performed to identify the biological processes involved. The bioconductor package clusterProfiler (5) was used to conduct gene ontology (GO) functional enrichment analyses for biological process (BP), for DEGs from different groups applicable. The plot was visualized using the ggplot2 package (v3.2.1). Analyses for DEGs were performed separately for highly expressed and low expressed genes. For CD45 positive and CD45 negative RNA sequencing, unpublished data was used: GEO NCBI, accession code: GSE211223 (https://www.ncbi.nlm.nih.gov/geo/info/linking.html).

Mice Experiments

The mice were injected with 1 x 10^6 viable 66cl4 and 67NR cancer cells into the mammary fat pad. Mice injected with 67NR cells were sacrificed after 3 weeks, while mice injected with 66cl4 were sacrificed after 4 weeks. Tumors were resected and snap frozen in liquid nitrogen and stored at -80ºC. All experiments involving mice were conducted in accordance with the European Convention for the Protection of Vertebrates used for Scientific Purposes. The mice were housed at the Comparative Medicine Core Facility at NTNU, and the animal studies were approved by the Norwegian Food Safety Authorities (FOTS: 17895 and FOTS: 26021)

Sample preparation and MS analyses

Small pieces of 67NR and 66cl4 tumors were thawed briefly in lysis buffer and homogenized using 1.4 mm ceramic beads (Precellys, 03961-1-103) in reinforced tubes (KT03961-1-403.2) for 4 cycles á 40 sec homogenization, 2 min break. Lysis buffer: 8 M urea (Merck Millipore, #1084870500) with 4 % CHAPS, 100 mM DTT, (Sigma, #646563), 1x Complete® protease inhibitor (Roche, #1187350001) and 2x phosphatase inhibitor cocktail II (Sigma, #P5726) and III (Sigma, #P0044). Homogenized tissue samples were shaken before centrifugation (15 000 g, 20 min, 4°C). Protein concentration was measured at 595 nm using BioRad protein assay dye reagent (Bio-Rad, #500-0006).15µg of each were added to 130µl 100mM ammonium bicarbonate. Proteins were reduced and alkylated with DTT (12mM) for 30min at 55°C. Samples were further alkylated with iodoacetamide (36mM) for 30min at room temperature and dark. Proteins were digested with 250ng trypsin at 37°C overnight and further acidified in acetic acid (0.5%) and desalted using Oasis HLB C^{18} solid phase extraction according to manufacturer's instructions. After elution of peptides from C^{18}, the samples were dried in speedvac and further dissolved in 18 µl 0.1% formic acid and LC-MS/MS were performed on a timsTOF Pro (Bruker Daltonics) connected to a nanoElute (Bruker Daltonics) HPLC. Peptides were separated using a Bruker15 (75µm*15cm) column with running buffers A (0.1% formic acid) and B (0.1% formic acid in acetonitrile) with a gradient from 0% B to 37%B for 100min. The timsTof instrument was operated in the DDA PASEF mode with 10 PASEF scans per acquisition cycle and accumulation and ramp times of 100 millisecond each. The 'target value' was set to 20,000 and dynamic exclusion was activated and set to 0.4 min. The quadrupole isolation width was set to 2 Th for m/z < 700 and 3 Th for m/z > 800.

Proteomics data analysis and bioinformatics analysis

Proteins were quantified by processing MS data using MaxQuant v.2.0.3.0 (6). The open workflow provided in FragPipe (7) was used to inspect the raw files to determine optimal search criteria and accordingly search parameters were set as follows: enzyme specified as trypsin with maximum two missed cleavages allowed; deamidation of asparagine/glutamine, oxidation of methionine, and protein N-terminal acetylation as variable modifications; precursor and fragment mass tolerance was set to 20

parts per million (PPM). These were imported in MaxQuant which uses m/z and retention time (RT) values to align each run against each other sample with a minute window match-between-run function and 20 mins overall sliding window using a clustering-based technique. These were further queried against the mouse proteome including isoforms downloaded from Uniprot (8) in 2021 along with MaxQuant's internal contaminants database using Andromeda built into MaxQuant. Both protein and peptide identifications false discovery rate (FDR) was set to 1%, only unique peptides with high confidence were used for final protein group identification. Peak abundances were extracted by integrating the area under the peak curve. Each protein group abundance was normalized by the total abundance of all identified peptides for each run and protein by calculated median summing all unique and razor peptide-ion abundances for each protein using label-free quantification (LFQ) algorithm (9) with minimum peptides ≥ 1. LFQ values for all samples were log-transformed with base 2. A correlation heatmap using R package pheatmap (10) was created using these transformed LFQ values and an outlier was removed. The rest of the values representing each condition were subjected to two-sided Student's t-Tests (11) as implemented in R(12) in order to check the consistency of change. The amount of change was estimated by subtracting the median of these values representing each group (log2 median change). Directionality of the change is encoded within the sign of log2 median change whereby a negative sign reflecting decreased and a positive sign reflecting the increased expression of the respective protein group. Further, to estimate the false-discovery rate (FDR), the T-test p-values were corrected using the Benjamini-Hochberg procedure (13). Differentially expressed (DE) protein groups were identified at FDR<0.1 and absolute log2 median change >1.5. The DE quantified only in one group were checked if their coefficient-of-variation of log2medianchange was within 5%. The Uniprot accession IDs of these DE were mapped to a volcano-plot using R package ggplot2 (14) . Volcano plots represented in the figures were drawn using the EnhancedVolcano R package (v 1.0.1), and the cut off was set to log2 median change ±1.5 and the corrected T-test p-value of < 0.05. log2 median change is represented as log2FoldChange throughout the paper.Functional enrichment analyses of all differentially expressed proteins were performed similar as for the transcriptome data.

