Exploiting tumor inflammation to increase the therapeutic impact of biguanides in oncology

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Abstract

Breast cancer is the most common cancer among Canadian women; 1 in 8 women are expected to develop breast cancer in their lifetime. Breast cancer is grouped into four distinct types, which informs treatment strategies and patient outcomes. Indeed, some of these breast tumor types are more likely to develop therapeutic resistance, leading to relapse for these women. Moreover, regardless of tumor type, treatments are rarely curative for women who are diagnosed with breast cancers at an advanced stage, including metastatic disease. For these reasons, identifying therapies that target essential vulnerabilities is necessary for improving survival outcomes of women with such hard-to-treat cancers.

Biguanides, including metformin and phenformin, suppress mitochondrial ATP production by inhibiting complex I of the electron transport chain and are typically used for the treatment of Type II diabetes. The repurposing of biguanides as anticancer agents has gained interest, but clinical trials examining the ability of metformin to improve survival in women with breast cancer have been disappointing. Even so, we have shown that when phenformin is used in combination with an inflammatory mediator, poly IC, to treat models of breast cancer in mice, we increase the cytotoxicity of phenformin by inducing an increase in oxidative stress. I hypothesize that this increase in sensitivity to biguanides is due to an increase in cytotoxic neutrophils, elicited by the combination therapy.

To this end, a monoclonal antibody was administered to deplete neutrophils from two models of breast cancer in mice, representative of Luminal B and Triple Negative breast cancers. These mice were then administered phenformin and poly IC and tumor volume was measured over time and compared to a control. Furthermore, the elicited neutrophil population was

functionally characterized through *in vivo* and *in vitro* assays looking at metastatic potential, maturation, cytotoxicity, and reactive oxygen species (ROS) production.

Neutrophils were required for the anti-neoplastic efficacy of the combination therapy consisting of phenformin and poly IC. The neutrophils were found to be cytotoxic *in vitro* and capable of killing cancer cells *in vivo*.

Abrégé

Le cancer du sein est le cancer le plus courant chez les femmes canadiennes ; on s'attend à ce qu'une femme sur huit développe un cancer du sein au cours de sa vie. Le cancer du sein est regroupé en quatre types distincts, qui déterminent les stratégies de traitement et les résultats pour les patientes. En effet, certains de ces types de tumeurs du sein sont plus susceptibles de développer une résistance thérapeutique, entraînant une rechute chez ces femmes. De plus, quelque soit le type de tumeur, les traitements sont rarement curatifs pour les femmes chez lesquelles un cancer du sein est diagnostiqué à un stade avancé, y compris une maladie métastatique. Pour ces raisons, l'identification de thérapies ciblant les vulnérabilités essentielles est nécessaire pour améliorer les résultats de survie des femmes atteintes de cancers aussi difficiles à traiter.

Les biguanides, y compris la metformine et la phenformine, suppriment la production d'ATP mitochondriale en inhibant le complexe I de la chaîne de transport d'électrons et sont généralement utilisés pour le traitement du diabète de type II. La réutilisation des biguanides en tant qu'agents anticancéreux a suscité de l'intérêt, mais les essais cliniques examinant la capacité de la metformine à améliorer la survie des femmes atteintes d'un cancer du sein ont été décevants. Néanmoins, nous avons montré que lorsque la phenformine est utilisée en association avec un médiateur inflammatoire, le poly IC, pour traiter des modèles de cancer du sein chez la souris, nous augmentons la cytotoxicité de la phenformine en induisant une augmentation du stress oxydatif. Je suppose que cette augmentation de la sensibilité aux biguanides est due à une augmentation des neutrophiles cytotoxiques, induite par la polythérapie.

A cette fin, un anticorps monoclonal a été administré pour épuiser les neutrophiles de deux modèles de cancer du sein chez la souris, représentatifs des cancers du sein Luminal B et Triple Négatif. Ces souris ont ensuite reçu de la phenformine et du poly IC et le volume de la tumeur a été mesuré au fil du temps et comparé à un témoin. En outre, la population de neutrophiles suscitée a été caractérisée de manière fonctionnelle par des essais *in vivo* et *in vitro* portant sur le potentiel métastatique, la maturation, la cytotoxicité et la production d'espèces réactives de l'oxygène.

Les neutrophiles étaient nécessaires pour l'efficacité anti-néoplasique de la thérapie combinée composée de phenformine et de poly IC. Les neutrophiles se sont révélés cytotoxiques *in vitro* et capables de tuer les cellules cancéreuses *in vivo*.

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Contribution of Authors

The following is my master's thesis written for the Department of Experimental Medicine for McGill University and is entitled "Exploiting tumor inflammation to increase the therapeutic impact of biguanides in oncology". It is composed of 4 chapters in accordance with the guidelines set in place by the Department of Graduate and Postdoctoral studies.

This project was completed in close collaboration with a colleague in the lab, Dr. Joey Heath, who initiated the project prior to the start of my master's research. Dr. Heath had generated the flow cytometry analysis, Ly6G depletion and maturation data prior to my arrival in the lab, but are included to rationalize the experiments I have completed. Dr. Heath also played a crucial role in the remainder of the experiments as well.

Phenformin/ poly IC treatment is a therapy published by the Ursini-Siegel lab in Nature Communications in 2021. Single-cell RNA sequencing was performed by Genome Quebec and analysis was done in collaboration with Dr. Claudia Kleinman (Samantha Worme and Alva Annett). Tumor lysates were prepared with the help of Young Im and Elias Maldonado, and ELISAs performed by EVE Technologies. Young Im also performed some of the drug treatments and tumor measurements.

Dr. Ursini-Siegel and Dr. Heath managed project conceptualization and Dr. Ursini-Siegel oversaw project supervision and revised and made suggestions for the editing of this thesis.

Drug preparation, tumor measurements, sample preparation for single-cell RNA sequencing (CD45 isolation), sample preparation for ELISAs (preparation of tumor lysates), neutrophil isolation, co-culture assays, lung and tumor harvesting, luminol/oxidative burst assays and HL-60 differentiation and oxidative burst measurement were performed by Sabrina Rezzara Richard.

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List of Abbreviations

2-DG	2-Deoxy- d-glucose
ADCC	Antibody-Dependent Cellular Cytotoxicity
AI	Aromatase Inhibitor
ALK5	Activin Receptor-Like Kinase 5
AMP	Adenosine Monophosphate
AMPK	AMP-activated protein kinase
AO	Acridine Orange
APC	Antigen Presenting Cell
ARG1	Arginase 1
ATP	Adenosine Triphosphate
ATRA	All-Trans Retinoic Acid
BCG	Bacillus Calmette-Guérin
BCL-2	B-cell Leukemia/Lymphoma 2
BPI	Bactericidal/Permeability- Increasing Protein
BSA	Bovine Serum Albumin
CCL2	Chemokine (C-C motif) ligand 2
CCL5	Chemokine (C-C motif) ligand 5
CCR2	CCR2 (C-C Motif Chemokine Receptor 2)
CD	Cluster of Differentiation
CGD	Chronic Granulomatous Disease
CR	Complement Receptor
CTLA-4	Cytotoxic T-lymphocyte Associated Antigen-4
DAPI	4',6'-Diamidino-2-Phenylindole
DC	Dendritic cell
DCA	Dichloroacetate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dsRNA	Double Stranded RNA
EDTA	Ethylenediamine Tetra-Acetic Acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked Immunosorbent Assay
ER	Estrogen Receptor
ERK1/2	Extracellular Signal-Regulated Protein Kinases
ETC	Electron Transport Chain
Fab	Antigen-Binding Fragment
FBS	Fetal Bovine Serum
Fc	Crystallizable Fragment
FcγRs	Fc Gamma Receptor
fMLP	N-Formyl-Methionyl-Leucyl-Phenylalanine
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte-Monocyte Colony-Stimulating Factor
GrzB	Granzyme B

GTP	Guanosine Triphosphate
H_2O_2	Hydrogen Peroxide
HBSS	Hank's Balanced Salt Solution
HER2	Human Epidermal Growth Factor Receptor 2
HL-60	Human Leukemia 60
HOCl	Hypochlorous Acid
ICAM-1	Inter-Cellular-Adhesion-Molecule-1
ICI	Immune Checkpoint Inhibitors
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IKKβ	IkappaB kinase
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IRS-1	Insulin Receptor Substrate-1
LFA-1	Lymphocyte Function-Associated Antigen 1
LPS	Lipopolysaccharide
MAC-1	Macrophage 1 Antigen
MDSC	Myeloid-Derived Suppressor Cells
MFP	Mammary Fat Pad
MHC	Major Histocompatibility Complex
MMP9	Matrix Metalloprotease 9
MPO	Myeloperoxidase
MT1	Metallothionein 1
mTOR	Mammalian Target of Rapamycin
NAC	N-Acetyl-L-Cysteine
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NE	Neutrophil Elastase
NET	Neutrophil Extracellular Trap
NF-κB	Nuclear Factor Kappa B
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NK	Natural Killer
NLR	Neutrophil to Leukocyte Ratio
NOD	Nucleotide-Binding and Oligomerization
NOX	NADPH Oxidase
NQO1	NAD(P)H quinone oxidoreductase 1
OCT	Organic Cation Transporters
OXPHOS	Oxidative Phosphorylation
PAD4	Peptidyl-Arginine Deaminase 4
PAMP	Pathogen-Associated Molecular Patterns
PARP1	Poly (ADP-ribose) Polymerase 1
PBS	Phosphate-Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PD-1	Programmed Death-1
PD-L1	Programmed Death Ligand-1

PI	Promidium Iodide
PI-3K	Phosphatidylinositol 3-Kinase
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
Poly IC	Polyinosinic-Polycytidylic Acid
PR	Progesterone Receptor
PrCR	Programmed Cell Removal
Prf1	Perforin-1
PROK2	Prokineticin 2
PRR	Pathogen Recognition Receptor
PSGL-1	P-Selectin Glycoprotein Ligand 1
PyMT	Polyoma Middle Tumor-Antigen
Rab	Ras-Associated Binding
Rac-2	Ras-Related C3 Botulinum Toxin Substrate 2
Retnlg	Resistin-like gamma
ROS	Reactive Oxygen Species
S100A8/9	S100 calcium-binding protein A8/A9
SNAP	Synaptosome-Associated Protein
SNARE	Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor
SOD	Superoxide Dismutase
STFA2	Stefin A2
STING	Stimulator of Interferon Genes
TAM	Tumor-Associated Macrophages
TAN	Tumor-Associated Neutrophils
TCR	T-cell Receptor
TGF-β	Transforming Growth Factor β
TLR	Toll-like Receptor
TME	Tumor Microenvironment
TNBC	Triple Negative Breast Cancer
TNF	Tumor Necrosis Factor
TP53	Tumor Protein 53
Tregs	Regulatory T cells
TRMP2	Transient Receptor Potential Cation Channel, subfamily M, member 2
VAMP	Vesicle-Associated Membrane Proteins
VEGF	Vascular Endothelial Growth Factor

Chapter 1: Review of the Literature

Breast Cancer

Morphology of the Human Mammary Gland

The human breast or mammary gland is a highly efficient organ mainly used to produce milk and is a mass consisting of glandular, fatty and fibrous tissues. The glandular tissue is composed internally of 15 to 25 lobes that radiate around the nipple(1). The lobes are separated by connective and adipose tissue. Each lobe consists of 20-40 lobules, smaller milk ducts, that contain alveoli, the clusters of cells where milk is produced. When breastfeeding, lactiferous ducts collect the milk from each lobule and drains it to a lactiferous sinus, where milk is stored, before being released from the nipple (2).

Mammary development begins during embryogenesis, where both males and females have a similar rudimentary mammary gland at birth. Further mammary development is triggered by the onset of puberty in females, which is highly dependent on high levels of estrogen produced by the ovary, as well as progesterone (2,3). The glandular structure is completely formed after puberty and begins to undergo sequential waves of proliferation and apoptosis marked by the menstrual cycle (4). However, breast development does not stop with puberty; significant developmental changes occur during pregnancy, lactation, and involution (5). The dynamic nature of this organ is supported by a subset of cells known as mammary stem cells. Mammary stem cells have a unique capacity for self-renewal as well as generating the three cell lineages that comprise the lobuloalveolar structure of the mammary gland: myoepithelial cells, ductal epithelial cells and alveolar epithelial cells (6). Under the regulation of fluctuating hormones, as well as local stromal epithelial interactions, these cells proliferate significantly

during pregnancy to produce structures specialized for high levels of milk production. After lactation is complete, a majority of these cells then undergo rapid apoptosis, and the remaining cells remodel into a structure that resembles the pre-pregnant state during a process termed involution (6,7). Understanding the factors and mechanisms that regulate breast development is crucial, because alterations in breast structure and function, specifically during the menstrual cycle and during involution, can predispose the tissue to malignant changes and thus, the development of breast cancer (8-10).

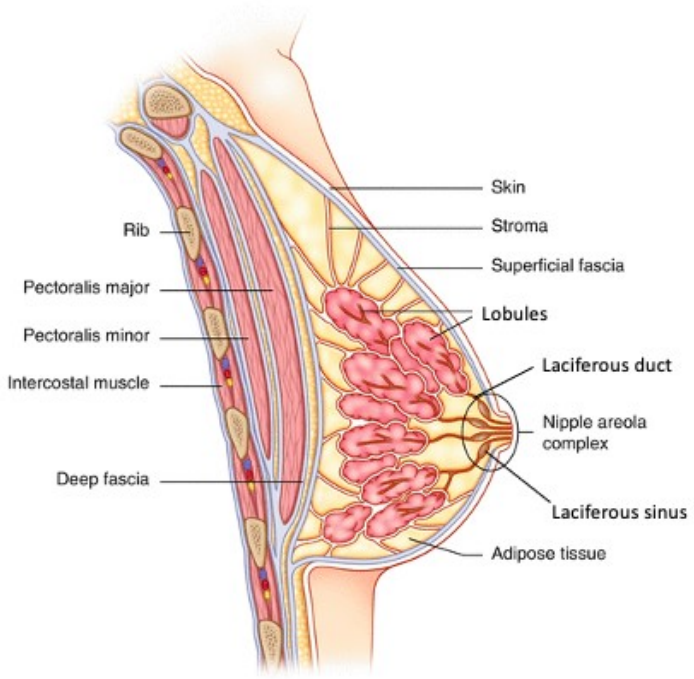

Figure 1. Schematic of the human mammary gland.
The human mammary gland contains 15 to 25 lobes; each lobe is subdivided into many smaller lobules which contain alveoli which produce milk. Lactiferous ducts collect milk from each lobule and drain it into the lactiferous sinus where it is stored before being released from the nipple. Adapted from Aydiner and McGuire 2016 (11).

Breast Cancer and Molecular Subtypes

Breast cancer is a genetically and clinically heterogenous disease characterized by the formation of tumors in the mammary tissue and is the most common cancer in women, excluding non-melanoma skin cancers (12). It is a disease with multiple subtypes that are typically classified from an immunohistochemical (IHC) perspective based on the expression of the hormone receptors estrogen (ER), progesterone (PR) and human epidermal growth factor (HER2) (13). Expression of the ER biomarker is an indispensable diagnostic factor as approximately 75% of breast tumors are defined by high ER expression (14,15). PR expression occurs in more than 50% of ER+ breast cancers. Its expression is positively correlated with increased overall survival and time to recurrence while its absence is associated with a more aggressive disease course (16). HER2 amplification or overexpression occurs in approximately 15-30% of invasive breast cancers (17,18). Its amplification is a significant predictor of both time to relapse and overall survival in patients with breast cancer (18). A lack of expression of these three markers (ER-, PR-, HER2-) is referred to as triple negative breast cancer (TNBC). TNBC constitutes about 20% of breast cancers and is most common in women under 40 and in African American women (19,20). Ultimately, expression of the forementioned biomarkers, or lack of expression, is a highly valuable prognostic tool that plays a key role in determining breast cancer subtype, grade as well as the treatment plan and predicted response rate.

Accordingly, there are five widely recognized molecular subtypes of breast cancer: Luminal A, Luminal B, HER2-positive, Normal-like and Basal-like (21,22). Most Luminal A tumors are identified as being ER+, PR +/- and HER2-, and have low expression of the cell proliferation marker Ki-67. Clinically, these tumors frequently have low histological grade, are slow growing, have a low relapse rate and the best prognosis. Patients diagnosed with Luminal A

breast cancer have a 5-year overall survival of 96.5% and benefit most from hormonal therapy with limited benefit from chemotherapy (23). Typically, ER+ breast cancer treatment may take on two approaches: inhibiting the production of estrogen so it may not bind to the receptor or targeting the receptor itself (24). Tamoxifen is used to treat breast cancer in both pre- and post-menopausal women or may be used as an adjuvant therapy. In breast tissue, it antagonistically competes with estrogen for binding to the estrogen receptor. Women suffering from ER+ breast cancer are most likely to benefit from this therapy and evidence indicates that long-term use of Tamoxifen may significantly reduce recurrence and mortality (25,26). In premenopausal women, estrogen is predominately produced by the ovaries, thus ovarian suppression drugs such as leuprolide or goserelin may be used to inhibit estrogen production (27). In postmenopausal women, estrogen is no longer produced by the ovaries and is produced from non-glandular sources via the aromatase enzyme (28). Consequently, aromatase inhibitors (AIs) are used to stop the production of estrogen by inhibiting the cytochrome P450 enzyme aromatase which is responsible for the conversion of androgens to estrogens (29). The first two generations of AIs were effective in treating breast cancer but had significant side effects. Thus, the use of third-generation AIs has become the gold standard of care for early and advanced breast cancer in postmenopausal women as they are more specific (30). These drugs can be categorized as steroidal (exemestane) and nonsteroidal (anastrozole, letrozole and vorozole) where steroidal lead to irreversible inhibition of enzymatic activity and nonsteroidal are reversible competitive inhibitors (31). Large clinical trials have been done to compare the use of various AIs and Tamoxifen alone and in combination and have found AIs to be much more effective in reducing recurrence rates and mortality rates in postmenopausal women (32-35).

Luminal B tumors are more aggressive than Luminal A tumors and are associated with poorer prognosis, higher recurrence rate and a higher grade (36). They comprise 15-20% of breast cancers and are characterized as being ER+, PR +/-, HER2+/- and Ki-67 high (37). Despite being ER+, a large majority of Luminal B cancers tend to not respond to hormonal therapy alone but seem to respond better to neoadjuvant chemotherapy than Luminal A breast cancers (38,39).

The HER2+ subtype is typically ER- and are highly proliferative (Ki-67 high). Tumors are characterized by gene amplification at chromosome 17q12-q21 where several genes associated with breast cancer development are upregulated. Among the genes that are located within this region, HER2 typically presents the highest level of amplification (40). Breast cancers can have up to 25-50 copies of the HER2 gene and 40-100-fold increase in HER2 protein (41). Patients have a worse prognosis compared to luminal tumors. In fact, HER2 amplification 3-fold or greater is associated with shorter disease-free survival (42). Fortunately, the discovery of monoclonal antibodies against the HER2 protein, such as trastuzumab, has been shown to increase survival (43). Since then, several second and third generation antibody-drug conjugates including trastuzumab emtansine and trastuzumab deruxtecan have been developed to deliver cytotoxic drugs specifically to cancer cells and have demonstrated remarkable results (44,45).

Normal-like tumors represent a small proportion of breast cancers, accounting for about 8% of all breast cancer cases and are poorly characterized. These tumors share a similar IHC status to Luminal A breast cancers are characterized by normal breast tissue profiling (46). Interestingly, several studies have shown that for this subtype specifically gene expression covariate analysis is more predictive of patient survival than clinical features covariate analysis (47).

The basal-like subtype is characterized by its aggressiveness, early relapse, high proliferation rate and its tendency to present itself in advanced stages (13). Basal-like cancers overexpress P-cadherin and epidermal growth factor receptor (EGFR) and are associated with frequent mutations in the tumor protein 53 (TP53) gene and inactivation of the retinoblastoma pathway(48). Importantly, these tumors do not express ER, PR and HER2. However, it is critical to recognize that the terms basal-like and triple negative are not completely synonymous. Triple negative refers to IHC expression whereas basal-like is defined via gene expression analysis, but there is approximately 80% overlap across studies. In clinical settings, molecular profiling is not frequently performed, thus basal-like cancers are often treated as TNBC. TNBC is an important area of research for both clinicians and researchers because unlike other subtypes, there is currently no specific targeted therapy available. Thus, the standard of care for nonsurgical TNBC remains nonspecific chemotherapy (49). This includes drugs targeting DNA repair complexes (platinum and taxanes) (50), P53 (taxanes) (51), cell proliferation (anthracycline) (52) and targeted therapies such as poly (ADP-ribose) polymerase 1 (PARP1) inhibitors (53). Unfortunately, though the most effective option currently available for treating TNBC, these drugs do have limited efficacy with TNBC patients having a decreased 3-year overall survival rate, increased risk of visceral metastases and shorter post recurrence survival than patients with non-TNBC (54). Overall, TNBC manifests itself as an aggressive disease for which a subset of patients with early stages may be cured with chemotherapy. The remaining patients usually experience early relapse and poor outcome, highlighting the need for more effective therapies for patients with such hard-to-treat cancers.

