Mechanisms of Macrophage-Mediated Chemoprotection in Breast Cancer

by

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A Dissertation

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Dedication

I would like to dedicate this thesis to my father, Robert Fredrick Olson.

Abstract

Tumor-associated macrophages (TAMs) are emerging as dynamic regulators of therapeutic response and the development of resistance in cancer treatment. TAMs have been shown to play critical roles in promoting cancer cell survival and regrowth of the tumor as well as suppressing the immune response associated with chemotherapeutic treatment. We have found that TAM accumulation in breast tumors increases in patients and mice treated with certain chemotherapeutic drugs. Furthermore these TAMs protect tumor cells against chemotherapy-induced death and we found that this protection was mediated in large part via specific members of the cysteine cathepsin protease family. Focusing on the widely utilized antimitotic agent Taxol, we also observed that co-culture of breast cancer cells with macrophages reduces the duration of mitotic arrest and accelerates the onset of mitotic slippage and failed cytokinesis. Macrophages reduce the levels of γH2AX and decrease phosphorylation of the tumor suppressor P53 in the newly tetraploid breast cancer cells and suppress subsequent cancer cell death. The ability of macrophages to affect the extent to which Taxol induces cancer cell mitotic arrest, and thereby promote survival of the newly aneuploid cells has significant implications for the potential role of the tumor microenvironment in interfering with chemotherapy-induced cancer cell death and promoting genomic instability. This research highlights the importance of the microenvironment in directing cytotoxic mechanism of action and cell fate in the context of therapeutic treatment.

Biographical Sketch

Oakley was born to Robert and Torie Olson on May 24th 1984 in Rockingham, Vermont. The entirety of Oakley's childhood was spent in Putney, Vermont and involved many outdoor and agricultural activities common to the region including cross-country and down hill skiing, mountain biking, maple sugaring, and dairy farming. In 2002 Oakley matriculated at Brown University in Providence, Rhode Island. As a university student Oakley studied bio- and organic chemistry and conducted undergraduate research with Wayne D. Bowen, PhD. This research involved the identification of structure-activity relationships for sigma-2 receptor agonists and culminated in an honors thesis. After receiving a Bachelor of Science (ScB) in 2006, Oakley accepted a research assistant position at Stanford University in Palo Alto, California. There, in the lab of Stephen K. Foung, MD, Oakley worked to map conformational epitopes of broadly neutralizing antibodies to the hepatitis C virus (HCV). In July of 2009 Oakley moved to New York, New York and matriculated into the Louis V. Gerstner Jr. Graduate School of Biomedical Sciences at Memorial Sloan Kettering Cancer Center.

Acknowledgments

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I would like to thank the postdoctoral fellows and graduate students with whom I worked on the research presented in this thesis. I would like to thank Dr. Tanaya Shree who initiated the study into the role of cathepsins in chemotherapeutic response. I would like to thank Dr. Hyunjung Kim, who has collaborated in assessing the effects of stromal cells on mitotic arrest. I would like to thank Dr. Daniela Quail, with whom I've collaborated on research into the effects of obesity on breast cancer metastasis, research not included in this thesis. I would also like to thank all the members of the Joyce lab, past and present, who have been to a person always eager to lend a hand, teach a technique, or discuss a problem. In particular I would like to thank Dr. Leila Akkari, whose generosity, both as a coworker and as a friend, is unparalleled.

I would like to thank my friends and family for their love and support through the course of this endeavor. I would like to thank my parents, Robert and Torie, who have always been so supportive and present. I would like to thank my sister, Jessamyn Olson Skarr, who like her newborn daughter can always put a smile on my face. I would like to thank my grandfather, Elihu Dale Albert, who was always the greatest champion of my education and my interest in science. I would also like to thank Drs. Shannon Yu and John Maciejowski, who befriended me upon my arrival at GSK and have been incredible friends throughout. Finally I would like to thank the doctors and nurses of MSKCC and MGH who cared for my father prior to his death from metastatic melanoma. Without