Quantitative real-time PCR

cDNA was synthesized from 500 ng total RNA by High-Capacity cDNA Reverse Transcription Kit (Invitrogen, #4368814). Quantitative real-time PCR (RT-PCR) was performed in 20 µl reactions containing 10 µl of 2X QuantiTect SYBR Green PCR master mix (Qiagen), 2 µl 10X QuantiTect Primer Assay and 8 µl of the sample containing 4 ng of cDNA. Primers are specified under Primer section. RT-PCR was performed on the StepOne plus system (Applied Biosystems) using the following cycling conditions: 95ºC for 15 min, 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. Relative gene expression levels were calculated with the 2^(-ΔΔCT) method. Transcripts were normalized to *Actb* for mouse and *RNA18SN5* for human cell lines.

Primers

QuantiTect Primer Assays were purchased from Qiagen: Mm_*Actb*_2_SG (QT01136772), Hs_*IFIT3*_1_SG (QT00100030), Mm_*Cxcl10*_1_SG (QT00093436), Mm_*Tmem173*_2_SG (QT01045618, for *Sting*). The following primers were purchased from Merck: mDloop1 Fw (AATCTACCATCCTCCGTGAAACC), Rev (TCAGTTTAGCTACCCCCAAGTTTAA); mDloop2 Fw (CCCTTCCCCATTTGGTCT), Rev (TGGTTTCACGGAGGATGG); mCOX1 Fw (GCCCCAGATATAGCATTCCC), Rev (GCCCCAGATATAGCATTCCC); m18SrRNA Fw (TAGAGGGACAAGTGGCGTTC), Rev (CGCTGAGCCAGTCAGTGT); mTert Fw (CTAGCTCATGTGTCAAGACCCTCTT), Rev (GCCAGCACGTTTCTCTCGTT); h18SrRNA Fw (GTAACCCGTTGAACCCCATT), Rev (CCATCCAATCGGTAGTAGCG).

Immunoblotting

Protein concentration was measured at 595 nm using BioRad protein assay dye reagent (Bio-Rad, #500-0006). Equal amounts of proteins (50µg) were run on Invitrogen NuPAGE Bis-Tris protein gels, transferred onto nitrocellulose membranes using iBlot dry blotting system, blocked in Intercept (TBS) blocking buffer (Li-Cor, mixed 1:1 with TBS containing 0.1% Tween 20 (TBST), and probed with antibodies as listed under antibodies section. Membranes were scanned and analyzed using an Odyssey CLx Infrared Imaging System and Image Studio v3.1 and v5.2 (Li-Cor). For normalization we used either Revert 700 total protein stain (Li-Cor) or antibody against ERK1/2.

Antibodies

The following antibodies were diluted in Intercept blocking buffer/TBST-mix 1:1 and used for immunoblotting: CXCL10 (Abcam, #ab9938, 1:1000), CCl5/RANTES (E9S2K) (Cell Signaling Technology (CST), #36467,1:1000). IRF7 (CST), #72073,1:1000), pIRF3 (CST, #29047,1:1000), STING/TMEM173 (D2P2F) (CST, #13647, 1:1000), pTBK1-Ser172 (CST, #D52C2, 1:1000), ERK1/2 (CST, #9107S, 1:2000) or IFIT3 (E-10) (Santa Cruz Biotechnology #sc-393396,1:500). Proteins of interest were detected with near-infrared fluorescent secondary antibodies (Li-Cor; IRDye 800CW and IRDye 680CW diluted 1:10 000 and 1:20 000, respectively, in Intercept blocking buffer/TBS-mix 1:1 (no Tween)).