Currently, immunotherapy has emerged as a novel therapeutic approach for the treatment of various cancers including breast cancer. It is well known that cancer cells create an

immunosuppressive environment to prevent it from being recognized by the immune system (55). Immunotherapy harnesses the power of the immune system to fight cancer progression by sensitizing the patient's immune system to cancer cells, thus aiding in tumor detection and destruction. Traditional immunotherapies, notably immune checkpoint inhibitors (ICIs) such as monoclonal antibodies against cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) (ipilimumab), programmed death-1 (PD-1) (nivolumab, pembrolizumab and cemiplimab), and programmed death ligand-1 (PD-L1) (atezolizumab, avelumab and duravalumab), rely heavily on the adaptive immune system for efficacy (56,57). Unfortunately, many patients do not respond to ICIs mainly due to primary or acquired resistance, limiting clinical efficacy (58,59). This requires the identification of novel ways of activating the immune system.

Biguanides

Biguanides (including metformin and phenformin) are a class of drugs often used as a treatment for type 2 diabetes. Biguanides suppress mitochondrial adenosine triphosphate (ATP) production by inhibiting complex I of the electron transport chain (ETC) (60). Complex I, also known as nicotinamide adenine dinucleotide hydrogen (NADH) ubiquinone oxidoreductase, is the first enzyme of the respiratory chain and is responsible for the oxidation of NADH, which is generated through the citric acid cycle, in the mitochondrial matrix (61). During this process, two electrons from NADH oxidation are used to reduce ubiquinone to ubiquinol in the mitochondrial inner membrane, supplying complexes III and IV with the electrons needed to convert O_2 to water. Furthermore, four hydrogen ions pass from the mitochondrial matrix to the intermembrane space, contributing to the electrochemical gradient (62). A direct consequence of the use of biguanides is a decrease in oxidative phosphorylation, resulting in a compensatory increase in glycolysis (63). This amounts to an increase in glucose uptake by cells, thus improving insulin

sensitivity. Moreover, metformin may also non-competitively inhibit mitochondrial glycerophosphate dehydrogenase, an enzyme responsible for the conversion of lactose to glucose, ultimately resulting in impaired hepatic gluconeogenesis (64).

Inhibition of complex I also leads to an increase in the adenosine monophosphate (AMP) to ATP ratio; an activator of AMP-activated protein kinase (AMPK) (65). AMPK regulates the activity of various metabolic enzymes and regulators in pathways that are critical for cancer cell proliferation. In fact, AMPK activation may act as a tumor suppressor by regulating energy levels, imposing metabolic checkpoints, and inhibiting cell growth (66,67). Furthermore, suppression of AMPK has been shown to promote cancer development (68,69), promote breast cancer metastasis (70), and down-regulation is associated with advanced breast cancer and poorer outcomes in certain contexts (70).

Evidence from retrospective population-based studies have demonstrated that drugs typically prescribed for other indications, such as diabetes, may be associated with reduced risk of cancer-related mortality (71). The repurposing of biguanides as anticancer agents has gained interest and preclinical studies have demonstrated their potential in oncology (72). Unfortunately, results using this class of drugs have been disappointing in breast cancer clinical trials. A phase III clinical trial looked at the effects of metformin on invasive disease-free survival in non-diabetic breast cancer patients and found that the addition of metformin to standard breast cancer treatment did not significantly improve invasive disease-free survival. Furthermore, metformin did not significantly ameliorate overall survival nor breast cancer-free interval (73). Ultimately, there is a clear requirement to identify drugs that can be used in combination with biguanides to increase efficacy. The lack of durable responses of biguanides as monotherapies in oncology can be explained by 2 factors. Firstly, most pre-clinical studies use

higher doses of metformin than what is clinically achievable (74,75). Secondly, cancer cells exhibit extreme metabolic flexibility, therefore during mitochondrial complex I inhibition, there is a compensatory increase in glycolysis in cancer cells, allowing them to survive (76). Drug combination strategies that lower the concentration of biguanides required for antineoplastic activity may revitalize the therapeutic potential of biguanides in oncology.

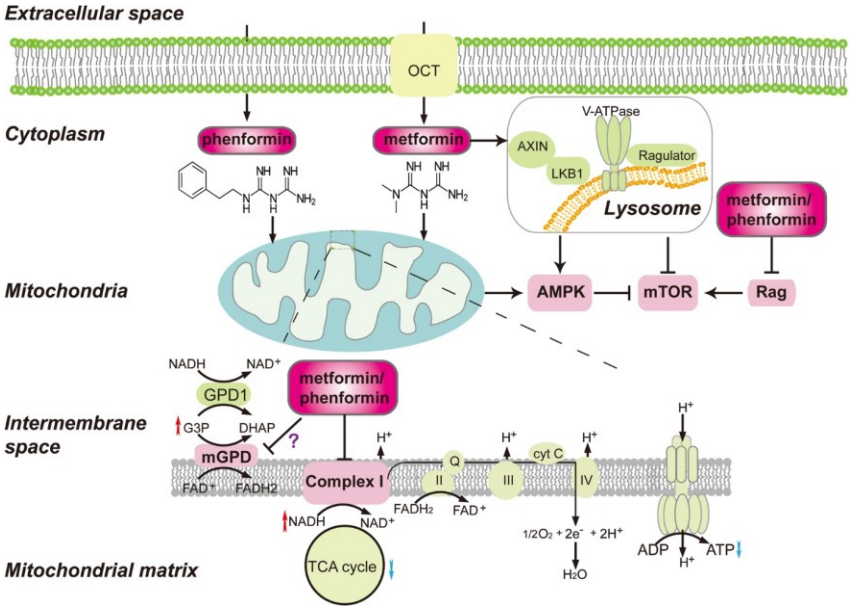

Figure 2. Mechanism of action of biguanides.
Biguanides (metformin and phenformin) are typically used for the treatment of Type II diabetes. They inhibit complex I of the electron transport chain which triggers an increase in the AMP: ATP ratio, leading to AMPK activation. Obtained from Zhao et al., 2021 (65).

Reactive Oxygen Species

Biguanides hold an underappreciated role as mitochondrial reactive oxygen species (ROS) generators (62). ROS is a collective term for oxygen containing species that are more reactive than molecular oxygen such as superoxide anion, hydrogen peroxide, and hydroxyl radical. They are formed by the partial reduction of oxygen and are highly reactive and unstable

molecules (77). Intracellular ROS are generated primarily from complexes I and III of the mitochondrial respiration chain. During respiration, electrons released from the electron transport chain incompletely reduce O_2 to form superoxide (78). Other endogenous sources of ROS include peroxisomes, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidases (NOXs) and the activation of inflammatory cells such as macrophages and neutrophils. Finally, environmental factors may act as ROS producers such as ionizing radiation, UV radiation, xenobiotics, smoking and pollution (79).

ROS play a pivotal role in biological processes and are a normal cellular metabolic by-product. Under a physiological state, the level of ROS is in a dynamic equilibrium where there is a balance between the amount of ROS being generated, through the mechanisms mentioned above, and the amount being removed, typically through antioxidant systems (80). Thus, in normal cells, ROS levels are maintained relatively low and fluctuate with changes in metabolic activity. These changes may induce shifts in signaling and gene expression pathways that control diverse cellular functions including cell proliferation (81), differentiation (82), circadian rhythms (83), vascular remodeling (84), and immunological functions (85).

Following changes in signalling, in healthy cells, homeostasis must be maintained through the elimination of ROS. Accordingly, cells have evolved numerous detoxification processes to neutralize extra ROS, predominantly utilizing antioxidant enzymes (86). Antioxidants are molecules that can counteract free radicals and neutralize oxidants and are categorized into three groups: enzymatic antioxidants (superoxide dismutase, catalase, glutathione peroxidase); hydrophilic antioxidants (urate, ascorbate, glutathione) and lipophilic antioxidants (tocopherol, carotenoid and ubiquinol) (87). Lack or inhibition of antioxidants may lead to the accumulation of oxidative stress and damage of organelles, DNA and proteins (88,89)

and has been implicated in the onset of diabetes (90), neurodegeneration (91), and carcinogenesis (92).

When it comes to cancer cells, ROS levels are highly regulated, given its paradoxical roles as both a tumor promoter and tumor suppressor, depending on the concentration. At moderate, yet chronically elevated ROS levels, ROS may contribute to an aggressive cancer phenotype. This may occur due to the ability of ROS to induce DNA damage, leading to mutations that can activate oncogenes or inactivate tumor suppressors (93,94). At these moderate levels, ROS may also significantly influence the tumor microenvironment (TME) and can initiate angiogenesis (95-97), metastasis (98,99), and survival of cancer cells (100). This is further supported by the fact that oncogene-induced ROS act as mitogenic signaling molecules that fuel cell hyperproliferation and fuel cancer progression (101). For these reasons, ROS, and the oxidative stress they may cause are typically associated with a poorer prognosis. However, beyond a certain threshold and in response to severe, acute oxidative stress, ROS will eventually cause cancer cell death through diverse means. For instance, ROS may trigger DNA damage response-mediated arrest resulting in halted cell proliferation and eventual apoptosis (102). Moreover, as a consequence of excessive cellular ROS, damage may arise to proteins, nucleic acids, membranes and organelles of cancer cells, inducing cell cycle arrest, senescence and autophagy (103,104). Additionally, hydrogen peroxide has been shown to induce apoptosis through the activation of various apoptotic pathways including activation of caspase-3 and the extracellular signal-regulated protein kinases (ERK1/2) pathway in leukemia, breast and bladder cancer cells (105-107). Given this fine balance between beneficial and detrimental ROS levels, cancer cells have evolved mechanisms for manipulating ROS to their advantage; notably, the upregulation of ROS scavengers and antioxidant mechanisms (108,109).

Therapeutic selectivity is essential in cancer treatment. Therefore, since cancer cells have elevated ROS generation and are under increased oxidative stress, it is plausible that malignant cells would be more vulnerable to therapies that further add oxidative insult through increased ROS generation or abrogate antioxidant systems (110). Given its potential as a tumor-killing agent, recent research has shifted towards uncovering ways of upregulating ROS to levels that elicit tumor cell death. In recent years, ROS generating agents have been found to be effective in treating certain cancers. For instance, arsenic trioxide has demonstrated its effectiveness in treating acute promyelocytic leukemia through ROS production (111,112). Furthermore, traditional chemotherapy agents such as doxorubicin can induce ROS formation and lead to apoptosis (113,114). Finally, given the dependence cancer cells have on a powerful antioxidant system, new therapies have also been found to diminish their efficacy in cancer cells, leading to apoptosis through ROS (115). Unfortunately, cancer cells have also shown resistance to ROS-inducing agents, limiting clinical efficacy (116,117). Thus, it is imperative to find novel ways that elicit ROS generation selectively in cancer cells with limited resistance to improve outcomes for cancer patients.

Inflammation

Despite the physical and chemical barriers that are set in place to protect us from infection, the body has evolved a network of cells responsible for recognizing and destroying foreign antigens. Inflammation is an ancient, evolutionarily conserved process which involves recruitment and activation of both the innate and adaptive immune systems to provide protective immunity and to maintain tissue homeostasis in response to stimuli such as microbes (bacteria, fungi, parasites), viruses, dead cells, toxins and cancer cells (118). Thus, following injury, acute

inflammation is initiated when there is an interaction between pattern recognition receptors (PRRs) on the surface of innate immune cells and pathogen-associated molecular patterns (PAMPs) (119). This interaction triggers the release of proteins and chemokines that promote the migration of leukocytes, primarily granulocytes along a chemotactic gradient to the infection site. The objective of this acute inflammatory response is to remove the inflammatory stimulus, initiate tissue healing and resolve damage (120). Persistent inflammation, lasting anywhere from months to years, which can occur due to prolonged exposure to an inflammatory stimulus or an inappropriate reaction to self-cells can lead to chronic inflammation. Chronic inflammation is referred to as a slow, long-term inflammation that does not seem to resolve itself. It is typically more prevalent in older individuals due to more circulating cytokines, chemokines and greater expression of genes associated with inflammation (121,122). An inflammatory response is characterized as normal when it is temporally restricted and marked by the upregulation of inflammatory markers when a threat is detected and resolves once the threat has passed (119). Thus, chronic inflammation is considered irregular and harmful and has been associated with increased disease risk and mortality (123). Furthermore, the clinical consequences of chronic inflammation are quite severe; it has been labeled as a contributing factor to the onset of a magnitude of diseases including, but not limited to, cardiovascular disease (124,125), arthritis (126,127), asthma (128), autoimmune diseases (129,130), diabetes (131,132), and of course, cancer (133-135). Given its varying functions, inflammation acts as a double-edged sword that must be tightly regulated to avoid its pathological consequences.

The immune response can be divided into two arms: the innate and adaptive immune responses. The innate immune system represents the first line of defense to any intruding pathogens and is activated immediately or within hours of antigen recognition, triggering a

proinflammatory response. It is an antigen-independent, non-specific response, meaning anything that is identified as foreign is a target. This system, as previously mentioned, relies on the ability of specific immune cells (typically macrophages and dendritic cells) that have PRRs, of which the family of Toll-like receptors (TLRs) is the most widely studied, to recognize PAMPs. Examples of PAMPs include lipopolysaccharide (LPS), double stranded ribonucleic acid (dsRNA) and peptidoglycan (136). Upon PAMP recognition, PRRs signal to the host to activate pro-inflammatory signalling pathways, notably through the upregulation of cytokines, and chemokines. Key inflammatory cytokines released in response to infection include interferons, interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF) (137). These cytokines, as well as others, play a key role in the rapid recruitment of innate immune cells, including neutrophils, macrophages, basophils, and dendritic cells, to the site of infection to fight and destroy any foreign pathogens.

The defense systems of the innate immune system are quite effective in fighting certain pathogens. However, this system does rely on recognition of surface molecules that are common amongst many microorganisms. Unfortunately, some microorganisms have evolved mechanisms to protect themselves. For instance, bacteria have evolved a protective capsule that enables them to remain concealed from innate immune cells and steer clear of phagocytosis (138). Consequently, the adaptive immune response has evolved to provide a broader and more finely tuned recognition to both self- and non-self-antigens. Adaptive immunity relies heavily on the interplay between a unique subset of innate immune cells with B and T cells, the two principal cell types of the adaptive immune system. Following pathogen destruction by innate immune cells, small fragments of the pathogen are kept by specific cells called antigen presenting cells (APCs) which include dendritic cells, macrophages and B cells. Once activated, APCs travel to a

nearby lymph node (139). APCs use a group of proteins known as major histocompatibility complex (MHC) to present this peptide on their surface. This peptide then gets recognized by T cells through their cell surface receptor (TCR) which triggers the rapid proliferation of T cells, all capable of recognizing the pathogen in question. Each T cell expresses a unique TCR capable of recognizing a specific antigen, ensuring each different antigen may be targeted specifically. The specificity of these receptors arises from a unique genetic mechanism that generates millions of different variants of the genes encoding the receptors (140). In turn, this ensures that millions of lymphocytes collectively carry millions of different antigen receptors capable of responding to specific pathogen infections (141). Following pathogen recognition, depending on the class of T cell, cluster of differentiation (CD)4 or CD8, different effector functions may occur. CD4 T cell activation may result in the activation of other immune cell populations. For instance, if a pathogen-specific peptide presented by a B cell (acting as an APC) is detected by a specific CD4 T cell, this may stimulate the B to produce antibodies (142). Alternatively, CD8 T cells are specialized to kill any pathogen-infected cell that they may recognize. Together, the innate and adaptive immune systems work synergistically to protect the host from any harmful entities. Thus, it is becoming increasingly popular to uncover novel ways we may manipulate the immune response to our advantage.

Inflammation in the Context of Cancer

The transformation from a normal cell to a malignant one has been summarized in a concept referred to as the hallmarks of cancer which comprise biological capabilities a cell must adopt that enable them to become cancerous (143). Of the many capabilities, one of particular interest is tumor promoting inflammation, which is typically within the TME. The correlation

between inflammation and cancer was first proposed by Rudolf Virchow in the 19th century when he observed that cancer typically originated in sites of chronic inflammation and tumor biopsies were rich in immune infiltrates. In fact, current estimates shows that 25% of tumors are correlated with chronic inflammation and sustained by chronic infections or inflammatory conditions, suggesting that in some contexts, inflammation may support tumorigenesis (144). Regardless of the cause and source of chronic inflammation, it plays a crucial role in the sculpting of the TME and influences cancer progression. Thus, our current understanding of the immune system in regard to carcinogenesis can be divided into two concepts. The immune system may adapt a pro-tumorigenic identity where inflammation promotes cancer by blocking anti-tumor activity and employing signals that promote various stages of tumorigenesis including initiation, proliferation, invasion and metastasis (145-147). Furthermore, pro-tumorigenic inflammation may shape the TME towards a more tumor-permissive state that nurtures cancer progression (148-150). On the contrary, the immune system may take on an anti-tumorigenic phenotype through immunosurveillance and through direct tumor cell killing (151-154). Thus, infiltrating immune cells can be divided into two types: tumor-antagonizing (or anti-tumorigenic) and tumor-promoting (or pro-tumorigenic) immune cells. Anti-tumor immune cells consist primarily of CD8+ cytotoxic T cells, CD4+ effector T cells, natural killer cells, dendritic cells, M-1 polarized macrophages and N-1 polarized neutrophils whereas pro-tumorigenic immune cells consist of regulatory T cells and myeloid-derived suppressor cells (MDSCs), among others.

Tumor cells wish to remain undetected by immune cells and favour their progression, thus create an immunosuppressive environment. This is achieved through a plethora of mechanisms including: the secretion of immunosuppressive, detrimental molecules including IL-10 and transforming growth factor β (TGF-β) (155-157); lowering the pH and the glucose levels

within the TME (158,159); downregulation of tumor antigen presentation (160); developing a resistance to apoptosis through B-cell leukemia/lymphoma 2 (BCL-2) upregulation (161); and releasing immunosuppressive vesicles including exosomes (162,163). Together, these defense mechanisms shape the TME and debilitate anti-tumor immune responses (164). Despite these mechanisms, tumor cells also protect themselves through the recruitment of immunosuppressive immune cells. For instance, MDSCs can induce profound anergy of effector immune cells (165). Additionally, they may recruit pro-tumorigenic regulatory T cells (Tregs) while simultaneously inhibiting effector T-cell proliferation and creating a stronger immunosuppressive environment. This is possible due to the upregulation of the cluster of differentiation CD40 receptor; this receptor is responsible for generating Tregs within the TME while inducing T cell tolerance (166). Tregs on the other hand not only suppress natural killer (NK) cell activity (167), but also mediate CD4 and CD8 proliferation and inhibit interferon (IFN)-gamma secretion (168-171), thereby leading to impairment of anti-tumor responses. Indeed, increased levels of Tregs has been linked to poorer prognosis in patients suffering from a multitude of cancers including breast, ovarian and gastric malignancies (172-174). Ultimately, several populations of immune cells, both innate and adaptive, have displayed pro-tumorigenic properties in certain contexts. However, eliciting ways to exploit the anti-tumorigenic properties of other immune cell subsets may increase prognosis.

Using the immune system to fight cancer has been one of the biggest breakthroughs in oncology, yielding the possibility of long-term clinical benefit and prolonged survival. Currently, ICIs are regarded as the most popular means for employing the immune system to fight cancer. ICI therapy is currently approved for 16 indications, however the number of responders to ICIs in 2018 was only at 12.46% (175). The success of immunotherapy relies greatly on the

immunogenic nature of the tumor; in fact, one important correlate of ICI success is the presence of a molecular signature of a pre-existing T cell response in the tumor (176). Thus, recently, researchers have gained an interest in identifying and targeting immune regulators beyond T cells, specifically through the innate immune cells.