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABP: Activity based probe

AIF1: Allograft inflammatory factor 1

AKT: Protein kinase B

ANGII: Angiotensin II

Athy/Nu: Athymic/Nude

ATM: Ataxia telangiectasia mutated

ATR: Ataxia telangiectasia and Rad3-related protein

BAK: Bcl-2 homologous antagonist/killer

BAX: BCL2-Associated X Protein

BCL-XL: B-cell lymphoma-extra large

BCL2: B-cell lymphoma 2

BID: BH3 interacting-domain death agonist

BMDM: Bone marrow derived macrophage

CAFs: Cancer associated fibroblasts

Cath-ABP: Cathepsin activity based probe

CC3: Cleaved caspase 3

CD163: Cluster of differentiation 163

CD45: Cluster of differentiation 45

CD68: Cluster of differentiation 68

CK: Cytokeratin

CM: Conditioned media

CRC: Colorectal cancer

CSF-1: Colony stimulating factor-1

CSF1R: Colony stimulating factor 1 receptor

CTSB: Cathepsin B

CTSC: Cathepsin C

CTSF: Cathepsin F

CTSH: Cathepsin H

CTSK: Cathepsin K

CTSL: Cathepsin L

CTSS: Cathepsin S

CTSZ: Cathepsin Z

DAPI: 4',6-diamidino-2-phenylindole

DMSO: Dimethyl sulfoxide

ECM: Extracellular matrix

EGF: Epidermal growth factor

ER: Estrogen receptor

ERK: Extracellular-signal-regulated kinase

FAK: Focal adhesion kinase

FRNK: FAK related non-kinase

HER2: Human epidermal growth factor receptor 2

HMW: High-molecular weight

HPV16: Human papilloma virus type 16

HSP70: Heat shock protein 70

IL-10: Interleukin 10

IL-1β: Interleukin 1 beta

IL-4: Interleukin 4

JAM-B: Junctional adhesion molecule B

kDa: Kilodalton

Li: Invariant chain

LMP: Lysosomal membrane permeabilization

LMW: Low-molecular weight

M6PRs: mannose-6-phosphate receptors

MAPK: Mitogen-activated protein kinases

MCL1: Myeloid cell leukemia 1

MEK: MAPK/ERK Kinase

MHC II: Major histocompatibility complex class II

MiT/TFE: microphthalmia/transcription factor E

MMP: matrix metalloproteinase

MMTV: Mammary tumor virus promoter

MOMP: Mitochondrial outer membrane permeabilization

MTD: Maximum tolerated dose

mTOR: Mammalian target of rapamycin

MZF1: Myeloid zinc finger 1

NOTCH1: Notch homolog 1

PanIN: Pancreatic intraepithelial neoplasia

PanNET: Pancreatic neuroendocrine tumor

PDX1: Pancreas and duodenum homeobox 1

PERK: Protein kinase R-like endoplasmic reticulum kinase

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

PR: Progesterone receptor

PyMT: Polyoma middle T antigen

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

RAB7: Ras-related protein Rab-7a

RGD: Arg-gly-asp integrin-binding motif

RIP1: Rat insulin promoter

RIPK1: Receptor-interacting serine-threonine protein kinase 1

SAC: Spindle assembly checkpoint

SCC: Squamous cell carcinoma

SPI2A: Serpin 2A

SRC: SRC proto-oncogene, non-receptor tyrosine kinase

STAT3: signal transducer and activator of transcription 3

Tag2: SV40 Large T antigen

TAMs: Tumor associate macrophages

TCGA: The cancer genome atlas

TGFβ: Transforming growth factor beta

TIMP: Tissue inhibitors of metalloprotease

TNF: Tumor necrosis factor

tPA: Tissue plasminogen activator

uPA: urokinase-type plasminogen activator

yH2AX: Phosphorylated H2A histone family, member X

Chapter 1

Introduction

Cancer has long been considered to be principally a genomic disease. The cancer cell is genomically unstable and clonal in origin, arising from somatically acquired mutations and chromosomal gains, losses, and rearrangements[1]. This is, however, a highly reductionist view and the tumor itself is comprised of a complex mixture of cancer cells, stromal cells, and extracellular matrix (ECM) molecules (Fig. 1.1). These stromal cells include the endothelial cells of the lymphatics and hematogenous vasculature, mesenchymal cells of the connective tissue such as fibroblasts, and infiltrating immune cells such as macrophages, T cells and B cells, which are predominantly recruited from the periphery. While cancer cells have acquired the ability to bypass the constraints regulating their proliferation and tissue homeostasis, the tumor still exists as a tissue environment. It has become well established that many elements of the tumor microenvironment provide critical functions supporting the growth, survival, and spread of cancer cells[2]. Indeed the ability of cancer cells to co-opt stromal cells and re-educate them to establish a pro-tumorigenic microenvironment has emerged as an important concept in the hallmarks of the disease[3].