Immunofluorescence

Cells were grown on high precision cover glass (thickness 0.17 ± 0.005mm; Marienfeld) until desired confluency, then fixed with ice cold methanol (10-15 min at -20 ºC) and permeabilized with 0.05% saponin in PBS for 5 min at room temperature (RT). Cells were then stained with the following antibodies to detect cGAS and Lamin A: rabbit anti-human cGAS (D1D3G) (Cell Signaling #15102; 1:100), rabbit anti-mouse cGAS (D3080) (Cell Signaling #31659; 1:500), mouse anti-Lamin A (abcam #ab8980;1:100), donkey anti-mouse Alexa Fluor 568 (Molecular Probes #A10037; 1:500), and donkey

anti-rabbit Alexa Fluor 488 (Jackson #711605152; 1:500). Primary and secondary antibodies were diluted in PBS containing 0.05% saponin and incubated for 1–2 h at RT for primary and 30min-1h for secondary antibodies. After antibody staining, the samples were mounted on microscope slides (Menzel–Glaser) with Mowiol (Sigma Aldrich #81381) containing 10 µg/ml Hoechst 33342 (Invitrogen #H3570) and kept in the dark and in a cold room until imaged. For quantifications, cells were imaged on a Nikon ECLIPSE Ti2-E inverted microscope (Nikon Corp, Tokyo, Japan) equipped with a CSU-W1 dual spinning disc (50 µm pinholes & 50 µm pinholes with microlenses) confocal unit (Yokogawa Electric Corp, Tokyo, Japan), a Prime BSI sCMOS camera (Teledyne Photometrics, Tucson, AZ, US), a laser unit with 405/488/561/638nm lasers (120/100/100/100mW), and BrightLine single-band bandpass filters (447/60nm, 525/50nm, 600/52nm, 708/75nm). ROI were randomly selected and Z-stacks with sectioning of 0.6 µm were collected with a 40X Plan Apo λ objective (NA 0,95, Air). The total number of cells was assessed by segmentation of nuclei using NIS-Elements AR (Nikon) and then the fraction of micronucleated cells and cGAS positive micronuclei were scored. More than1000 cells were counted for each experiment. Representative images were taken at Zeiss LSM 780 confocal microscope a laser diode 405–430 CW (405 nm), a DPSS-561 10 (561 nm), and (Argon laser (488 nm). The objective used was a Zeiss plan-apochromat ×63/1.40 oil DIC M27.

Analysis of mitochondrial membrane potential:

The cells were cultured until 80% confluency, and then stained with 200nM of tetramethylrhodamine, ethyl ester, perchlorate (TMRE, Invitrogen) and 300nM Mitotracker Green (MTG, Invitrogen) for 30 minutes at 37°C. The cells were harvested by trypsinization, and then stained with Fixable viability stain 780 (FVD, Invitrogen, 1:1000 in PBS) for 30 min on ice. The cells were washed twice and resuspended in FACS buffer (PBS with 2% FCS and 0.2 mM EDTA) and run on a BD LSR II flow cytometer in biological and technical triplicates recording 50,000 events per well. The fcs files were analyzed in FlowJo10.2 software. Cell gates were set after the exclusion of duplets and dead cells, followed by gating on TMRE and MTG positive cells. Mitochondrial abundance and mitochondrial membrane potential (MMP) was measured with median fluorescence intensity (MFI) of MTG and TMRE, respectively. The MFI of TMRE was normalized to MFI of MTG to determine the mitochondrial activity of the total mitochondria present within each cell population.

Detection of mitochondrial reactive oxygen species

The cells were cultured until 80% confluency, and then stained with 5µM of MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen) for 1 hour at 37°C. The cells were harvested by trypsinization, washed twice in PBS and stained with Fixable viability stain 780 (FVD, Invitrogen, 1:1000) for 30 mins on ice. The cells were washed twice and resuspended in FACS buffer (PBS with 2% FCS and 0.2 mM EDTA) and run on a BD LSR II flow cytometer in biological quadruplicates and technical triplicates recording 50,000 events per well. The fcs files were anlayzed in FlowJo 10.2

software. Cell gates were set after the exclusion of duplets and dead cells, followed by gating on MitoSOX positive cells. The percentage of cells positive for MitoSOX was used to determine the mitochondrial ROS within each cell population.