Tumor-associated macrophages (TAMs) represent one of the main tumor-infiltrating immune cell types and play critical roles in both the innate and adaptive immune responses. TAMs are generally characterized into two categories: M1 and M2 macrophages where the former are responsible for overseeing all anti-tumorigenic roles. Macrophages rely on activation signaling to detect cancer cells and become cytotoxic. When stimulated by LPS or/and IFN-γ, macrophages differentiate into the M1 phenotype, which typically activate antimicrobial functions (177). Interestingly, it has been shown that using bacteria may activate macrophages that can fight cancer as well. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) immunotherapy was found to polarize a cytotoxic macrophage population towards killing bladder cancer cells (178). M1 macrophages may directly and indirectly kill cancer cells through various mechanisms. For instance, a live coculture model of activated macrophages and mouse mammary cancer cells found that macrophages displayed elevated phagocytic activity against the cancer cells (179). Similarly, macrophages may engage in macrophage-mediated programmed cell removal (PrCR) to eliminate diseased or damaged cells. The use of TLR agonists in macrophages, in combination with an antibody that blocks CD47, an antiphagocytic signal, on tumor cells worked synergistically to enhance PrCR and resulted in cancer cell phagocytosis by macrophages (180). Finally, macrophages may also mediate tumor cell death through nitric oxide dependent and independent apoptosis (181).

NK cells are an innate lymphoid cell, thus do not require APC-dependent antigen presentation for activation of their cytotoxic activity. NK cells target cancer cells through direct, contact-dependent cytotoxicity as well as through cytokine production for immune modulation. Target-cell cytotoxicity is mediated primarily through perforin (Prf1)- and granzyme B (GrzB)-mediated apoptosis (182). This is supported by data suggesting that Prf1-knockdown mice had an increase in circulating tumor cells, increased lung metastases and larger metastases than control mice in a human colon cancer xenograft mouse model. Furthermore, computer modelling showed that Prf1-dependent killing by NK cells decelerates the growth of primary tumors and kills 80% of circulating tumor cells (183). To this effect, NK cells may also eliminate cancer cells through the release of cytotoxic granules containing GrzB or by activating death receptors that initiate caspase cascades. In fact, it was observed that NK cells induce GrzB-mediated death at first cancer cell killing, then switch to caspase-8- mediated killing for subsequent tumor cell encounters (184).

Dendritic cells (DCs) are commonly viewed as messengers of the immune system, acting as APCs to activate T cells. A less conventional role for this immune cell subset is as a direct cytotoxic effector cell against tumors. In fact, DCs with cytotoxic activity have been referred to as killer DCs (185). DC cells isolated from human blood were activated with granulocyte-monocyte colony-stimulating factor (GM-CSF) and IL-4 and had very potent cytotoxicity against a panel of tumor cell lines including tumors of hematopoietic and epithelium origin *in vitro* (186). Various mechanisms have been identified in DC-mediated tumor cell killing. It has been proposed that activated neutrophils may induce tumor cell death through peroxynitrite production due to direct cancer cell contact (187,188). This is further supported by the fact that $iNOS^{-/-}$ and $gp91^{-/-}$ DCs' cytotoxicity was severely impaired. Inducible nitric oxide synthase

(iNOS) is an enzyme capable of producing detrimental ROS whereas gp91 is the glycosylated subunit of NOX, responsible for the production of superoxide ion (187). Peroxynitrite-mediated tumor cell death by DCs was also shown through the isolation of DCs from patients with advanced cancers which were capable of killing several human tumor cell lines ex vivo (189) following LPS activation.

Innate immune cells are finally beginning to receive the recognition they deserve as cytotoxic effector cells. This being said, more research is needed to decipher mechanistically what is going on as well as identify other immune cell subsets that may display potent anti-neoplastic activity.

Neutrophils

Neutrophils are a cell of the innate immune response and are the most abundant circulating leukocytes in humans, accounting for 50-70% of all leukocytes and a dominant leukocyte population in mice (190,191). They are a short-lived cell, with a half-life of 8 to 12 hours in circulation and 1 to 2 days in tissue, thus require constant replenishment from the bone marrow where they are produced (192). Neutrophils are generated at a rate of 10^{11} per day, which may increase to 10^{12} during infection, accordingly it is not surprising that roughly 60% of the bone marrow is dedicated to their production (193). Neutrophils are important effector cells of the innate arm of the immune system; they are the first line of defense that protect the host from bacteria, viruses, and fungi. In fact, a decrease in neutrophil numbers in the blood is associated with severe immunodeficiency in humans (194,195). Neutrophils are constantly patrolling the host for any signs of infection and upon detection respond quickly to destroy any invaders. In comparison to the adaptive immune response which is dependent on previous

pathogen interactions and required time to develop, the ability of neutrophils to kill pathogens is immediate, non-specific and does not rely on previous microbial interactions (196).

Neutrophil Recruitment

After their development in the bone marrow, mature neutrophils reach sites of inflammation via the vasculature, primarily through the postcapillary venules (197). The recruitment of neutrophils into tissue, known as the neutrophil extravasation cascade, is a multistep process. Neutrophil recruitment is initiated by changes on the surface of endothelium cells (198). This occurs in response to stimulation from inflammatory molecules such as histamine and cytokines that are released from tissue-resident leukocytes in response to pathogen detection (199). Vascular endothelial cells can also be activated directly through PRRs that detect foreign invaders (200). Upon activation, endothelial cells immediately begin upregulating molecules of the selectin family of adhesion molecules consisting of P- and E-selectins (201). P-selectin is rapidly translocated to the membrane from its storage site in Weibel-Palade bodies (202). Alternatively, E-selectin must be synthesized *de novo* in response to molecules such as IL-1, LPS and granulocyte colony-stimulating factor (G-CSF) (203). P- and E-selectin are structurally very similar, suggesting they may have overlapping functions (201). Ultimately, these two adhesion molecules work synergistically to maximize neutrophil recruitment by binding to their glycosylated ligands such as P-selectin glycoprotein ligand 1 (PSGL-1) on the surface of neutrophils, leading to the tethering of neutrophils and subsequent rolling in the direction of blood flow along the endothelium (204). Neutrophil rolling along the endothelium requires rapid formation and cleaving of adhesive bonds, thus the P-selectin to PSGL-1 bond at the rear of the cell must be disengaged at the same time as a new bond forms at the front end of

the cell (205). As neutrophils roll along the endothelium, they encounter chemokines that may act as chemoattractants, particularly those of the CXC family such as CXCL8, CXCL2 and CXCL5 (206). These chemokines signal via CXCR2 on neutrophils, which triggers the change of conformation of integrins on the neutrophil surface (207,208). Integrins act as cell surface receptors for constituents of the extracellular matrix. Thus, following neutrophil rolling is a firm arrest of neutrophils which is mediated by activated β2-integrins (LFA-1 and MAC-1) on the neutrophil interacting with inter-cellular-adhesion-molecule-1 (ICAM-1) on endothelium (209). Once firmly attached, neutrophils then flatten in an effort to minimize their surface exposure to blood flow, shear force, and collisions with circulating blood cells. They then crawl along the endothelial lumen surface in search for a permissive site for transmigration across the endothelial barrier. This crawling is directed by gradients in adhesion receptors, chemokines and endothelial cell stiffness and is mediated by MAC-1 interacting with ICAM-1 (210). In fact, in MAC-$1^{-/-}$ mice, it was found that neutrophils adhered to the endothelial surface but failed to crawl whereas LFA-$1^{-/-}$ neutrophils had extreme difficulty attaching, but the few that did had no issues with crawling (210). Once an optimal site has been found, neutrophils must emigrate from the vasculature and into the tissue. This may occur in one of two fashions: either through a paracellular route (in between endothelial cells) or a transcellular route (through an endothelial cell), which occurs 5% to 10% of the time (211). Neutrophils predominantly opt for the paracellular route, particularly at the intercellular junctions (212), but may engage in transcellular emigration depending on the vascular bed of the tissue and the intensity of tissue inflammation (213).

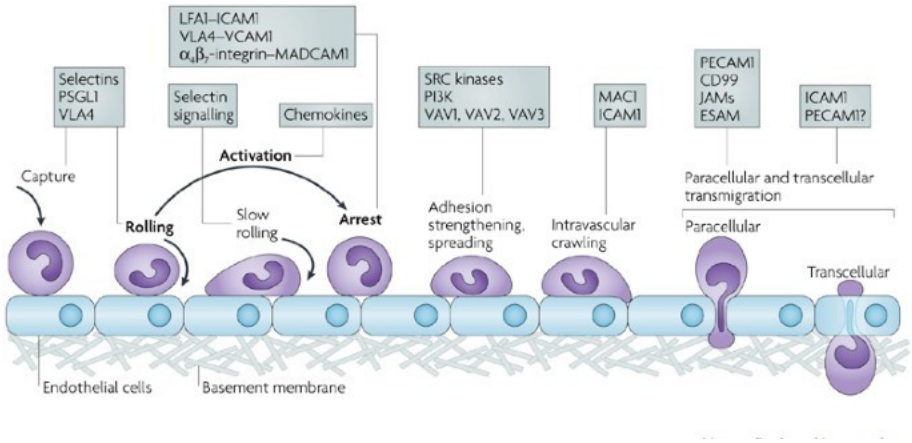

Figure 3. Neutrophil extravasation cascade.
Neutrophils may enter an inflamed tissue through a multistep process which includes capture, rolling, arrest, adhesion, spreading, crawling and transmigration. Obtained from Ley et al., 2007 (199).

Neutrophil Activation

Circulating neutrophils are quiescent in nature requiring proper signaling for activation. After entry into the infected tissue, neutrophils respond to pro-inflammatory stimuli in the tissue to become fully activated. The current belief is that neutrophil activation is a two-step process; priming occurs first, which allows neutrophils to be able to respond to activating stimuli and then become fully activated (214,215). This would make sense if priming molecules were involved in migration while PAMP detection was involved in activation. However, in the literature there is no clear distinction between priming and activating stimuli. For instance, LPS has been reported as both a neutrophil primer and activator despite being a PAMP (216-218). Thus, current evidence suggests that efficient neutrophil activation requires at least two simultaneous inflammatory stimuli (including G-CSF, N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP), TNF and LPS) rather than a priming and activating phase (219).

Neutrophils utilize various classes of cell surface and intracellular receptors for the detection of pathogen and host proteins opsonizing the microbe. PRRs are used to detect microbial motifs including LPS, double-stranded viral RNA and bacterial DNA. In brief, the primary non-phagocytic PRR on a neutrophil is the TLR. Human neutrophils express TLR1, 2, 4, 5, 6, 7, 8, 9 and 10 (220) and TLR engagement primes neutrophils to increase respiratory burst and ROS production (221), stimulate cytokine release (222) and even delay spontaneous neutrophil apoptosis (223). Other signaling PRRs include nucleotide-binding and oligomerization domain (NOD)-1 and -2, which respond to bacterial peptidoglycan recognition (224), and Dectin-1 which recognizes fungal β-glucan and in turn internalizes and eliminates fungal pathogens (225).

The complement system plays a crucial role in the innate defense against various microbes by opsonizing pathogens and inducing a multitude of inflammatory processes that help fight infection. Inactivated complement proteins circulate the blood in search of pathogen and become rapidly activated once in contact with a foreign surface. Complement activation involves a cascade of proteins that react with one another to tag microbial surfaces with plasma-derived host proteins (226). All three major complement pathways converge at the formation of C3 convertases that cleave C3 molecules to C3b (227). Depending on the environmental conditions, C3b may also be cleaved to another opsonin, iC3b, through a process mediated by serum complement factor I (228). Unstimulated neutrophils express relatively low numbers of complement receptors. However, upon activation, they upregulate expression of several complement receptors, notably complement receptor (CR) type 1 (CR1) and CR type 3 (CR3) which bind C3b and iC3b respectively (229). Together, these two receptors recognize opsonized pathogens and act as strong stimulators of neutrophil-mediated killing mechanisms, particularly

phagocytosis (227,230). Ultimately, neutrophils do not rely solely on the detection of pathogen-associated motifs to become activated; they may also recognize host proteins that are associated with infection to engage their full killing capacity.

Pathogen Killing by Neutrophils

Given the key role neutrophils play in fighting infection, it is no surprise that neutrophils have evolved several antimicrobial mechanisms to prevent pathogen infection. Thus, the process by which neutrophils kill invading pathogens depends on four primary mechanisms: phagocytosis, ROS production, degranulation, and neutrophil extracellular trap (NET) formation.

Phagocytosis

Phagocytosis is defined as the engulfment of cells, cell fragments or pathogens leading to sequestration and elimination of these entities (231). Neutrophil phagocytosis is a rapid process; uptake can occur in as quickly as 20 seconds (232). The process can be divided into four main steps: recognition of the target particle, signaling to activate the internalization machinery, phagosome formation and phagolysosome maturation (233). In neutrophils, particle recognition to initiate phagocytosis may occur through a multitude of specialized receptors located on the cell surface including the previously mentioned Dectin-1 (234), CR1 (227), CR3 (235) as well as receptors for the Fc portion of IgG, termed Fc gamma receptor (FcγRs), which recognize pathogens coated in antibody (236). Following pathogen recognition, a series of signaling events is triggered to activate phagocytosis. Notably, signaling induces changes and rearrangements of the actin cytoskeleton, causing the formation of a phagocytic cup as well as the formation of pseudopods, which are protrusions that extend around a foreign particle and engulf it (237). In

complement receptor-mediated phagocytosis, the actin cytoskeleton is used to engulf pathogens in a sinking-mediated method, followed by the guanosine triphosphate (GTP)ase RhoA activating signaling leading to F actin polymerization and ultimately phagosome formation (238). Finally, the phagosome alters its composition into a phagolysosome, a vesicle that may destroy ingested particles through a process termed phagosome maturation. During this process, the phagosome undergoes a series of fusions and fissions with endocytic organelles (including early or sorting endosomes, late endosomes, and lysosomes) to acquire the necessary proteins and conditions to destroy engulfed pathogens (239). These changes include an acidified pH (240), acquisition of hydrolytic enzymes from lysosomes (including cathepsins, proteases, lysozymes, and lipases) (241), acquisition of scavenger molecules that sequester molecules needed by bacteria (242) and acquisition of ROS generators (243). Ultimately, phagosome formation and phagocytosis is a key mediator in neutrophil killing; impaired phagosome maturation in neutrophils is associated with bacterial accumulation and inefficient clearing (244).

ROS Production

Neutrophils may also evoke their cytotoxicity through the production of ROS. This is typically manifested in the form of an oxidative burst, which is a rapid release of high levels of ROS generated primarily by the activation of NOX (245). NOX is a multicomponent enzyme system consisting of two transmembrane proteins $gp91^{phox}$ and $p22^{phox}$ as well as four cytosolic proteins $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac2 (246). Upon stimulation, $p47^{phox}$ undergoes phosphorylation and the entire subunit translocate to the membrane to form the active oxidase (247). $p47^{phox}$ phosphorylation occurs on serine residues located between Ser303 and Ser379 (248) and were shown to be phosphorylated by protein kinase C (PKC) in vitro and in

neutrophil-like HL-60 cells (249,250). Upon complete assembly, NOX transfers electrons across the phagosomal membrane from cytosolic NADPH to molecular O_2 generating O_2^- (251). Neutrophils lacking components of NOX are significantly impaired in their ability to kill pathogens (252). Furthermore, patients with chronic granulomatous disease (CGD), a rare genetic disease characterized by a lack of functional NOX, are highly compromised in their ability to clear infections, highlighting the importance of this enzyme in innate immunity (253). NOX activation can produce large amounts of O_2^-, however, these anions are very short lived, therefore are typically enzymatically converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 may further be converted to other potent ROS forms; for instance, the enzyme myeloperoxidase (MPO) may convert H_2O_2 to hypochlorous acid, a strong oxidant that exhibits high toxicity against bacterial, fungal, and viral pathogens (254-256). The antimicrobial potential of ROS generated by the neutrophil stems from its ability to cause irreversible oxidative damage to DNA, proteins, and lipids, resulting in pathogen death. NOX activation must be tightly regulated as prolonged activation can be detrimental; it may cause oxidative damage to tissue and is associated with autoimmune diseases and prolonged inflammation (257). Additionally, reduced NOX capacity caused by single nucleotide polymorphisms has been associated with the development of several illnesses including rheumatoid arthritis (258) and systemic lupus erythematosus (259).

Degranulation

Neutrophils utilize both oxidative and non-oxidative methods to kill invading microorganisms. One method of non-oxidative killing includes the use of antimicrobial proteases that are packaged into intracellular vesicles called granules which are acquired during neutrophil

maturation in the bone marrow (260). Neutrophil granules can be broadly categorized into four main types based on their protein content: primary (azurophilic), secondary (specific), tertiary (gelatinase), and secretory. The differences in protein content are not dependent on the sorting of proteins, but rather, according to the targeting-by-timing hypothesis, are dependent on the proteins being produced during the time of granule formation (261). In other words, as different granule proteins are synthesized at different stages of neutrophil differentiation, this will give rise to the varying granule types. In fact, some granule proteins are present in several granule types whereas others are unique to a particular subset suggesting that granules may actually exist in a continuum containing various amounts of different proteins (262). Primary granules, as the name implies, are the earliest to be formed in promyelocytes (263). They contain the most pro-inflammatory and many antimicrobial peptides, including MPO, bactericidal/permeability-increasing protein (BPI), serine proteases (neutrophil elastase (NE), proteinase 3, cathepsin G, and azurocidin), and α-defensins (264). Proteomic analysis has identified 852 proteins associated with primary granules, speaking to the true heterogeneity of this population (264). Primary granules fuse primarily with phagosomes, aiding in pathogen degradation through protease secretion (265). Secondary and tertiary granules on the other hand are smaller than primary granules and are formed throughout myelocyte and metamyelocyte stages (266). These granules share similar protein content and function, but some distinguishing features include that secondary granules are lactoferrin and neutrophil gelatinase-associated lipocalin (NGAL) positive and matrix metalloprotease 9 (MMP9) negative whereas tertiary granules are lactoferrin and NGAL negative and MMP9 positive (264). Secondary and tertiary granules are not very well studied but are thought to be involved in recruitment of neutrophils and other immune cell subsets alike and aid in their survival (267-269). Finally, secretory vesicles are neutrophil

intracellular storage granules formed by endocytosis during the banded and segmented stages (270). These granules contain plasma proteins, supporting their origin as endocytic granules. Additionally, secretory vesicles contained cytoskeletal proteins such as α- and β- actin as well as chemokine receptors, adhesion molecules and membrane components of NOX (264,271). These granules are suggested to be important in the initial steps of neutrophil recruitment, being mobilized during the onset of neutrophil rolling. Essentially, mobilization of secretory vesicles may play a role in transforming a neutrophil from a passive cell to one that is well suited for migration into tissues (272).

The process by which neutrophils release their granules is termed degranulation. Degranulation can occur at the plasma membrane for killing extracellular pathogens or at the phagosome for intracellular delivery. In brief, the process of neutrophil degranulation can be divided into four distinct steps. Upon receptor stimulation, granules get recruited to the phagosomal or plasma membrane where Rac2 GTPases orchestrate actin cytoskeleton remodelling (273). $Rac2^{-/-}$ mice lack the ability to release primary granules without affecting the degranulation of secondary and tertiary granules, suggesting a role limited to primary granules (274). However, all granule types rely on actin reorganization, thus likely relying on other granule-specific mechanisms (275). Next, the outer surface of the granule must be in close contact with the inner surface of the desired membrane. Docking and tethering must be initiated primarily through Rab GTPase and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes. Interestingly, depending on the type of granule, the complexes used for tethering may vary. Primary granules are enriched in synaptosome-associated protein (SNAP)-23 and vesicle-associated membrane proteins (VAMP)-1 and VAMP-7 whereas secondary and tertiary contained VAMP-1, VAMP-2, and SNAP-23 (276). Granule priming then

follows to ensure granules acquire fusion competence. Finally, an increase in intracellular calcium encourages the formation of a fusion pore between the granule and the membrane allowing fusion to occur and allows the granule to release its contents. Each type of neutrophil relies on different calcium concentrations for exocytosis with secretory vesicles requiring the most, followed by tertiary granules, then secondary granules and finally primary granules (277).

Neutrophil Extracellular Trap Formation

Upon sensing the entry of pathogens, neutrophils may release a mesh-like structure consisting of DNA and histones which is decorated in antimicrobial peptides and enzymes, a process termed NETosis, which captures and destroys the invading pathogen. The molecular mechanisms involved in NET formation and linked to the production of ROS. ROS generated by NOX during infection oxidizes DNA bases, inducing DNA damage. Following DNA damage, DNA repair mechanisms including proliferating cell nuclear antigen (PCNA) are activated, resulting in the full opening of chromatin and subsequent NETosis (278). The magnitude and duration of ROS play a crucial role in the formation of NETs and seem to be dependent on NOX. For instance, neutrophils from p47$^{phox-/-}$ mice, thus mice lacking a functional NOX enzyme, are not capable of producing NETs (279). Furthermore, neutrophils isolated from CGD patients are incapable of producing NETs, yet gene therapy that complements NOX function was able to restore the potential of these neutrophils to produce NETs (280).

Another important step in NETosis is the release of particular granule proteins into the cytosol. Azurophil or primary granules contain a protein complex called the azurosome which contains 8 granule proteins including NE, MPO, azurocidin, cathepsin G, eosinophil cationic protein, defensin-1, lysozyme, and lactoferrin. ROS production causes dissociation of this

complex, releasing several of these proteins into the cytosol (281). Once in the cytosol, these proteins, especially NE play a critical role in breaking down cytoskeletal elements, allowing NETs to form (282). Subsequently, peptidyl-arginine deaminase 4 (PAD4) gets transferred to the nucleus to catalyze the hypercitrullination of histones, leading to chromatin decondensation (283). Lastly, pores are formed in the plasma membrane allowing NETs to be released into the environment. Antimicrobial proteins released from the granules may strongly bind to the NET surface due to electrostatic interactions. Together, these mechanisms ensure neutrophils capture invading pathogens and destroy them in a special form of programmed cell death, resulting in neutrophil and pathogen death alike.

Neutrophils in Cancer

Whereas researchers once believed that neutrophils were present only during the acute phase of inflammation, functioning only as pathogen killers, more recently it has been shown that neutrophils are also found infiltrating many types of tumors (284). Early studies believed that given the short lifespan of these cells, neutrophils were likely mere bystanders within the TME, having no effect on the progression of a chronic and progressive disease like cancer. However, the study of tumor-associated neutrophils (TANs) has become a widespread area of research since it has become clear that TANs have relevant roles in cancer progression and tumorigenesis.

Elevated numbers of neutrophils have been seen in many patients with advanced cancer in both the tumor and the blood (285,286), likely due to tumor secretion of molecules that may instigate neutrophil release from the bone marrow such as GM-CSF (287). This neutrophilia is typically associated with poor prognosis in several cancer types including lung and renal

carcinomas (288,289). The ratio of neutrophils to other leukocytes (NLR) in the blood serves as a prognostic factor for cancer. Given its simplicity and inexpensive nature, NLR is a good marker of ongoing cancer-related inflammation and an indicator of prognosis of solid tumors. Furthermore, in oncology, NLR correlates with tumor size, stage of tumor, metastatic potential and lymphatic invasion (290). Unfortunately, a high NLR, typically one greater than 3.0, is associated with worse disease-free and overall survival in many cancers including breast (291) and lung cancer (292). However, in some types of cancer, such as gastric cancer, many neutrophils is indicative of a positive prognosis and can be used as a tool for the early detection of cancers that are difficult to identify (285,293). Ultimately, neutrophilia is not always a bad indicator of cancer progression, and their role needs to be explored in more detail in each specific context.

Protumor Function of Neutrophils

A large body of clinical evidence indicates neutrophils are involved in various stages of cancer development and tumor progression. The protumoral functions of neutrophils are numerous and have only recently started to be elucidated. Perhaps the most well characterized mechanism of neutrophil-induced tumor progression is aiding in angiogenesis. Angiogenesis is a hallmark of malignant disease as the formation of new blood vessels is required for tumors to acquire the oxygen and nutrients necessary for their continued growth. Neutrophils can enhance tumor angiogenesis through the production of many pro-angiogenic factors including MMP9 and vascular endothelial growth factor (VEGF). Furthermore, neutrophil depletion in these mouse models of melanoma and fibrosarcoma significantly inhibited tumor growth (294). It has also been shown that MMP9 levels are elevated in cancer patients, specifically, neutrophils from head and neck squamous cell carcinoma tissue expressed MMP9 at higher levels than all other cell

types within the TME (295). TANs were responsible for producing MMP9 in hepatocellular carcinoma as well and this molecule was found to stimulate the proangiogenic activity of hepatoma cells (296). Finally, in a model of colorectal cancer, tumors were resistant to anti-VEGF therapy under situations of increased inflammation. These tumors were found to be enriched in neutrophils that supported angiogenesis through the upregulation of Bv8/ Prokineticin 2 (PROK2) and suppression of this neutrophil population resulted in response to anti-VEGF therapy (297).

Neutrophils may also contribute to tumor initiation through several mechanisms. Although neutrophil-derived ROS may be able to destroy cancer cells, they can also cause genotoxicity in circumstances when a cell is not killed. Neutrophil-produced ROS, at chronic, low levels, may induce DNA damage and promote tumorigenesis in lung epithelial cells (298). Additionally, neutrophils contribute directly to carcinogenesis by amplifying the genotoxicity of urethane, a carcinogen, via ROS (299). Finally, neutrophils may also induce DNA damage in a ROS-independent mechanism. In inflamed areas, such as the colon, it was observed that neutrophils may release nanoparticles that carry pro-inflammatory microRNAs (miR-23a and miR-155). These microRNAs promote the accumulation of double-stranded breaks by targeting key proteins involved in DNA repair such as RAD51 and lamin B1 (300).

Neutrophil granules contain a multitude of proteins typically used for the degradation of pathogens. Unfortunately, some of these molecules may adopt other functions including enhancing tumor growth and creating an immunosuppressive environment favorable for cancer development. NE, as previously mentioned, is a major protein of primary neutrophil granules. Interestingly, in a mouse model of lung adenocarcinoma, NE directly enhanced tumor cell proliferation of A549 cells in a co-culture system. This proliferation was reduced when cancer

cells were co-cultured with $NE^{-/-}$ neutrophils or a NE inhibitor was used. NE could penetrate into the cells and degrade insulin receptor substrate-1 (IRS-1), leading to more phosphatidylinositol 3-kinase (PI-3K) available to enhance proliferation (301). Alternatively, Arginase 1 (ARG1) is also present in neutrophil granules and is used to degrade arginine. Arginine is an essential amino acid required for proper T cell proliferation and activation (302). In non-small cell lung cancer, tumor cells secreted IL-8, triggering the release of ARG1 from neutrophils (303). This results in the degradation of arginine, thus inhibiting the proliferation of antigen-specific T cells, crucial mediators of anti-tumor immunity (304).

Lastly, neutrophils can influence the migration potential of cancer cells and promote metastasis. Neutrophils secrete numerous cytokines which may augment cancer cells' aggressiveness. For instance, neutrophils secrete TNF-α and TGF-β1 which increased transmigration and metastasis in pancreatic ductal adenocarcinoma cells (305). G-CSF, a molecule involved in neutrophil proliferation, differentiation and recruitment has also been shown to be a key mediator in neutrophil-mediated metastasis. Tumors which overexpress G-CSF mobilized neutrophils to the tumor, but also to the premetastatic site, priming this region and subsequently facilitating metastasis of cancer cells (306). Furthermore, cancer-cell derived G-CSF was found to not only recruit neutrophils, but also engage in NETosis, which promoted breast cancer metastasis to the liver (307). Together, these findings provide clear evidence that neutrophils can promote cancer progression and display a protumorigenic phenotype in multiple contexts.

Antitumor Function of Neutrophils

Despite the large amount of evidence in support of the tumor-promoting roles of neutrophils during carcinogenesis, there is also clear evidence demonstrating their antitumor activity. A neutrophil's ability to kill cancer cells was discovered decades ago both *in vitro* and *in vivo* (308,309), however the mechanisms used to orchestrate such cytotoxicity are only recently beginning to be uncovered.

Neutrophils potentiate this antitumor effect once they have been activated. For instance, in a model of TNBC, it was shown that following neutrophil activation by IFN- γ producing monocytes, neutrophils displayed enhanced killing capacity in vitro through the upregulation of stimulator of interferon genes (STING). Furthermore, these monocytes recruited neutrophils specifically to the pre-metastatic organ of the lung where they were cytotoxic and prevented metastatic outgrowth (310). Similarly, in another model of breast cancer as well as in a model of renal carcinoma, neutrophils prevented tumor cells from colonizing the lung by protecting the pre-metastatic niche and creating an unfavourable environment for metastatic development (311,312). In this breast cancer model, neutrophil-derived H_2O_2 mediated tumor cell killing in the lung, preventing metastatic outgrowth (311) while in the renal carcinoma model, IL-8 secreted by tumor cells elicited a cytotoxic neutrophil population (312). Neutrophil-secreted H_2O_2 has also been found to induce a lethal influx of Ca^{2+} in tumor cells through the upregulation of transient receptor potential cation channel, subfamily M, member 2 (TRPM2), a ubiquitously expressed H_2O_2-dependent Ca^{2+}-permeable channel, thus suggesting that not only ROS, but other molecules may play crucial roles in tumor cell cytotoxicity (313).

Previously, the role of G-CSF as a molecule that may increase neutrophil-mediated metastasis was discussed. Interestingly, several publications have shown the contrary; both G-

CSF and GM-CSF may induce a cytotoxic neutrophil population. For instance, in a murine model of colon adenocarcinoma, cells transduced with G-CSF lost tumorigenic activity following a massive influx of neutrophils into the tumor site. Additionally, neutrophils were found to discriminate between G-CSF producing and non-producing cells and only inhibit G-CSF expressing ones (314). Similarly, neutrophils activated by GM-CSF exert antitumor activity against melanoma cells in a ROS-dependent mechanism (315).

Finally, neutrophils can destroy cancer cells by antibody-dependent cellular cytotoxicity (ADCC). ADCC is the process by which antibodies coat a target cell and subsequently recruit effector target cells to engage cytotoxic mechanisms. Antibodies can bind to their antigen via their antigen-binding fragment (Fab) portion and interact with effector cells via their fragment crystallizable region (Fc) portions. This allows antibodies to act as bridges that link effector cells to their target (316). Neutrophils express Fc receptors on their surface, enabling them to behave as an effector cell in ADCC-mediated killing (317). Neutrophils express different Fcγ receptors including FcγRI, FcγRII, FcγRIII which have different binding affinities for immunoglobulin G (IgG), however FcγIIα was found to be the predominant Fc receptor inducing ADCC in breast cancer (318). One mechanism by which neutrophils kill antibody opsonized cancer cells is through trogoptosis. Trogoptosis is the repeated process of trogocytosis, where one cell, in this case the neutrophil, endocytoses cytoplasmic fragments of the target cell's membrane, resulting in membrane destruction and necrotic cell death (319).

Continuum of neutrophil states

The previous two sections highlight some of the pro- and anti-tumor roles a neutrophil may adopt in various contexts. Interestingly, it has been presented that certain molecules such as

ROS, IL-8 and G-CSF can drive both pro- and antitumorigenic neutrophil activity and neutrophils can both support and discourage metastasis. These conflicting reports fuel a debate regarding the role of neutrophils in cancer and bring forward the hypothesis that neutrophil function may be context-dependent, and heterogeneity may rely on more than just the influence of the TME.

Neutrophil plasticity and phenotypic diversity can be directly affected by the TME, however neutrophil heterogeneity is also influenced by neutrophil maturity. Previously, a neutrophil population was often described as being either immature, where neutrophils supported tumorigenesis or mature, where neutrophils were anti-tumor (320). Premature release of neutrophils from the bone marrow, which occurs primarily during situations of emergency granulopoiesis, is considered the main reason for the presence of immature neutrophils in circulation and is thought to occur primarily by the increased production in G-CSF (321). An increase in immature neutrophils is commonly observed in cancer patients (322). On the contrary, mature neutrophils are kept in the bone marrow to develop completely before entering circulation and are much more cytotoxic in nature (323).

Recently, it has been proposed that neutrophils do not exist as either an immature or mature homogeneous populations. Studies using single-cell transcriptomics of neutrophils have provided insights into their transcriptional heterogeneity during maturation (324). In brief, because neutrophils are constantly undergoing evolution and progression as a function of time and environmental cues, there are not just immature and mature neutrophil populations that exist, but rather there are various sub-populations with different polarizations that exist along a transcriptional, chronological continuum with varying phenotypic and functional organization. Ultimately, it is crucial to recognize that neutrophils are a heterogeneous population with varying

functional properties. Thus, it is imperative to find therapies that may functionally reprogram the majority of the neutrophils and skew them toward an antitumor phenotype.

Therapeutic Approaches

Despite the clinical evidence that support the notion of neutrophil presence having a negative effect in cancer, these cells can clearly kill tumor cells. Thus, several novel therapeutic approaches are being investigated that skew a neutrophil's phenotype to enhance their antitumoral capacity.

Given the prominent role TGF-β has in supporting a pro-tumor neutrophil phenotype, as well as being secreted directly from tumor cells, it has become an intriguing target in cancer therapy. Thus, a mathematical model was first developed to predict how use of a TGF-β inhibitor and IFN-β could enhance the anti-tumor neutrophil phenotype and combat tumor progression. This model was quite promising and even estimated drug dosage, total drug amount, infusion time and relative cost (325). The results were so convincing that this advanced to pre-clinical and clinical research. *In vitro*, anti-TGF-β treatment increased the cytotoxicity of TANs and decreased the levels of metastatic chemoattractants secreted by TANs. Additionally, anti-TGF-β therapy increased colorectal cancer cell apoptosis and suppressed the migration of tumor cells (326). *In vivo*, treatment with a TGF- β inhibitor slowed tumor growth, an effect that was lost when neutrophils were depleted (326). Following these findings, several clinical trials have been initiated looking at Galunisertib, an ALK5 (TGF- β type I receptor) inhibitor for the treatment of a multitude of cancers. These phase I and II clinical trials have assessed the role of Galunisertib in combination with chemotherapeutic agents and results have been mixed (327-329). Similarly, LY2109761, a TGF-β receptor type 1 and 2 dual inhibitor has also been the center of several

human and murine studies. LY2109761 was found to inhibit liver metastasis both in vitro and in vivo in a model of pancreatic cancer (330). Furthermore, this drug was found to be good at sensitizing cancer cells to the anti-neoplastic effects of radiotherapy (331) and oxaliplatin, a conventional chemotherapy (332). Together, these findings highlight the potential of using TGF-β inhibition as a means of phenotypically switching neutrophils towards a more antitumor phenotype, resulting in cancer cell death. Although therapies that target TGF- β are promising, it is important to recognize that TGF- β is highly pleiotropic, thus acting on multiple cell types within the TME. Accordingly, the observed cytotoxicity may be due to a combination of distinct phenotypic switches in multiple cell types, including neutrophils, resulting in tumor cell death.

In recent years, it has been shown that specific therapies may directly alter neutrophils to eradicate tumors. A neutrophil-activating therapy consisting of three molecules; TNF, which mobilizes neutrophils to the tumor, an anti-CD40 monoclonal antibody, which augments a neutrophil's killing capacity, and anti-gp75, a tumor-binding antibody, were able to eradicate B16 melanoma cells, as well as LL/2 lung carcinoma, 4T1 mammary carcinoma, and Sparkl.4640 colon carcinoma (333). The same combination was able to activate human neutrophils *in vitro*, enabling them to lyse human cancer cells. Mechanistically, it was revealed that this therapy relied on complement activation, particularly the upregulation of the complement component C5a, to induce ROS production by neutrophils via xanthine oxidase which generates oxidative damage to tumor cells and results in cancer clearance (333).

The TME is typically an inflammatory milieu that potentiates cancer progression. Thus, lately, it has been asked whether converting the TME from a chronically inflamed state to one of acute microbial inflammation would influence cancer progression. Intriguingly, when microbial bioparticles were injected intratumorally, there was a substantial increase in activated neutrophils

within the tumor (28-fold increase). The therapy elicited a primarily mature neutrophil population within the tumor; they were the main immune subset to phagocytose microbial particles and upregulated iNOS (334). In comparison, neutrophils from unmanipulated tumors expressed high levels of VEGF, which is typically a pro-tumor molecule involved in angiogenesis. Furthermore, recurrent microbial treatment with *S.aureus* bioparticles repressed tumor growth in a ROS-dependent manner as treatment with N-acetyl-L-cysteine (NAC), a ROS scavenger, reversed this phenotype (334). This treatment also amplified CD8 T-cell function and increased the efficacy of checkpoint inhibitor therapy in the AT-3 mammary carcinoma tumor model. These data indicate that finding novel ways to reprogram neutrophils from a wound healing and tumor-promoting phenotype to an activated cytotoxic phenotype may be crucial for ameliorating outcomes for cancer patients.

Rationale and Objectives

Though many treatments are available for women with ER+ and HER2+ cancers, there is still 20% to 30% of patients that relapse, particularly when diagnosed at an advanced stage. Furthermore, women with triple negative cancers are only treated with standard chemotherapy, whereby >50% develop intrinsic or acquired resistance. For these reasons, identifying therapies that target essential vulnerabilities to treat tumors is necessary for improving survival outcomes of women with such hard- to-treat cancers.

Our lab has previously shown that inflammatory mediators such as IFNγ and Polyinosinic-polycytidylic acid (Poly IC) may potentiate the cytotoxicity of phenformin in vitro and in vivo for the treatment of murine and human breast cancer models (335). Additionally, this therapy results in decreased expression of NAD(P)H quinone oxidoreductase 1 (NQO1), a key

ROS scavenger, within breast cancer cells, resulting in the accumulation of oxidative DNA damage, leading to cancer cell death.

Hypothesis

I hypothesize that neutrophils are responsible for mediating the cytotoxic effects exhibited during phenformin and Poly IC treatment in murine models of breast cancer. The combination therapy repolarizes neutrophils towards a more antitumorigenic phenotype, capable of killing cancer cells in a ROS-dependent manner.

Aim 1: To functionally validate the importance of specific immune cell subsets in potentiating the cytotoxic response of biguanides in breast cancer.

Our research shows that poly IC-induced inflammation profoundly sensitized two syngeneic models of HER2+ (MT4788) and TNBC (4T1) breast cancers to the cytotoxic effects of phenformin and in a ROS-dependent manner. Single-cell RNA sequencing has shifted our attention to focusing on innate immune cell subsets which may be responsible for the observed cytotoxicity. Multiplex flow cytometry studies reveal that neutrophils are selectively enriched in the tumors of poly IC/phenformin treated mice relative to vehicle controls. Depletion of neutrophils from these mouse models using a monoclonal antibody result in loss of therapy effectiveness, suggesting a crucial role for this cell type. *In vitro* co-cultures between neutrophils isolated from combination-treated mice and syngeneic cancer cells display extensive cancer cell death in comparison to cancer cells co-cultured with control-mouse neutrophils.

Aim 2: To functionally characterize the neutrophil population elicited by the phenformin/poly IC therapy.

Functional characterization of the therapy-elicited neutrophil population was crucial for identifying how these neutrophils may be killing the cancer cells. A spontaneous metastasis experiment identified that the combination therapy did not increase metastatic burden in the lungs in comparison to vehicle control mice. Through microscopy it was possible to visualize nuclear segmentation of the neutrophil's isolated from treated mice. The phenformin/poly IC therapy induced a primarily mature neutrophil population. There was no change in the oxidative burst generated from neutrophils isolated from control- and combination-treated mice using a chemical stimulant and *ex vivo* drug treatment did not augment the strength of the oxidative burst.

Chapter 2: Methods

Mammary Fat Pad Injections

Cells (0.05×10^6 for 4T1-537 and 0.5×10^6 for MT4788) were injected into the fourth mammary fat pads of 8- to 10-week-old female mice. Cells were resuspended in a 1:1 phosphate-buffered saline (PBS) and Matrigel mixture. Upon palpation, tumor growth was monitored using caliper measurements. Volume was determined following the equation: Volume = $4/3 \times (3.14159) \times (\text{length}/2) \times (\text{width}/2)^2$. Drug treatments were initiated when tumors reached an initial volume of approximately 100 to 150 mm^3 and measured every two days until the control group reached an approximate volume of 750 mm^3.

Drug preparation and treatment

Phenformin hydrochloride (Toronto Research Chemicals) powder was dissolved in PBS, filter sterilized, and stored at 4°C. Phenformin hydrochloride was made fresh for each experiment and administered intraperitoneally at a dose of 50 mg/kg daily. PBS was administered as a vehicle control.

IACS-010759 (Selleckchem) was dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C. IACS-010759 (or DMSO for control mice) was administered via oral gavage at a dose of 5 mg/kg daily.

Poly IC high molecular weight form (InvivoGen) was prepared according to the manufacturer's recommendations and stored at -20°C. Mice were treated with 50 μg of Poly IC by intraperitoneal injection (50 μL) every 2 days or with a saline control. Poly IC treatment was commenced 2 days prior to starting phenformin/ IACS-010759 treatment.