Detection of total and cytosolic mtDNA

The cells were cultured in 6-well plates until 80% confluency was achieved. For isolation of cytosolic DNA, cells were washed once in PBS, scraped and lysed on ice using 100 µl 0.1% NP-40, transferred to eppendorf tubes and incubated for 15-20 min on ice, and centrifuged at 16,000 × g for 20 min at 4°C (15). The supernatants were transferred to new tubes and stored at -80ºC until isolation of DNA. For isolation of total cellular DNA, the cells were harvested by trypsinization, centrifuged at 340 × g for 5 min, the supernatants were removed and the pellets were stored at -80ºC until isolation of total (nuclear, mitochondrial and cytosolic) DNA. All DNA samples were isolated by QIAamp DNA mini kit (#51306). To avoid clogging of the columns, we used only half of the sample material for the isolation procedures. For isolation of total DNA, the cell pellets were dissolved in 185 µl of PBS, the exact final volume was determined, half of this was removed and kept as backup while the remaining was adjusted up to 200 µl with PBS. The further protocol including proteinase K (20 µl), AL-lysis buffer (200 µl) and ethanol (200 µl) was as described by the manufacturer, and the samples were eluted with 200 µl water. For isolation of cytosolic DNA, the exact sample volumes were determined, half was removed and kept as backup while the remaining was adjusted up to 100 µl with 1% NP-40 lysis buffer. To get samples as comparable as possible to the samples used for isolation of total DNA, we added PBS (200 µl), AL-lysis buffer (100 µl) and ethanol (200 µl) before proceeding as described by the manufacturer and eluting in 200 µl water. Quantitative PCR was performed on equal volumes of all DNA samples from both cell lines using primers specific for mitochondrial DNA (COX1, Dloop1, Dloop2) and nuclear DNA (18S rRNA and Tert) (primer sequences above under primer section). Using this extraction protocol, nuclear DNA was present only in very low amounts in the cytosolic fraction (CT-values for 18S rRNA ranging from 29.8 – 31.4 and Tert from 29.8 to undetermined), indicating that nuclear rupture occurred at minimum. The relative abundance of both total and cytosolic mtDNA was calculated relative to the non-metastatic cell line 67NR (see explanation below).

<u>Calculations used on qPCR data to determine content of mitochondrial DNA in total and cytosolic DNA</u>

The following calculations were done, based on (16) with small adjustments:

For total DNA →

ΔCT_{tot} = CT (mitochondrial primer) – CT (nuclear primer = 18S rRNA or Tert) (to relate the amount of mitochondrial DNA in the total DNA to cell numbers)

Average ΔCT_{tot} = average of ΔCT_{tot} values obtained for the total DNA from your sample (67NR and 66cl4)

$\Delta\Delta CT_{tot}$ = ΔCT_{tot} (for your sample of interest; 67NR or 66cl4) - average ΔCT_{tot} 67NR (to evaluate if there is a difference in mitochondrial DNA per cell in total DNA from 66cl4 as compared to 67NR)

Relative abundance of mitochondrial DNA in the total DNA sample = $2^{\wedge(-\Delta\Delta CTtot)}$

For cytosolic DNA →

Here the average ΔCT_{tot} for each cell type (see above) is included in the calculations.

ΔCT_{cyt} = CT (mitochondrial primer; 67NR or 66cl4) – average ΔCT_{tot} (for 67NR or 66cl4) (the amount of mitochondrial DNA in cytosolic DNA from each cell line is related to the total amount of mitochondrial DNA per cell for that particular cell line)

Average ΔCT_{cyt} = average of ΔCT_{cyt} values obtained for the reference sample = 67NR

$\Delta\Delta CT_{cyt}$ = ΔCT (for your sample of interest; cyt DNA 67NR or 66cl4) – average ΔCT_{cyt} 67NR (to evaluate if there is a difference in mitochondrial DNA per cell in cytosolic DNA from 66cl4 as compared to 67NR)

Relative abundance of mitochondrial DNA in the cytosolic DNA sample = $2^{\wedge(-\Delta\Delta CTcyt)}$

Use of public databases

Kaplan-Meier plotter (17) is an online database that utilizes data from multiple cDNA microarrays for examining prognostic markers in several cancer types, including breast cancer (18, 19). Relapse-free survival (RFS) and overall survival (OS) of IFN-I markers were analyzed in estrogen receptor (ER) positive (ER positive subtype), ER-negative, progesterone receptor (PR)-negative and human epidermal growth factor receptor 2 (HER2) negative (Triple negative breast cancer (TNBC) subtype and HER2 enriched subtype (ER negative, PR negative, Her2 positive) (20). A set off 11 IFN-I genes were analyzed using the mean expression of 11 different trancripts. Broad Institute Cancer Cell Line Encyclopedia (CCLE), (https://portals.broadinstitute.org/ccle) (21) was used for gene expression analysis in human breast cancer cell lines.cBioPortal (22) is an open access database that allows visualization and analysis of large-scale cancer genomics data sets(23, 24).Gene exepression (mRNA expression) data were used to identify the correlation between expression of various genes within the Molecular taxonomy of breast cancer international consortium (METABRIC) cohort (25-28).

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Paper II

Local arginine restriction dampens IFN-I response via autophagy in breast cancer

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Paper III

A role of Arginase-1-expressing myeloid cells in cachexia

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ISBN 978-82-326-7080-2 (printed ver.)
ISBN 978-82-326-7079-6 (electronic ver.)
ISSN 1503-8181 (printed ver.)
ISSN 2703-8084 (online ver.)

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Science and Technology