Single-cell RNA sequencing

MT4788 tumors were extracted from control- and combination- treated mice and were physically dissociated using razor blades. Enzymatic tissue digestion was performed as previously described by Rodriguez de la Fuente et al (336). Following digestion, tissue was mashed through a 70μm cell strainer, washed, red blood cells were lysed with Red Blood Cell Lysing Buffer Hybri-Max (Sigma) and washed again. Cells were counted using acridine orange (AO)/propidium iodide (PI) staining. 5.0 x 10^7 cells were taken and stained with anti-mouse CD16/CD-32 (Fc block) (clone 2.4G2, BD Pharmingen) for 10 minutes on ice. Samples were then stained with biotin anti-mouse CD45 antibody (clone 30-F11, BioLegend) for 5 minutes on ice and washed with buffer (1x PBS with 0.2% Bovine serum albumin (BSA) and 2mM Ethylenediamine tetra-acetic acid (EDTA)). Samples were then stained for 15 minutes on ice with anti-biotin microbeads (Miltenyl Biotec). CD45+ cells were isolated using LS columns (Miltenyl Biotec) following the manufacturer's recommendations. Single-cell RNA sequencing was performed by Genome Quebec at the CHU Sainte-Justine. Samples were sequenced with the NovaSeq 6000 system (Illumina). Analysis was performed in collaboration with the Kleinman laboratory (Lady Davis Institute). Immune cell populations were identified using single-sample gene set enrichment analysis with murine tumor immune cell signatures described by Sinha et al (337).

ELISA

MT4788 and 4T1-537 plasma and tumor lysates were analyzed using a multiplex cytokine array which measures cytokine and chemokine levels. Discovery assays used include the Mouse Cytokine/Chemokine 31-plex Discovery assay (MD31) and the Mouse IFN 2-Plex Discovery Assay (MDIFNAB) (Eve Technologies, Calgary, AB).

Flow cytometry

Fc receptors were blocked with CD16/CD32 (clone 2.4G2, BD Pharmingen) and subsequent staining was performed in FACS buffer (2% fetal bovine serum (FBS) in PBS) for 20 to 30 minutes on ice. Antibodies used for flow cytometry include: BV711 CD45.1 (clone A20, BioLegend), BUV496 B220 (Clone RA3-6B2, BD Horizon), eF450 CD3 (clone 500A2, Invitrogen), PE-eF610 CD11b (clone M1/70, BD Horizon), APC-eF780 CD11c (clone N418, Invitrogen), BV785 F4/80 (clone BM8, BioLegend), BUV 737 I-A/I-E (MHC II) (clone M5, BD OptiBuild), BV605 Ly6C (clone HK1.4, BioLegend), BUV395 Ly6G (clone 1A8, BD Horizon), AF647 CD206 (clone C068C2, BioLegend) and BV510 CD86 (clone GL1, BD Horizon). Dead cells were identified by staining with Fixable Dye eFluor 780 (eBioscience). Data was acquired on a LSR Fortessa (BD Biosciences) cytometer and analysis was performed using FlowJo software. Exclusion of debris, doublets and dead cells were applied to all analyses.

Neutrophil Depletion

FVB and Balb/c mice received 50 μg of anti-mouse Ly6G (clone 1A8, BioXCell) 2 days prior to mammary fat pad (MFP) injection. The next day (1 day prior to MFP injection), mice received 50 μg anti-rat Kappa Immunoglobulin light chain (clone MAR 18.5, BioXCell). This cycle was repeated until mice were euthanized. Control mice received 50 μg of mouse IgG2a isotype control (clone C1.18.4, BioXCell) by intraperitoneal injection daily. Saphenous blood was drawn 12 days post injection, 2 days post treatment to confirm Ly6G depletion.

Neutrophil Isolation

Mice were anesthetized using 2% Isofluorane and blood was drawn from the heart by cardiac puncture using a 0.5M EDTA-coated syringe. Red blood cells were lysed using Red Blood Cell Lysing Buffer Hybri-Max (Sigma) on a shaker for approximately 5 to 10 minutes. After centrifugation at 500 x g for 5 min and 3 washes with FACS buffer, cells were counted. Cells were then stained with CD16/CD-32 (Fc block) (clone 2.4G2, BD Pharmingen) for 10 minutes on ice, followed by Biotin anti-Ly6G (clone 1A8, BioLegend) for 10 minutes on ice. Lastly, cells were stained for 20 minutes on ice with anti-biotin microbeads (Miltenyl Biotec). Ly6G+ cells were isolated using LS columns (Miltenyl Biotec) following the manufacturer's recommendations.

In-vitro cytotoxicity studies

4T1-537 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1x penicillin/streptomycin and 10mmol/L HEPES. They were then plated in a 24-well, flat bottom plate approximately 5 hours prior to neutrophil addition to allow for attachment. Neutrophils isolated from the blood of control-, monotherapy- and combination- treated mice were added directly to the cancer cells in a 1:10 ratio of cancer cells to neutrophils and left in a 37°C incubator overnight. Following incubation, media was aspirated, and samples were fixed with 100% methanol. Cancer cells were stained with crystal violet and scanned using Epson scanner. Analysis was performed using ImageJ software.

Metastasis studies

4T1-537 cells were implanted orthotopically into the mammary fat pad of Balb/c mice. Mice were treated with either PBS/saline or phenformin/poly IC once tumors reached 100mm^3. Tumors were allowed to grow until approximately 500mm^3 when which they were resected. 3 weeks post-resection, mice were euthanized, and lungs were harvested. Lungs were stored in 70% ethanol before being embedded in paraffin and then stained with hematoxylin and eosin (H and E) for the visualization of metastases. Analysis was performed using ImageJ software.

Neutrophil Cytospins and Assessing Maturation

Neutrophils were spun down on microscope slides using the Universal 32 centrifuge (Hettich) and fixed in ice-cold methanol for 5 minutes. The slides were then mounted and stained with ProLong Diamond Antifade Mountant with 4',6'-diamidino-2-phenylindole (DAPI) (Invitrogen) to visualize nuclear segmentation. Slides were scored using confocal microscopy to determine the percentage of immature and mature neutrophils in each treatment group.

Luminol Assay

Neutrophils or Human Leukemia 60 (HL-60) cells were resuspended in Hank's balanced salt solution (HBSS) and plated in a 96-well white bottom microplate at a concentration of 2.0 x 10^5 cells per well. Cells were left to rest in 37°C incubator for 30 minutes with or without drug treatments (Phenformin, IFNβ, 2-Deoxy- d-glucose (2-DG), Poly IC or LPS). Following incubation period, luminol sodium salt (50μM, Sigma) was added to each well. Plated neutrophils or HL-60 cells were stimulated with Phorbol 12-myristate 13-acetate (PMA) (15.6 ng/ml, Sigma) to induce the generation of an oxidative burst. Measurements were taken every 2

minutes over the course of 1 hour using the PerkinElmer EnSpire multimode plate reader. All values were subtracted from a baseline value (value obtained prior to PMA addition) and analysis was performed using the Prism 8 software.

HL-60 cell line

Cells were cultured in RPMI supplemented with 10% FBS, 1 x penicillin/ streptomycin and 25 mM HEPES and split every 2 to 3 days ensuring the cell concentration does not exceed 2 million cells/mL. Once in exponential growth phase, cells were differentiated towards a neutrophil-like phenotype using 2 differentiation agents: 1.25% DMSO or 1μM all-trans retinoic acid (ATRA) (Sigma) supplemented in the media for 4 to 5 days with a media change on days 2 or 3. Differentiation was assessed using an AF594 anti-CD11b antibody (clone M1/70, BioLegend). Oxidative burst assay was performed as described above.

Statistical Analysis

Statistical tests were performed in Prism 8 software. See details on statistical tests performed in figure legends. Flow cytometry analyses and statistics (percentages) were determined with the FlowJo Software 10.

Chapter 3: Results

Complex I inhibition is necessary for phenformin/poly IC therapy efficacy

We have previously identified that when murine models of luminal B breast cancer (MT4788) and TNBC (4T1-537) were co-administered phenformin and poly IC, a double stranded RNA analog which engages TLR3, breast tumors were extremely sensitive to this treatment and demonstrated impaired growth potential in comparison to the vehicle control or mice treated with either drug alone (Figure 4a-b). Alone, phenformin induced a partial sensitivity in breast tumors whereas as a monotherapy, poly IC had no significant effect on tumor growth. This suggested to us that a significant trigger of inflammation was required for biguanides to effectively display tumoricidal properties.

Though we suspected that the anti-neoplastic effects of phenformin were due to complex I inhibition, biguanides may have other off-target effects by not only affecting complex I of the electron transport chain. For instance, metformin may inhibit gluconeogenesis independently of complex I inhibition (64). Thus, we sought to identify if complex I inhibition was responsible for the tumorigenic phenotype we observed when using phenformin. A potent, and specific complex I inhibitor, IACS-010759 was administered by oral gavage with and without poly IC. It was observed that alone IACS-010759 had a partial effect in diminishing tumor growth, as was observed when phenformin is used as a monotherapy. Moreover, combining IACS-101759 with poly IC significantly increases the antitumorigenic effects of this therapy, similarly to the phenformin/poly IC treatment (Figure 4c). Ultimately, inhibition of complex I of the ETC is responsible for the anti-neoplastic effects observed in breast tumors when biguanides are used in conjunction with poly IC.

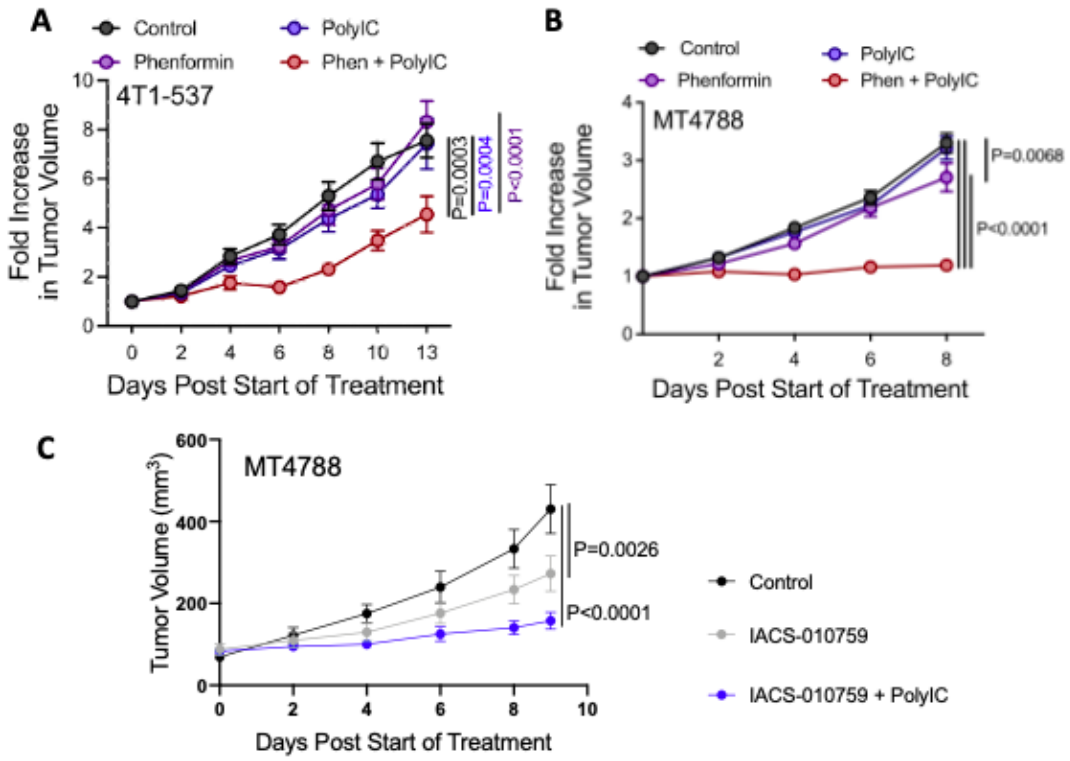

Figure 4. Complex I inhibition is necessary for phenformin/poly IC therapy efficacy.

(A-B) MFP injection of MT4788 and 4T-537 breast cancer cells into FVB and Balb/c mice respectively. At approximately 100mm^3, mice were treated with poly IC (50 μg) or saline by intraperitoneal injection. Two days later, phenformin (50 mg/kg, daily) or PBS treatment was started, in combination with poly IC or saline every 2 days. Tumors were measured by caliper measurements every 1-2 days and displayed in fold increase in tumor volume over time post start of treatment. *Graphs obtained by Totten et al., 2021*. (C) MFP injection of MT4788 breast cancer cells into FVB mice. Treatment was as described in (A-B) with the exception that phenformin was replaced by IACS-010759 (5 mg/kg daily) (or DMSO) administered daily by oral gavage. Data is displayed as increase in tumor volume over time post start of treatment. One independent experiment with Control: *n*= 7 tumors, IACS-010759: *n*= 9 tumors, IACS-010759 + Poly IC: *n*= 8 tumors. Significant P values are in the Figure and were calculated using a two-way ANOVA with a Tukey's multiple comparisons test (Prism 8 Software).

Innate immune cells are among key immune cell subsets altered by phenformin/poly IC therapy

Poly IC is a strong inducer of inflammation, given its function as a dsRNA analog. We hypothesized that since poly IC is necessary to augment the cytotoxicity of phenformin, we may be inducing alterations to one or many immune cell subsets within the tumor during treatment. To confirm this, single-cell RNA sequencing was performed on all immune cells (CD45+) isolated from the tumors of control-, phenformin-, poly IC- and combination-treated mice. Gene expression profiles were identified for each cell isolated from the tumor. Using these profiles, as well as lists of canonical cell type markers, genes that were enriched were then used to identify clusters of cells and determine the identity of each population.

Firstly, it was identified that tumors were populated with over 20 different immune cell subsets (Fig 5a). Cluster size on the UMAP is representative of how many cells from each immune cell subtype are present within the tumor. Thus, clusters 0 and 2, two of the largest subsets were found to be macrophage and monocyte populations respectively. Moreover, cluster 1 was found to be a CD8+ T cell population. Furthermore, it was possible to identify what proportion of the cells from each cluster come from which treatment group, enabling the possibility to identify which immune cell subsets are most impacted by the combination therapy (Figure 5b).

Looking at the identity of the first 12 clusters, which are the ones where enough cells were present to make a strong assumption regarding the cell type (greater than 500 cells/sample), we see that many of the cells are in fact innate immune cells rather than adaptive ones. In fact, 64% of immune cells within the tumor are innate immune cells (clusters 0,2,4,8,9,10,11,12) while only 36% are adaptive immune cells (clusters 1,3,5,7) (Figure 5c). This was surprising

considering MT4788 tumors are a model of Luminal B breast cancer, which is typically immune cold and highly immunosuppressive. Clusters 13 to 21 had fewer than 500 cells detected, thus further independent flow cytometry analysis would need to be conducted to confirm identity.

Lastly, focusing on the largest immune cell subsets, we sought to identify which immune cell subsets had the largest differential gene expression profile between the control- and combination-treated tumors. Cluster 8 identified as the neutrophil population showed very little differential gene expression between the control- and phenformin-treated tumors on the level of transcription (Figure 5d). In fact, in cluster 8, there are only 3 transcripts that are differentially expressed in phenformin tumors when compared to the controls (Prok2, Gm5416 and Mt1). Thus, it seems as though phenformin is not transcriptionally altering this population. This was observed in many of our immune cell subsets, meaning phenformin alone is not inducing any transcriptional shifts within our immune infiltrate. However, when we look at poly IC or the combination therapy in comparison to the control, we see there are very dramatic transcriptional shifts. There are 79 transcripts differentially expressed in the poly IC-treated tumors and 69 transcripts in the combination therapy-treated tumors when compared to the control tumors. Some differentially expressed genes between the control and combination groups include Stfa2, S100a8, S100a9, CCL5 and Retnlg, amongst many. Elevated differential gene expression is indicative that a driver of inflammation, such as poly IC, may play a critical role in inducing functional alterations to immune cell infiltrate within the tumor.

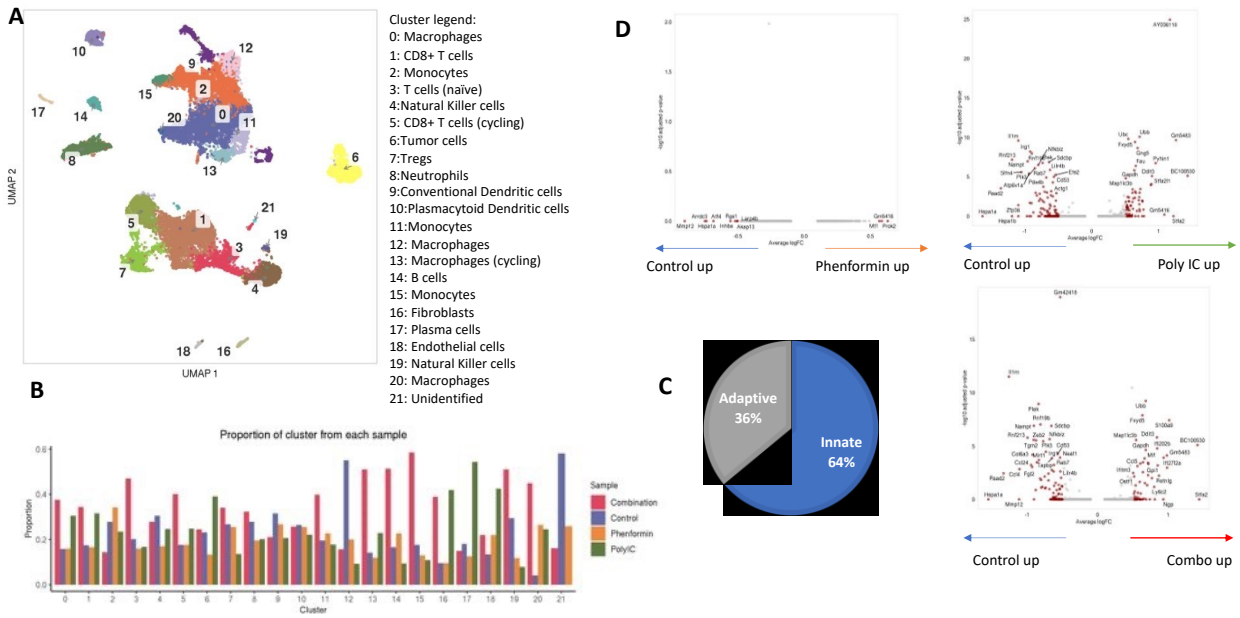

Figure 5. Innate immune cells are among key immune cell subsets altered by phenformin/poly IC therapy

(A) Single-cell RNA sequencing identifies 21 different immune cell subsets present within MT4788 breast tumors using gene enrichment analysis. Cluster size is indicative of the number of cells within each subset. (B) Diagram displaying proportion of cluster coming from each cluster. (C) Percentage of innate and adaptive immune cells present in tumors based on first 12 clusters (clusters with more than 500 cells/sample). (D) Differential gene expression between control and phenformin, poly IC and combination therapy in cluster 8 (neutrophils). All analyses were performed by the Kleinman lab.

Phenformin/poly IC therapy drives NF--κB inflammation within the tumor

Poly IC has previously been identified as a robust inducer of type I IFN-driven inflammation (338). IFNs are potent promoters of the anti-tumor response; IFNs may alter immune cell activation and function within the tumor microenvironment, allowing elimination of malignant cells as described by Fenton et al (339). We wished to elucidate whether type I IFN-driven inflammation was responsible for the cytotoxic phenotype observed in tumors during phenformin/poly IC administration. To assess this, ELISAs were performed on MT4788, and 4T1-537 tumors isolated from mice following drug treatment. Despite the use of poly IC in our combination therapy, ELISAs show relatively low levels of IFN within the tumors (Figure 6a-c). In fact, there were no detectable levels of IFNβ within the MT4788 tumors. Moreover, there was no significant difference in IFNα and IFNβ levels between the treated and control tumors. Thus, type I IFN-driven inflammation does not seem to be responsible for the observed cytotoxicity intratumorally.

Following these observations, it was assessed whether any other indicators of inflammation were present in the tumor. Notably, there was an increase in TNFα, IL-1β and IL-6 in MT4788 and 4T1-537 tumors (7a-c). These cytokines are indicative of the activation of the nuclear factor kappa B (NF-κB) pathway. NF-κB regulates multiple aspects of the innate and adaptive immune responses including regulation of the survival, activation and differentiation of innate immune cells and T cells (340). Furthermore, NF-κB activation has been found to drive an accumulation of innate immune cells in certain contexts (341). Moreover, cytokines downstream of NF-κB, particularly CCL2 and CCL5 were also upregulated in combination-treated tumors (Figure 7d-e). Ultimately, the combination therapy seems to drive a non-conical, NFκB-driven inflammation within the TME. This may influence immune cell polarization and function.

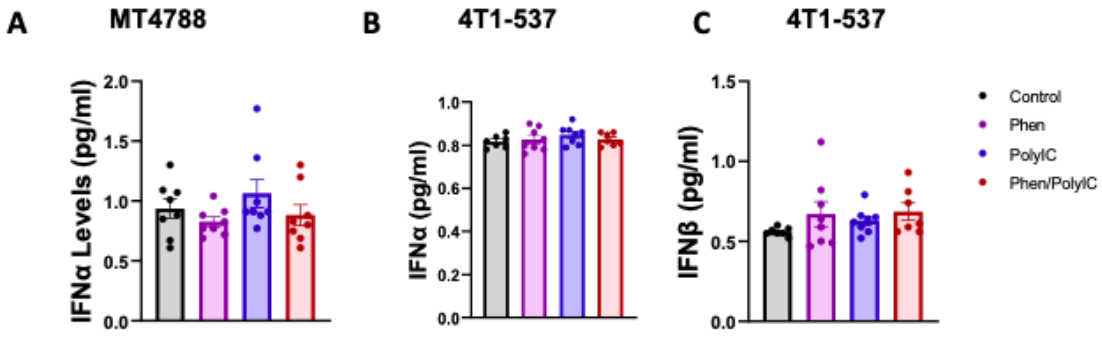

Figure 6. Phenformin/poly IC therapy does not drive type I IFN inflammation in the tumor.

Tumor lysates were generated from MT4788, and 4T1-537 breast tumors isolated from mice treated with pbs/saline (control), phenformin, poly IC or phenformin/poly IC. Tumor lysates were sent to EVE technologies where ELISAs were performed, and various cytokine and chemokine levels were measured. (A) IFNα levels (in pg/ml) were measured in MT4788 tumors. (B) IFNα levels (in pg/ml) were measured 4T1-537 tumors. (C) IFNβ levels (in pg/ml) were measured in 4T1-537 tumors. No significant differences were observed. Statistical analysis performed using Prism 8 software using 2-way ANOVA with Tukey's multiple comparisons test.

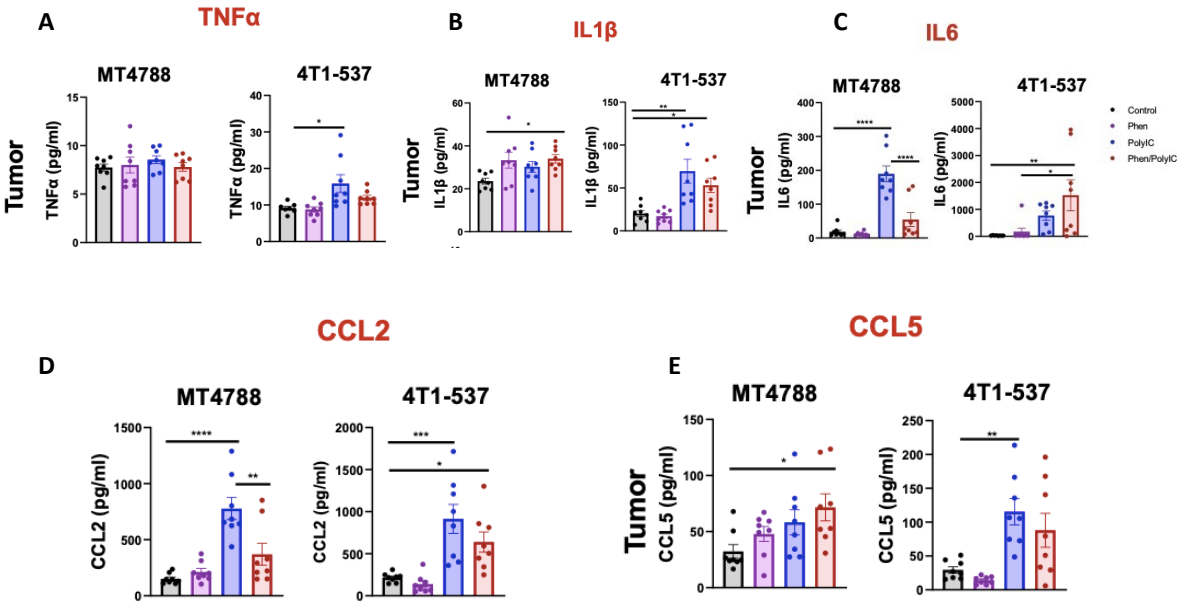

Figure 7. Phenformin/poly IC therapy induces NFκB-driven inflammation in the tumor.

Tumor lysates were generated from MT4788, and 4T1-537 breast tumors isolated from mice treated with pbs/saline (control), phenformin, poly IC or phenformin/poly IC. Tumor lysates were sent to EVE technologies where ELISAs were performed, and various cytokine and chemokine levels were measured. (A) TNFα levels (in pg/ml) were measured in MT4788 and 4T1-537 tumors. (B) IL-1β levels (in pg/ml) were measured in MT4788 and 4T1-537 tumors. (C) IL-6 levels (in pg/ml) were measured in MT4788 and 4T1-537 tumors. (D) CCL2 levels (in pg/ml) were measured in MT4788 and 4T1-537 tumors. (E) CCL5 levels (in pg/ml) were measured in MT4788 and 4T1-537 tumors. Statistical significance is shown. Statistical analyses were performed using Prism 8 software using 2-way ANOVA with Tukey's multiple comparisons test.

Phenformin/poly IC treatment recruit neutrophils to the tumor and blood

We next sought to identify in more detail whether there were alterations to the numbers of various immune cell subsets following administration of the combination therapy. To do this, an extensive flow cytometry panel was developed with the goal of immune profiling the tumors of control- and combination-treated tumors. PyMT breast cancer tumors (MT4788) were dissociated and enzymatically digested before being stained. Looking specifically at the immune cells within the tumor (CD45+), we observed no significant differences in the CD3+ (T cell) population, nor the B220+ (B cell) population or the CD11c+ (dendritic cell) population. However, there was a statistically significant increase in the CD11b+ (myeloid cell) population which is a marker for monocytes, macrophages, and granulocytes (Figure 8a). Thus, we looked more extensively into this population and added specific markers for each of these immune cell subsets. There were no significant changes in the monocyte population (Ly6C+), nor the macrophage populations (F480+). There was a significant increase in the neutrophil population (Ly6G+) in the combination-treated tumors relative to the vehicle controls, with an increase of about to 2% to 5% of all CD45+ cells in the tumor (Figure 8b). The therapy also induced an expansion of the neutrophil population in the blood, but not the spleen, suggesting a mobilization of neutrophils (Figure 8c). Moreover, tumor volume and Ly6G infiltrate are inversely correlated meaning a higher number of Ly6G+ cells are associated with smaller tumors, as determined by a correlation coefficient of -0.4814(Figure 8d). This suggested that neutrophils are potentially mediating a cytotoxic response. These findings were recapitulated in the 4T1-537 model of TNBC where the number of Ly6G+ cells were the only significant change and increased significantly from about 23% in control tumors to 32% of all immune cells in the combination-treated tumors (Figure 9a-b). Furthermore, there was an inverse correlation between tumor

volume and percentage of CD45+ that are neutrophils as well, with a correlation coefficient of -0.4750 (Figure 9c). Ultimately, the phenformin/poly IC therapy seems to alter the immune landscape within the TME, primarily by driving an accumulation of neutrophils in the tumors and blood of combination-treated mice. Thus, it is likely that this cell type is involved in driving the cytotoxicity associated with this therapy.

Neutrophils are required for therapeutic effectiveness

Given the neutrophil expansion observed by flow cytometry, we next asked whether the elicited neutrophil population was necessary for the decrease in tumor growth. To do this, a monoclonal anti-Ly6G and anti-rat kappa immunoglobulin light chain antibody were administered 2 days and 1 day respectively prior to cancer cell injection. This cycle was repeated every 2 days whereas vehicle control mice received a non-specific IgG2a antibody daily. A blood draw completed 12 days post-injection, 2 days post start of treatment confirmed that neutrophils accounted for 21.6% of all CD11b+ cells in the control group and 39.9% in the combination-treated and went down to 0.025% and 0.018% respectively following anti-Ly6G administration (Figure 10a). In both the MT4788 and 4T1-537 models, neutrophil depletion resulted in a loss of sensitivity to the treatment (Figure 10b-c). The phenformin/poly IC combination therapy no longer seems to have a cytotoxic response, especially in the 4T1-537 model where there are typically many more neutrophils present. Thus, it seems that the presence of neutrophils is required for optimal cytotoxicity with the combination treatment, suggesting this immune population may be responsible for the observed cancer cell killing.

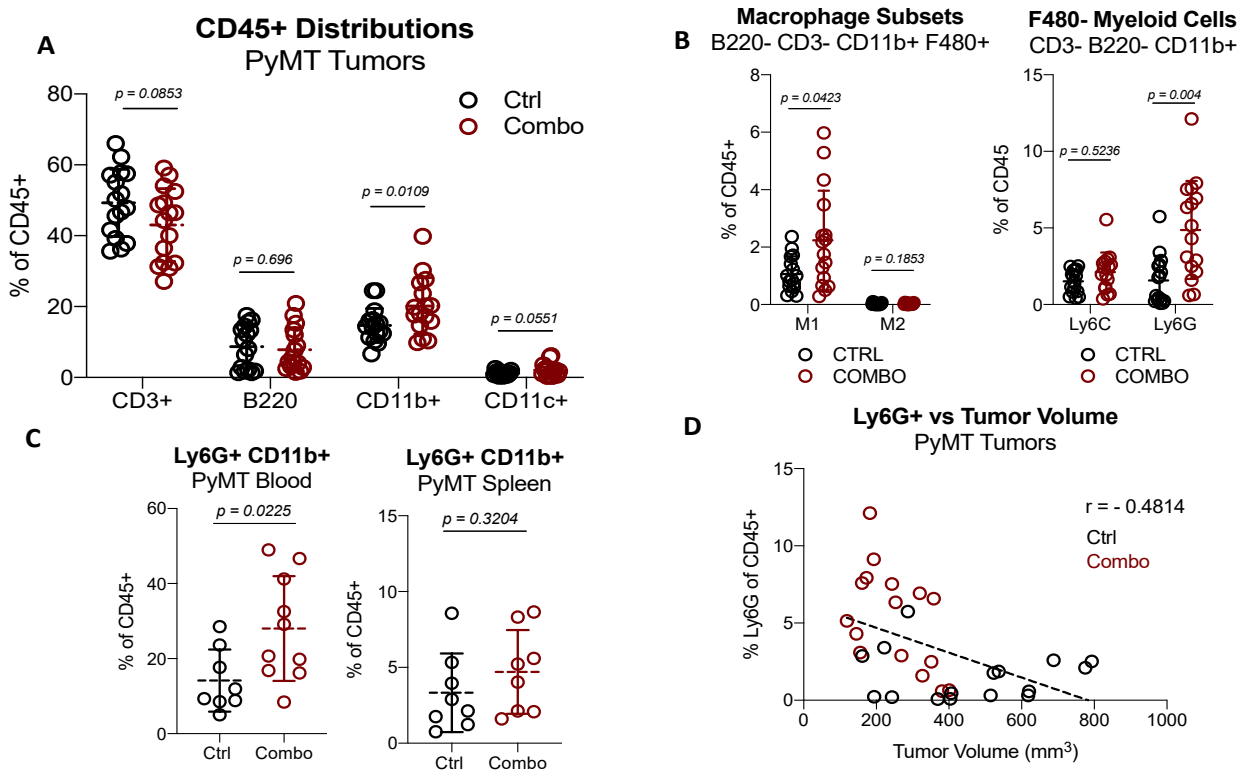

Figure 8. Phenformin/poly IC treatment recruit neutrophils to the tumor and blood of MT4788 tumor-bearing mice.

(A)PyMT tumors were dissociated and a percentage of all immune cells (CD45+) was determined for various immune cell subsets including T cells (CD3+), B cells (B220+), monocytes (CD11b+) and dendritic cells (Cd11c+). (B) % of all CD45+ cells that are either macrophages (CD11b+, F480+), monocytes (CD11b+, Ly6C+) or neutrophils (CD11b+, Ly6G+) are shown. For (A-B) *n*= 16 tumors per group, 2 independent experiments. (C) % of CD45+ cells that are neutrophils in blood and spleen of control- and combination-treated mice. *n*= 8 per group, 2 independent experiments. For (A-C) P values are shown on figures and calculated using Prism 8 software unpaired T-test. (D) Inverse correlation between number of neutrophils in the tumor and tumor volume. Correlation coefficient (*r*) determined using Prism 8 software, simple linear regression analysis.

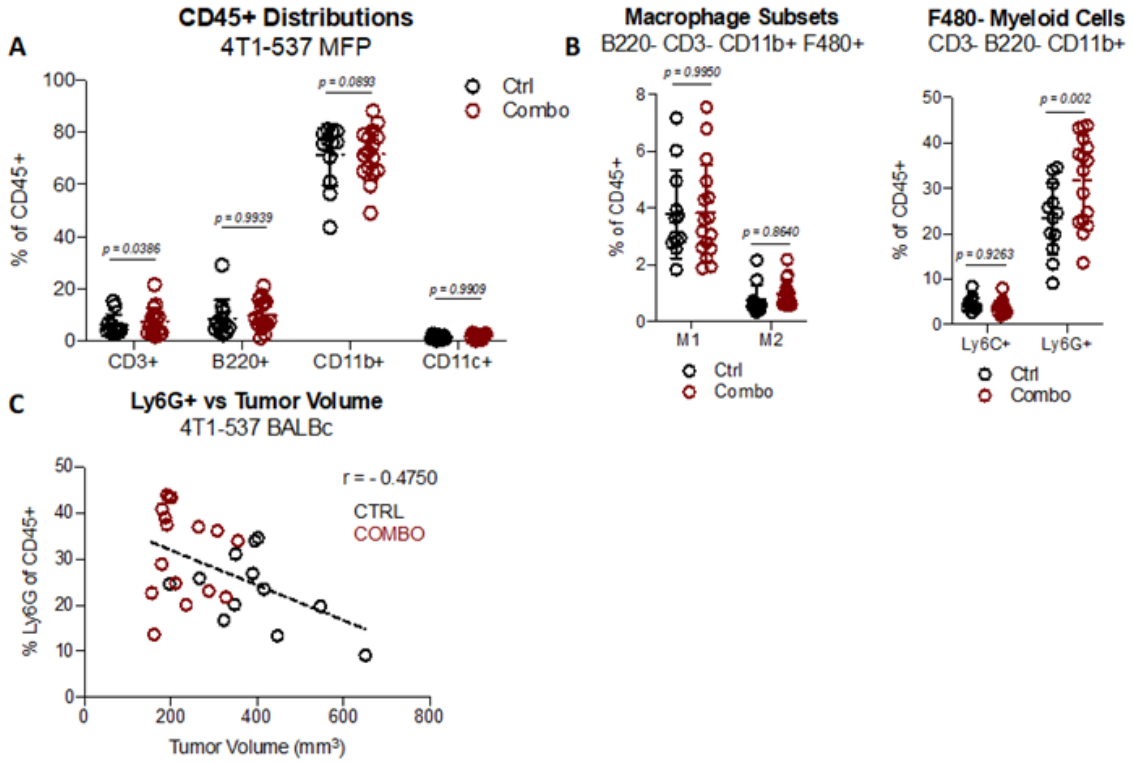

Figure 9. Phenformin/poly IC treatment recruit neutrophils to the tumor of 4T1-537 tumor-bearing mice.

(A) 4T1-537 tumors were dissociated and a percentage of all immune cells (CD45+) was determined for various immune cell subsets including T cells (CD3+), B cells (B220+), monocytes (CD11b+) and dendritic cells (Cd11c+). (B) % of all CD45+ cells that are either macrophages (CD11b+, F480+), monocytes (CD11b+, Ly6C+) or neutrophils (CD11b+, Ly6G+) are shown. For (A-B) *n*= 16 tumors per group, 2 independent experiments. P values are shown on figures and calculated using Prism 8 software unpaired T-test. (C) Inverse correlation between number of neutrophils in the tumor and tumor volume. Correlation coefficient (*r*) determined using Prism 8 software, simple linear regression analysis.

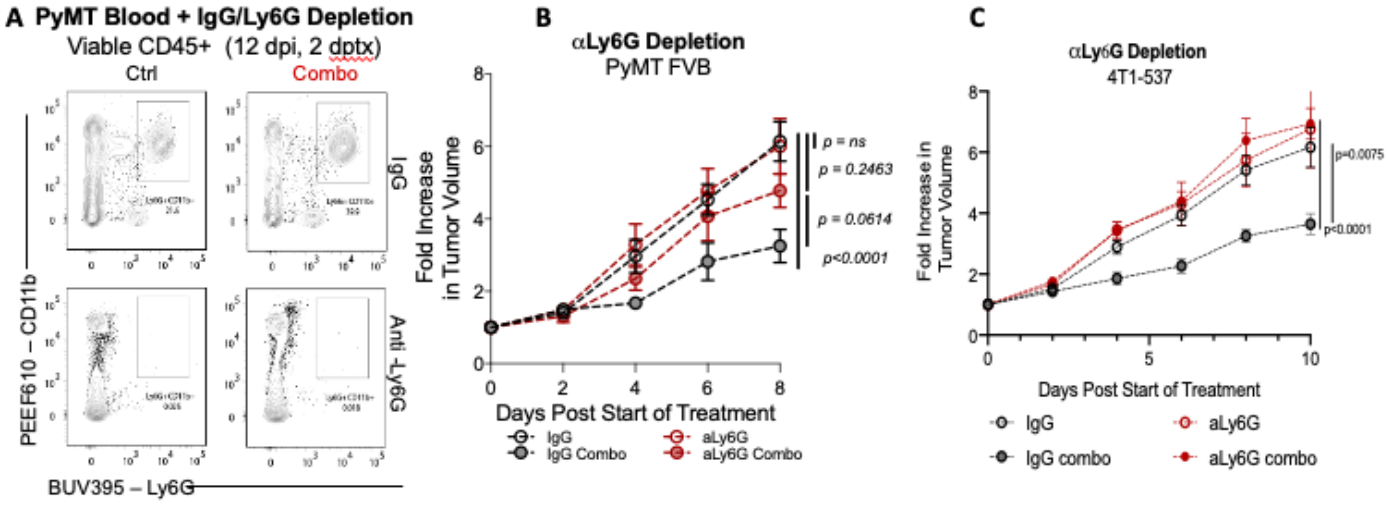

Figure 10. Neutrophils are required for therapeutic effectiveness

(A)Neutrophils were depleted using anti-Ly6G and anti-rat monoclonal antibodies in control and combination-treated mice (or IgG2a isotype control). Blood was drawn 12 days post-injection; 2 days post commencement of drug treatment and neutrophils were quantified using flow cytometry. (B-C) Control and combination drug therapies were administered to mice receiving either IgG2a or anti-Ly6G antibodies and tumor volumes were measured with calipers every 2 days in MT4788 (B) and 4T1-537 (C) mice. *n*= 8 to 10 tumors per group, 1 independent experiment per mouse model. Significant p values are shown and calculated using Prism 8 software 2-way ANOVA with Tukey's multiple comparisons test.

Combination-treated neutrophils acquire a cytotoxic phenotype and kill tumor cells *in vitro*

As previously described, neutrophils may have pro- and anti-tumor capabilities in cancer, depending on the context. Since neutrophils are armed with a variety of toxic peptides and molecules, we postulated that they may be able to kill tumor cells. Thus, knowing neutrophils are required for the therapy's effectiveness, we next sought to identify whether neutrophils are in fact responsible for the observed cytotoxicity. To directly evaluate the antitumor activity of neutrophils following treatment, we isolated neutrophils from the blood of control-, phenformin-, poly IC- and phenformin/poly IC- treated mice and performed an *in vitro* co-culture with 4T1-537 breast cancer cells. Neutrophils were plated in a 1:10 ratio of cancer cells to neutrophils and incubated for 24 hours. Neutrophils isolated from control-treated, and poly IC-treated mice had no significant cytotoxic effect (Figure 11). In fact, these neutrophils very minimally affected tumor cell viability. In contrast, neutrophils isolated from either the blood of phenformin alone or from the combination therapy mediated potent tumor cell killing. Neutrophils from phenformin-treated mice and combination-treated mice decreased tumor cell viability to about 53% and 46% respectively. This suggests phenformin may induce a cytotoxic neutrophil population responsible for the killing of tumor cells, which may be potentiated slightly more with the addition of poly IC. Ultimately, these findings suggest that neutrophils are likely the main immune cell subset affected by the treatment and are polarized towards an antitumor phenotype.

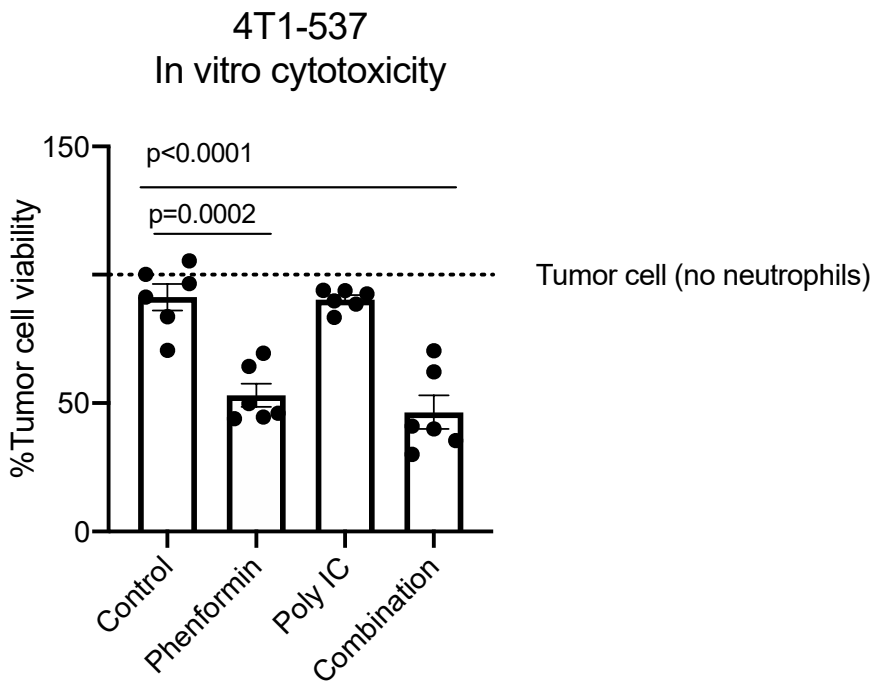

Figure 11. Combination-treated neutrophils acquire a cytotoxic phenotype and kill tumor cells *in vitro*

Neutrophils isolated from the blood of mice treated with either PBS/saline, phenformin, poly IC or phenformin/poly IC were placed above 4T1-537 breast cancer cells in a 10:1 ratio of neutrophils to tumor cells. Following a 24-hour incubation, cells were fixed with methanol and stained with crystal violet dye. % tumor cell viability was determined by quantifying the number of remaining tumor cells following incubation using the ImageJ software. *n*= 6 per treatment group, 3 independent experiments. Significant p-values are shown and calculated using Prism 8 software 2-way ANOVA with Tukey's multiple comparisons test.

Phenformin/poly IC therapy does not increase metastatic burden

Studies have shown that neutrophils may both support or inhibit metastasis, as described extensively previously. Despite evidence suggesting neutrophils may protect the premetastatic niche, clinically, the presence of neutrophils is typically associated with poor prognosis and increase in metastatic burden. Thus, it was imperative to decipher whether the treatment we were using and the neutrophil population we were eliciting may increase metastasis to the lung. To do this, 4T1-537 cancer cells were injected into Balb/c mice. This model is quite aggressive and has been developed to spontaneously metastasize from the primary tumor in the mammary gland to the lung. Following tumor development, mice were treated with phenformin/poly IC (or PBS/saline) until primary tumors reached a tumor volume of $500mm^3$. Control tumors reached the desired volume by 5 to 10 days post-injection while combination-treated ones took approximately 18 days (Figure 12a). Thus, combination-treated mice were tumor bearing for a longer duration than control-treated mice.

Primary tumors were resected and 3 weeks later, lungs were harvested and analyzed for lung metastases. It was observed that there was no significant difference in lung metastatic burden between the control and combination groups (Figure 12b). In fact, the combination group seems to trend downward, despite having tumors for a longer time point. Accordingly, the combination therapy does not increase metastasis to the lung and the elicited neutrophil population does not seem to be one that supports metastatic potential.

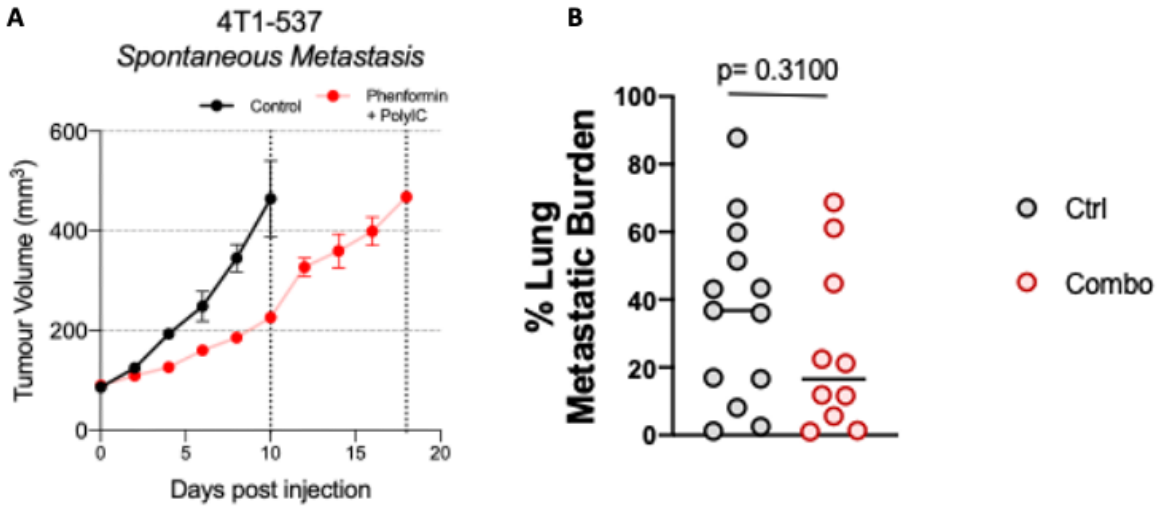

Figure 12. Phenformin/poly IC therapy does not increase metastatic burden

(A)Balb/c mice were injected with 4T1-537 breast cancer cells on one side and treatment with phenformin/poly IC (or PBS/saline) was initiated when tumors reached 100 mm^3. Primary tumors were resected at 500 mm^3. Control tumors took about 5 to 10 days to reach the desired volume whereas combination-treated tumors took 12 to 18 days. (B) 3 weeks post-resection, mice were euthanized, and lungs harvested. Lungs were stained with H and E and lung metastatic burden was assessed using ImageJ. Control: *n*= 13 mice per group, Combination: *n*= 10 mice per group, 1 independent experiment. P value was determined using Prism 8 software unpaired T-test.

Phenformin/poly IC therapy elicits a primarily mature neutrophil population

A neutrophil's maturation state may influence its functional behavior. Neutrophils that are prematurely released into the bone marrow, particularly during situations of emergency granulopoiesis are identified by their unsegmented nucleus. These neutrophils are typically associated with protumor phenotypes and poorer prognosis. Alternatively, mature neutrophils develop completely in the bone marrow and leave with a segmented, multi-lobulated nucleus, making them easily distinguished. Since these neutrophils are fully developed, they typically have evolved cytotoxic functions, rendering them effective killer cells against cancer. However, as previously described, neutrophils likely exist in a continuum of various sub-populations with different polarizations.

We wished to characterize the maturation state of the neutrophil population we were eliciting. To do this, a cytospin was performed on neutrophils isolated from the blood of 4T1-537 tumor-bearing mice treated from all 4 groups to create a monolayer of neutrophils on a microscope slide. Slides were then stained with DAPI, and each neutrophil was scored based on nuclear segmentation. An immature neutrophil was characterized as one where there was no visible nuclear segmentation (Figure 13a) whereas a mature neutrophil was one where nuclear segmentation had occurred (Figure 13b). It was observed that mature neutrophils constituted 76%, 86%, 72% and 92% of control-, phenformin-, poly IC- and combination-treated mice respectively (Figure 13c). The combination therapy induces a primarily mature neutrophil population, capable of killing cancer cells. This is in line with the concept that mature neutrophils may be more cytotoxic in nature, as is described in the literature. This finding provides further evidence that the phenformin/poly IC therapy elicits a cytotoxic neutrophil population.

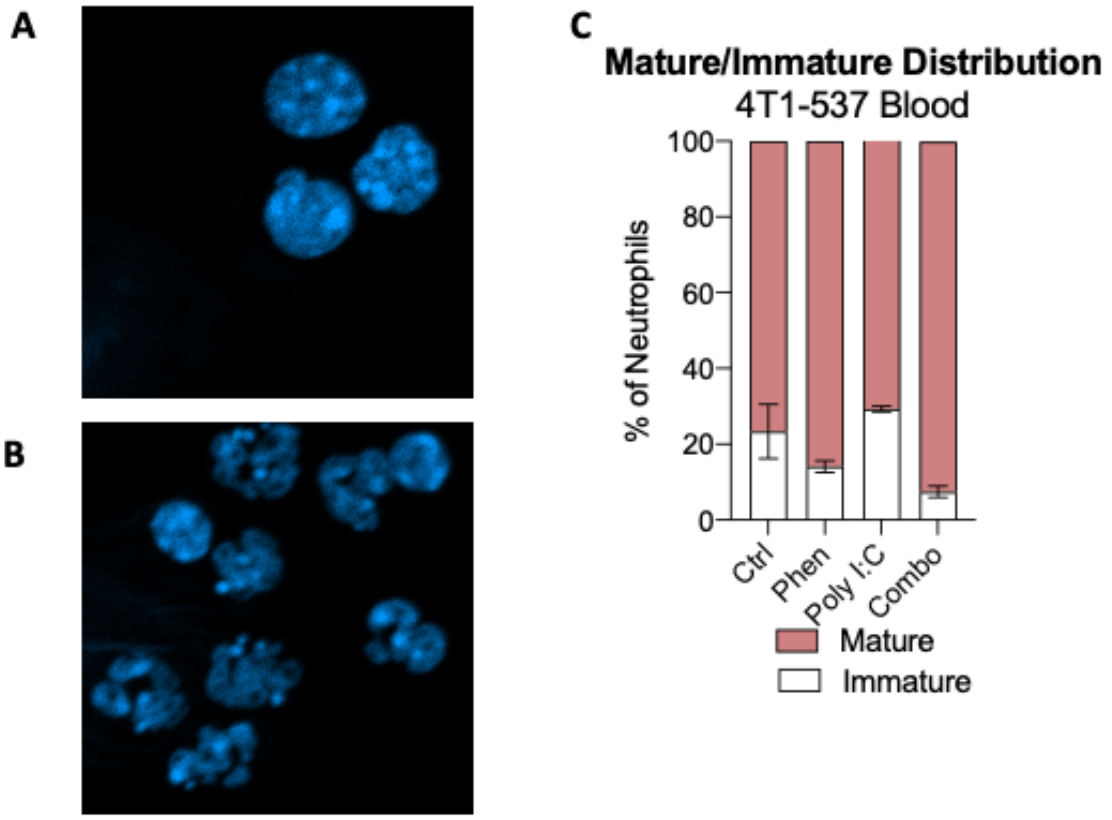

Figure 13. Phenformin/poly IC therapy elicits a primarily mature neutrophil population

Neutrophils isolated from the blood of control (PBS/saline) and combination (phenformin/poly IC)-treated 4T1-537 tumor-bearing mice underwent a cytospin to form a monolayer of cells on a microscope slide. Following fixation with cold methanol, cells were stained with fluorescent DAPI and scored based on nuclear segmentation. (A) Immature neutrophils, characterized by lack of nuclear segmentation. (B) Mature neutrophils, evident by the multi-lobular, segmented nucleus. (C) Relative proportion of immature and mature neutrophil populations present in the blood isolated from mice of all 4 treatment groups.

Phenformin/poly IC therapy is not sufficient to increase a neutrophil's ability to potentiate an oxidative burst

Neutrophils may kill pathogens through various mechanisms including degranulation, ROS production, phagocytosis and NETosis, as described previously. Given the roles phenformin and poly IC play in producing ROS, we hypothesized that neutrophils may engage in cancer cell killing through the production of an oxidative burst. To assess this, neutrophils were isolated from the blood of mice using anti-biotin microbeads. Blood samples were then passed through a magnetic column and neutrophils were eluted from the column. Luminol sodium salt was added to react with any H_2O_2 that is produced by neutrophils and produce luminescence that can be measured. PMA is added as a chemical stimulus to commence the full assembly of NOX, a complex required for the activation and generation of an oxidative burst. It was observed that there was no significant difference in the oxidative burst produced by neutrophils isolated from the blood of all 4 treatment groups (Figure 14a-b). Under stimulation by a strong chemical inducer of an oxidative burst, neutrophils from combination-treated mice do not differentially produce more H_2O_2 than neutrophils from any other treatment group.

Moreover, we wished to assess whether neutrophils isolated from tumor-bearing mice could be treated *ex vivo* to potentiate a larger oxidative burst. Thus, isolated neutrophils were treated with various drugs that have previously been described as robust ROS inducers (Phenformin, IFNβ, Poly IC or LPS). Additionally, we treated neutrophils with 2-DG, a pharmacological inhibitor of glycolysis to evaluate whether neutrophils required glucose to produce an oxidative burst. Following the addition of PMA, we observed that the addition of phenformin, poly IC or LPS did not further potentiate the oxidative burst generated by neutrophils. It seems that exposure to further inflammation once the neutrophils have been

isolated from the blood and have already been exposed to an inflammatory environment *in vivo* does not augment their ability to produce an oxidative burst (Figure 15c-d). Alternatively, it was observed that *ex vivo* treatment with IFNβ significantly reduces the ability of a neutrophil to produce an oxidative burst (Figure 15c-d). This further supports the notion that type I IFN-driven inflammation is simply not responsible for the cytotoxic phenotype observed in the elicited neutrophil population. Lastly, treatment with 2-DG severely impacted the neutrophils' capability to potentiate an oxidative burst (Figure 15e-f). This suggests that neutrophils are likely reliant on glucose for the generation of ROS and dependent on glycolysis. In sum, though ex vivo treatment does not seem to augment a neutrophil's ROS production, it is possible to decrease it via the use of certain inflammatory stimuli and metabolic inhibitors.

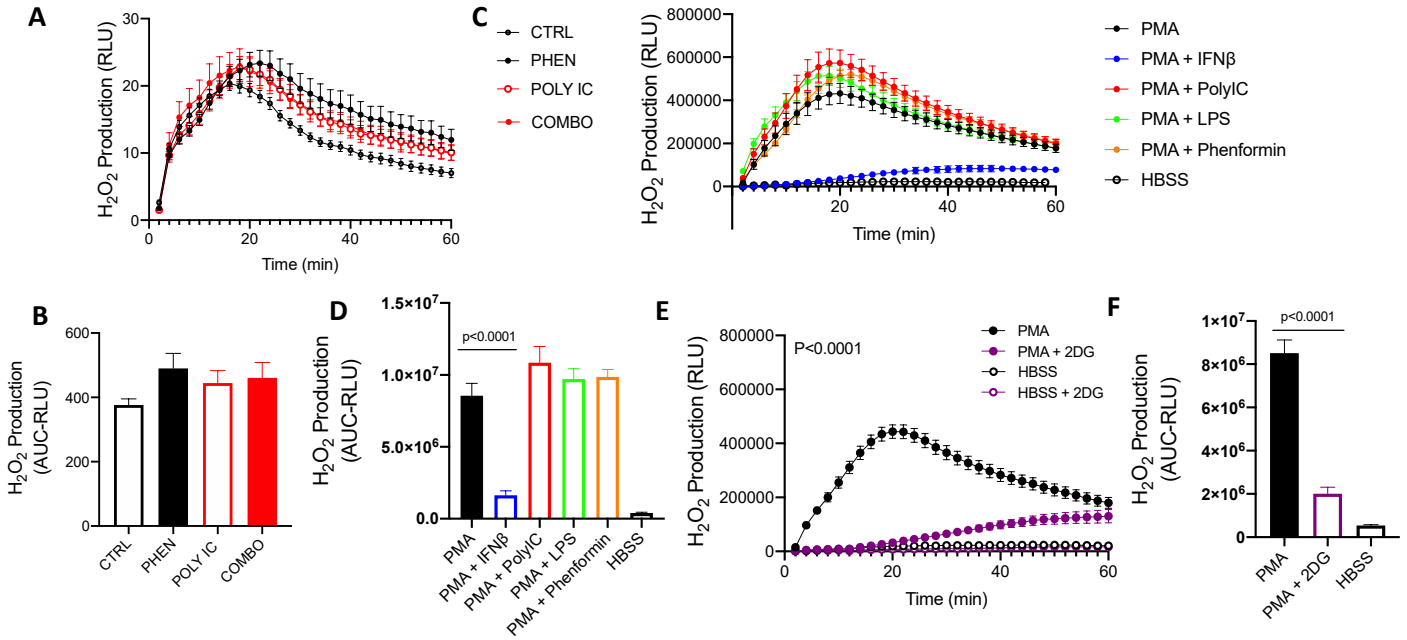

Figure 14. Phenformin/poly IC therapy does not increase a neutrophil's ability to potentiate an oxidative burst

(A)Neutrophils were isolated from the blood of mice treated with pbs/saline, phenformin, poly IC, or phenformin/poly IC and incubated for 30 minutes at 37°C in a 96-well plate. Following incubation, luminol sodium salt was added to each well and a baseline value was measured. Immediately after, PMA was added to each well. Luminescence was read over a time frame of 60 minutes and values were normalized to baseline value. (B) Area under the curve of luminescence emitted for each *in vivo* treatment group. (C) Neutrophils were isolated from tumor-bearing mice. During incubation period, neutrophils were treated with either IFNβ, poly IC, LPS, phenformin or none. After baseline reading, PMA was added or not and luminescence was measured over the course of 60 minutes. All values were normalized to baseline values. (D) Area under the curve of luminescence emitted for each *ex vivo* treatment group. (E) As described in (C), however neutrophils were treated with 2-DG. (F) Area under the curve of luminescence emitted for 2-DG *ex vivo* treatment. In (A-F) RLU: relative luminescence. All significant p-values are shown and calculated using Prism 8 software 2-way ANOVA with Tukey's multiple comparison test.

HL-60 cell line can be differentiated to neutrophil-like cells with similar functional behavior

The isolation of neutrophils to perform *in vitro* assays is a long process which often results in limited numbers of neutrophils available for use. This impedes the number of experiments that can be completed with each mouse cohort, as well as the number of replicates per condition. To circumvent these limitations, we sought to identify whether a cell line could be manipulated to differentiate towards a neutrophil-like cell type. To do this, the HL-60 cell line, a human leukemia cell line, was used. Following expansion, culture media was replaced with a differentiation media consisting of either 1.3% DMSO or 1μM of ATRA. To establish differentiation, CD11b expression was measured using flow cytometry on days 3 and 5 following the initiation of differentiation. It was observed that after 3 days in differentiation media, little CD11b was expressed by cells in normal growth media (Figure 15a-b). In comparison, approximately 30% of cells treated with either DMSO or ATRA were CD11b+. Similarly, following 5 days in differentiation media, approximately 70% and 75% of HL-60 cells were CD11b+, confirming that differentiation was successful.

We next wished to assess whether neutrophil-like cells could potentiate an oxidative burst similarly to mature neutrophils. Thus, control-, DMSO- and ATRA-treated cells were stimulated with PMA identically to how we would stimulate isolated neutrophils. We discovered that DMSO and ATRA differentiated cells could potentiate a larger oxidative burst than the vehicle control (Figure 15c). It seems ATRA may potentiate a larger oxidative burst, likely due to the increase of cells that are CD11b+. This data suggests that HL-60 cells may be differentiated towards a mature neutrophil-like cell type capable of generating an oxidative burst.

Finally, we wished to examine how neutrophil-like cells would respond to in vitro treatment with various inflammatory stimuli and metabolic drugs. Thus, differentiated HL-60 cells were treated with either poly IC, LPS, phenformin or 2-DG. Similarly to the observations made previously with mouse neutrophils, treatment with poly IC, LPS or phenformin did not significantly influence the oxidative burst produced by neutrophil-like cells (Figure 15d). Moreover, treatment with 2-DG significantly reduced the oxidative burst potentiated by neutrophil-like cells, suggesting a requirement for glycolysis. Together, these finds indicate that neutrophil-like cells share a similar phenotype to neutrophils isolated from tumor-bearing mice, suggesting they could likely be used for in vitro studies, as a replacement for neutrophils, a limiting resource.

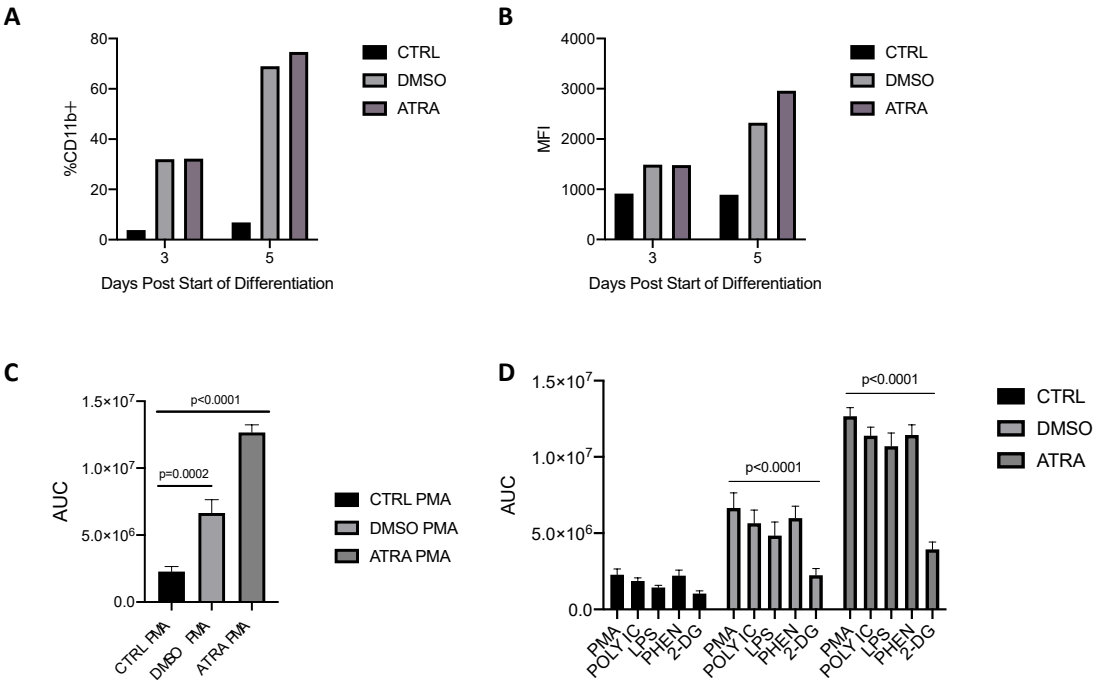

Figure 15. HL-60 cell line can be differentiated to neutrophil-like cells with similar functional behavior

HL-60 cells were treated with differentiation media consisting of either 1.3% DMSO or 1 μM of ATRA (or standard growth media for vehicle control) for up to 5 days. On days 3 and 5, an aliquot of cells was stained with a CD11b antibody and samples were analyzed via flow cytometry. (A) shows the % of cells that are CD11b+. (B) shows the mean fluorescence intensity. (C) Differentiated HL-60 cells were plated at a density of 2 x 10^5 cells/well on a 96-well white bottom plate and stimulated using PMA. Luminescence was measured every 2 minutes for 1 hour and area under the curve (AUC) is displayed. Significant p-values are shown and calculated using Prism 8 software 1-way ANOVA Dunnett's multiple comparisons test. *n* = 12, 2 independent experiments. (D) Differentiated HL-60 cells were treated for 30 minutes with either poly IC, LPS, phenformin or 2-DG in the 37°C incubator. Following incubation, PMA was added to all wells. Luminescence was measured every 2 minutes for 1 hour and area under the curve (AUC) is displayed. Significant p-values are shown and calculated using Prism 8 software 2-way ANOVA Tukey's multiple comparisons test. *n* = 12, 2 independent experiments.

Chapter 4: Discussion

In this project, we aimed to identify how phenformin and poly IC worked synergically to reduce tumor growth in murine models of breast cancer. Our lab has previously shown that these two molecules target a selective vulnerability in breast cancer cells, resulting in cancer cell death. This strategy focuses on the underappreciated role of phenformin as a ROS producer, exposing cells to elevated levels of oxidative stress (335).

Most clinical studies using biguanides in oncology focus on metformin, since it is widely used for the treatment of type 2 diabetes, better tolerated and has a lower risk of lactic acidosis(72). Moreover, several studies using metformin alone, or in combination with other therapies, have shown its potential in a variety of cancers (342-344). However, as previously described, alone, metformin did not increase survival in non-diabetic women suffering from breast cancer (73).Thus, perhaps shifting our focus towards the use of phenformin may be beneficial in oncology. Phenformin has been found to be much more potent than metformin and has been shown to have higher antitumor effectiveness (345). Studies of several cancer types, including breast and liver, demonstrate that phenformin is more potent in inhibiting tumor growth than metformin, both *in vivo* and *in vitro* (346,347). Unlike metformin, phenformin does not rely on organic cation transporters (OCTs) for entry into the cell; this permits a higher phenformin concentration within the tumor cell and may also achieve successful treatment of tumors with no OCT overexpression (348). Ultimately, more potent complex I inhibition using phenformin displays increased cytotoxicity.

Several studies have shown that a combination of phenformin with other anticancer agents may be beneficial for the treatment of various cancers. For instance, combined treatment with SCH772984 and phenformin inhibited proliferation of mutant melanoma cells through a

combination of complex I and extracellular signal-regulated kinase (ERK) inhibitors (349). Moreover, the combination of phenformin and oxamate/dichloroacetate (DCA) may be a promising therapeutic option. While phenformin blocks complex I, increases ROS production and decreases the activation of oxidative phosphorylation (OXPHOS), DCA or oxamate may decrease glycolysis (350,351). Cancer cells have metabolic flexibility in choosing either OXPHOS or glycolysis for ATP generation. Thus, this powerful combination results in metabolic catastrophe for tumor cells, due to cellular ATP pool depletion, thus causing tumor cell death. Our results using both phenformin and IACS-010759 contribute to accumulating evidence that complex I inhibitors should be exploited therapeutically and set the stage for clinical trials that look for the best combinations of complex I inhibitors with ROS stimulators. Combination strategies may decrease the therapeutic dose of biguanides necessary to achieve the desired results, bypassing the current limitations associated with biguanides in oncology therapies.

Breast cancers have typically been viewed as immunologically silent, limiting the efficacy of ICIs. A major cause for the failure of ICIs can be attributed to the inability of T cells to infiltrate within the TME (352). Accordingly, it has been shown that a higher number of tumor- infiltrating lymphocytes can been observed in TNBC, and these are the breast tumors that respond best to ICIs (353). In fact, each 10% increase in intratumoral lymphocytic infiltration was associated with a 17% reduced risk of recurrence and 27% reduced risk of death (354,355). Even though the combination therapy described in this paper is dependent on neutrophils, which are innate immune cells, we still observe an overall increase in immune cell infiltrate within the tumor, when amount of immune infiltrate is normalized to tumor size. As such, combination with T-cell targeted therapies such as ICIs represents an attractive avenue for future studies which

may further increase therapeutic efficacy. While the combination therapy may elicit a cytotoxic innate immune cell population, it may also act as a means for attracting T cells into the tumor, eliminating a barrier associated with immunotherapies, specifically in immune "cold" tumors. Thus, when combined with phenformin/poly IC, ICIs may activate a cytotoxic adaptive immune response within the tumor. Together, these two approaches may work synergically to potently eradicate tumors through the activation of simultaneous cytotoxic innate and adaptive immune responses.

The current findings reported here propose a model by which neutrophils can be harnessed to eliminate breast tumors. Most previous studies that look at neutrophils in cancer focus on ways to either block the development and/or recruitment of pro-tumorigenic neutrophils or find ways to diminish their suppressive properties. Tumor-promoting neutrophil formation is induced primarily by tumor-derived cytokines including GM-CSF, G-CSF and VEGF (356). Thus, inhibition of these molecules/pathways has been found to impair the development of this neutrophil population. For instance, the use of an anti-GSF antibody or IL-12 has been found to reduce the number and activity of tumor-promoting neutrophils (357,358). Moreover, TANs are recruited via specific chemokine signaling pathways, making inhibitors of chemokine receptors a promising therapeutic approach. For instance, CCR2 blockade with a small molecule CCR2 inhibitor reduced tumor burden in a model of pancreatic adenocarcinoma by blocking the recruitment of TANs to the tumor. Furthermore, when combined with chemotherapy, there was a significant increase in survival (359). Lastly, various strategies have been described to dampen the immunosuppressive properties of TANs. One example is the use of cabozantinib, a receptor tyrosine kinase inhibitor, in combination with a dual PI3K/mTOR inhibitor, dactolisib, significantly downregulated immunosuppressive genes in tumor-promoting neutrophils including

Arg1, Ncf1 and Ncf4. Additionally, these molecules synergized with ICIs to eliminate primary and metastatic prostate tumors (360).

Here we show that biguanides, when used in combination with molecules that stimulate systemic inflammation such as poly IC, can elicit an increase in neutrophils in both the tumor and blood of mice bearing breast tumors. However, it has not been identified whether this drug combination is acting directly or indirectly on the neutrophil population. Furthermore, it is demonstrated that these neutrophils are required for the therapy to be effective, and that the neutrophil population is polarized towards a mature, cytotoxic phenotype capable of killing cancer cells directly *in vitro*. Ultimately, our findings lay the groundwork for a means of activating cytotoxic neutrophils for cancer therapy rather than focusing on ways to diminish their suppressive effects. Neutrophils are numerous in the body, particularly during cancer progression, as has been described previously by an elevated NLR in cancer patients (286). Thus, exploiting the abundance of neutrophils, as well as their plasticity, offers an attractive opportunity to use neutrophils as powerful anti-cancer effector cells whereas depletion or inhibition of their immunosuppressive properties would obliterate their potential. Currently, we present a mechanism by which neutrophils can be utilized to minimize tumor growth, but future work would be needed to design therapeutic approaches ready to enter the clinic.

In the current study, we examine whether neutrophils are required for therapeutic effectiveness using a Ly6G monoclonal antibody, as previously described. It is crucial to address that anti-Ly6G agents are not specific to neutrophils. In mice, Ly6G is also expressed by granulocytic MDSCs. These cells have emerged as important contributors to cancer progression, notably through mechanisms of immune suppression, as well as contribution to angiogenesis and metastasis (361). Furthermore, MDSCs have been shown to reduce the efficacy of anti-PD-1 and

anti-CTLA4 immunotherapies (362). Given that the Ly6G depletion completed in this study results in a loss of anti-tumorigenic activity, it is thus not likely that the population we are targeting and eliminating is a traditional granulocytic MDSC. This population does not typically act as a cytotoxic cell type, whereas neutrophils are commonly identified as cancer-killing cells.

Activation of the NF-κB pathway acts as a double-edged sword in the context of cancer. On the one hand, it is constitutively activated in many types of cancer and has been associated with pro-tumorigenic functions. For instance, NF-κB activation may promote tumor angiogenesis through the upregulation of VEGF in colorectal cancer (363). Moreover, it has been shown that NF-κB activation can promote tumor cell cycle progression and apoptosis, promoting cellular proliferation in a model of bladder cancer through the upregulation of survivin, a well-known cancer specific molecule and a marker of poor prognosis (364). On the other hand, the activation of NF-κB-driven inflammation does play a key role in mediating the immune response. Consequently, it may activate antitumor immune responses; for example, NF-κB was found to be required for conventional dendritic cell maturation and subsequent tumor cell killing (365). Furthermore, genetic loss of IKKβ, an NF-κB pathway regulating kinase was found to result in more rapid growth of melanoma tumors, due to an inhibition of macrophage tumor cell killing (366).

Here, we provide further evidence that NF-κB-driven inflammation may be responsible for anti-tumor immune responses. The phenformin/poly IC therapy induces an upregulation of TNFα, IL-1β and IL-6 in mammary breast tumors, a cytokine signature associated with NF-κB inflammation. Moreover, we observe increases in CCL2 and CCL5 in tumors, 2 cytokines downstream of NF-κB. Interestingly, CCL5 was also noted to be differentially expressed in combination tumors, in comparison to control-treated tumors by single cell RNA sequencing.

CCL5 is a cytokine that has been shown to be involved in neutrophil recruitment via activation of macrophages (367). Similarly, using a transgenic mouse model that overexpresses CCL5 in the lung, it was observed that CCL5 overexpression displayed a significant increase in neutrophil infiltration compared to control mice (368). This may be important in preventing circulating tumor cells from entering the lung during the metastatic cascade. Finally, CCL5 has been shown to increase neutrophil H_2O_2 production, resulting in an increase in breast tumor cell death (311). Here, we provide further evidence to support that the NF-κB pathway may be beneficial in activating tumor-killing immune cell subsets. However, further work would need to be done to confirm whether this pathway is required for the observed neutrophil cytotoxicity. Moreover, we provide some evidence showing an association between CCL5 and cytotoxic neutrophil activation. While the literature primarily focuses on the role of CCL5 in neutrophil recruitment, we focus on the role it may play in inducing a cancer-killing neutrophil population, a topic that remains poorly described.

Moreover, we observe an increase in CCL2 production in tumors from mice treated with either poly IC alone or in combination with phenformin. Interestingly, CCL2 is a critical chemokine for the recruitment of NK cells. In fact, antibody-mediated neutralization of CCL2 in a mouse model of invasive aspergillosis resulted in an impaired recruitment of NK cells to the lungs during infection (369). In the context of cancer, using a model of hepatocellular carcinoma, it was identified that antibody-mediated neutralization of CCL2, but not CCL3, CCL4 or CCL5 prevented NK cell recruitment and infiltration into tumors, while also reducing tumor elimination (370). Several studies have shown that NK cells are incredibly sensitive to poly IC treatment (371,372). Importantly, NK cells have been shown to respond to poly IC through the production of IFN-gamma (373). IFN-gamma may enhance neutrophil functions. For instance, in

a cohort of patients with CGD, it was discovered that IFN-gamma administration resulted in the release of O_2^- from neutrophils. Additionally, IFN-gamma treatment altered gene expression in over 2700 neutrophil genes, including the upregulation of genes involved in generating NOX, as well as proteins involved in MHC I and II (374). Finally, the presence of both NK cells and IFN-gamma has been found to convert tumor promoting neutrophils to tumor suppressing ones (375). Ultimately, the therapy presented in this study upregulates CCL2; a key chemokine involved in the recruitment of NK cells. Moreover, NK cells are extremely sensitive to poly IC and respond through the secretion of IFN-gamma, a molecule shown to play a role in neutrophil activation. Though further investigation is required, it is possible that NK cells play a critical role in bridging the gap between poly IC administration and the observed neutrophil cytotoxicity.

After successful extravasation, tumor cells must survive in a new environment in a distant organ to form metastasis. The contribution of neutrophils to the progression of metastasis and the formation of a favorable premetastatic niche remains controversial. On the one hand, several studies have shown that the accumulation of neutrophils within the premetastatic niche may support metastasis. For example, CXCR2-dependent accumulation of neutrophils in the pre-metastatic niche has been shown to be required for liver metastases to form in pancreatic ductal adenocarcinoma (376). Similarly, another group has found evidence to support that neutrophils are the main driver of metastatic establishment within the premetastatic lung in mouse models of breast cancer. Mice deficient in G-CSF were unable to accumulate neutrophils in the lung and exhibited a robust reduction in spontaneous lung metastases (377). Finally, it has been observed that neutrophils specifically attracted by the CXCL12-CXCR4 signaling pathway increase progressively during breast cancer progression and accumulation was closely related to the formation of the premetastatic niche (378).

On the other hand, a few studies have identified cytotoxic neutrophils within the premetastatic niche that may prevent metastases from forming. Despite remaining a relatively poorly studied phenomenon, it was identified that IFNγ-producing monocytes got recruited to the lung where they prime neutrophils towards an antitumor phenotype, inhibiting metastasis by killing circulating tumor cells attempting to reside in the lung (310). Similarly, another group identified that a neutrophil depletion resulted in increased metastatic burden in comparison to controls. In fact, despite there being no change to the size of the metastases, there was a 3-fold increase in the number of metastases formed in the lungs of neutrophil-deficient, tumor-bearing mice (311).

Here we show that despite an increase in abundance of neutrophils in the blood and tumors of mice treated with phenformin and poly IC, we do not observe any significant increases in metastases. In fact, it seems that the neutrophil population may prevent metastatic seeding in the lung, given the trend downwards in the number of metastases observed. It is imperative to recall that this slight trend downwards is observed even though combination-treated mice had primary mammary tumors for almost double the amount of time that control-treated mice had tumors. This finding may be incredibly beneficial in a clinical setting; not only does the combination therapy disrupt the growth of the primary tumor, but it may also slow metastatic onset. This is extremely relevant considering that, according to the American Cancer Society, the 5-year survival rate for women suffering from localized breast cancer is 99% yet drops to 30% following metastasis. Further investigation would need to be done to characterize the neutrophil population inhabiting the lung and identify a mechanism responsible for inhibiting the colonization of circulating tumor cells within the lung. However, the identification of therapies

that may simultaneously impact the primary tumor as well as the premetastatic niche remains a promising approach that should be explored more deeply.

Neutrophils rely on various mechanisms for killing pathogens, including degranulation, ROS production, phagocytosis and NETosis. We hypothesized that neutrophils may be killing cancer cells through the production of ROS, given the significant role both therapeutic drugs play in ROS generation. Interestingly, it was observed that there were no changes in H_2O_2 production between neutrophils isolated from the blood of control- and combination-treated tumors. A few possible explanations may explain why no changes can be observed. Firstly, luminol bioluminescence is dependent on MPO. MPO is a member of the XPO subfamily of peroxidases and produces hypochlorous acid (HOCl) from H_2O_2 and chloride anion (Cl^-) during the neutrophil's respiratory burst. Ultimately, it is possible that MPO might be the limiting factor; if there isn't enough of it to convert all H_2O_2 to HOCl, then it might not be possible to capture the full picture of all the H_2O_2 being produced by combination-treated neutrophils. This would explain the lack of variation between treatment groups. Another possibility is that neutrophils are killing cancer cells through ROS, but not H_2O_2. Neutrophils may produce a variety of ROS, not only H_2O_2. One limitation associated with the use of luminol is that it may only convert H_2O_2 to measurable luminescence, not other forms of ROS. Thus, if neutrophils are using other forms of ROS as well, we may not be capturing those during our analyses. Using other agents, such as lucigenin, that may pick up levels of other forms of ROS may be beneficial in identifying which ROS sources are being generated by neutrophils during an oxidative burst. Moreover, all analyses presented in this work use PMA, a chemical stimulus to trigger the potentiation of an oxidative burst. This is likely less representative of what is occurring *in vivo*, where neutrophils are activated through the detection of cancer cells. Thus, it is possible that neutrophil activation

with PMA results in a different level of oxidative burst than what occurs in our mouse model. Using cancer cells as a means of activating neutrophils *in vitro* may be a way to circumvent this possibility and provide a more accurate representation of our neutrophil populations' oxidative burst. Finally, it is possible that the observed neutrophil cytotoxicity is not due to ROS production at all, but rather another mechanism of neutrophil killing. Ultimately, further work would need to be done to identify which method of killing is being employed by neutrophils during phenformin/poly IC therapy.

It was possible to generate HL-60 cells that acquire a neutrophil-like phenotype using ATRA, and DMSO to a lesser extent. Moreover, these neutrophil-like cells were found to respond similarly to *in vitro* drug treatment to mouse neutrophils. This provides a novel means to further characterizing a neutrophil's functional behavior without having to endure the limitations associated with neutrophil isolation. This will allow for further research to be done in potentially investigating whether phenformin/poly IC induces metabolic alterations to neutrophils and how blocking of the NF-κB pathway/ CCL5 may influence neutrophil cytotoxicity, amongst many questions.

Despite the promising findings presented in the current study, further work is necessary for translation into the clinic to occur. While mouse neutrophils do express TLR3, the poly IC receptor, human neutrophils do not(220). Thus, alternative inflammatory stimuli that activate neutrophils with a similar cytotoxic phenotype would need to be identified. For instance, studying molecules that may activate the NF-κB pathway, in a TLR3 independent fashion, may be a promising approach. Moreover, identifying key inflammatory cytokines that are required for therapeutic efficacy may bypass the need for poly IC administration. For instance, looking at TNFα, IL-6, CCL2 and CCL5 (all cytokines found to be upregulated by poly IC therapy in this

study) and pinpointing which combination of these molecules is necessary for neutrophil cytotoxicity. If keys cytokines may be identified, then these agents could be used directly, in combination with phenformin, to eradicate tumor cells in human patients.

Conclusions

Altogether, we present a novel therapy that polarizes neutrophils towards a primarily mature, cytotoxic neutrophil population capable of killing breast cancer cells both *in vitro* and *in vivo.* Despite an increase in the abundance of neutrophils in the blood and tumor of phenformin/poly IC-treated mice, the elicited neutrophil population does not increase metastatic burden, seeming to potentially protect the lung for circulating cancer cell colonization. This study positions neutrophils as an attractive immune cell subset that could be harnessed therapeutically in oncology, particularly for the treatment of tumors with minimal response to ICIs due to low immune cell infiltrate.

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