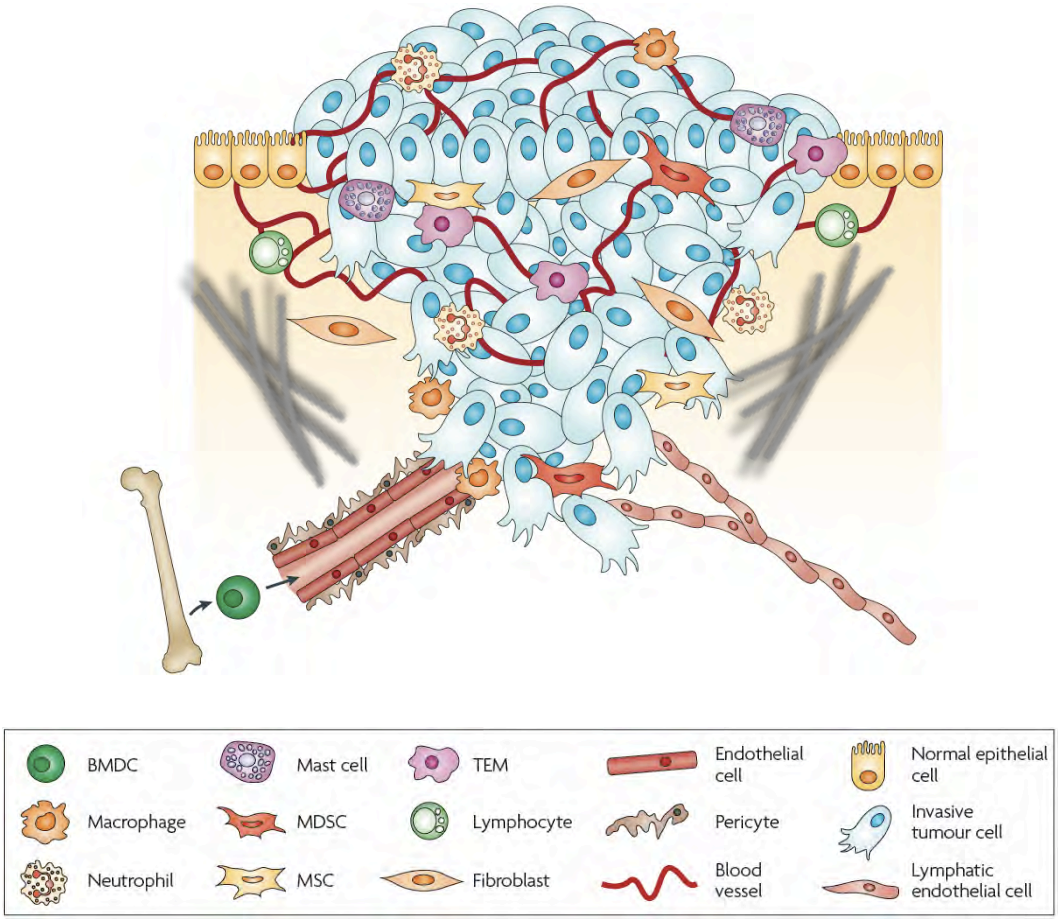

Figure 1.1: Complexity within the tumor microenvironment. Cancers develop within a complex tissue microenvironment, where tumor cells recruit stromal or non-cancerous cells from the surrounding tissue and the bone marrow. These stromal cells include fibroblasts and other mesenchymal cells, infiltrating immune cells, as well as the endothelial cells and pericytes that comprise the hematogenous vasculature and tumor lymphatics. The extracellular matrix (grey lines) and other non-cellular components of the tumor microenvironment can also significantly impact tumor biology and disease progression. Adapted from Joyce, J.A. and Pollard, J.W. (2009). Microenvironmental regulation of metastasis. *Nature Reviews Cancer 9*, 239-252.

One stromal cell type that has emerged as a particularly potent pro-tumorigenic effector within the microenvironment is the tumor-associated macrophage (TAM). Macrophages are a part of the mononuclear phagocyte system and as such serve a critical function in removing dead and dying cells from the tissue[4]. Tissue-resident macrophages also patrol their microenvironment and in this manner are often the point of first contact with the immune system[4]. However what makes macrophages ideal for co-option by cancer cells is their plasticity and ability to respond heterotypic signaling from cells within the tumor microenvironment[5]. In this manner, education of macrophages within the tumor environment toward a “wound healing” phenotype strongly supports tumor growth and metastasis. TAMs have been shown to promote angiogenesis, remodel the ECM, and promote tumor cell proliferation, invasion and metastasis[5].

Research into the tumor microenvironment has also identified proteases as an important class of enzymes responsible for reshaping the tumor microenvironment and promoting disease progression. Secretion of proteases into the extracellular space allows for remodeling of the ECM, activation of growth factors and cytokines, disruption of tumor cell-cell junctions and invasion into the surrounding tissue[6]. Cysteine cathepsin proteases in particular have become recognized as a functionally important family of enzymes in tumorigenesis[7]. Cathepsins promote many hallmarks of cancer progression including angiogenesis, proliferation, apoptosis and invasion[8, 9]. Cathepsin proteases are often upregulated in cancer cells, but are also abundantly expressed by TAMs[10]. Indeed the intersection of TAMs and cathepsins in tumor

biology has been a major area of research in the Joyce laboratory and is the focus of the research presented here in this thesis.

Investigation into the functional contributions of the tumor microenvironment has largely focused on its role in tumor initiation, growth and metastasis. Clinical management of cancer patients, however, often involves radical intervention including surgery, radiation and chemotherapy. Thus the functional contribution of the tumor microenvironment to therapeutic response is critical to understanding this disease and developing new treatment strategies. Pioneering work in this field found that tumors in vivo can develop chemotherapeutic resistance that is not tumor cell-intrinsic[11]. Early investigations also identified mechanisms of tumor cell adhesion-mediated resistance as well as resistance induced by stromal-secreted factors[12]. More recently it has also been reported that in response to chemotherapy reactive systemic processes induce mobilization of endothelial progenitor cells which aid in revascularization of the tumor[13]. These findings underscored the dynamic nature of the tumor microenvironment and how chemotherapy could alter its composition. Our research has sought to further these studies by investigating the roles of macrophages and cathepsin proteases in the response to chemotherapy.

Cysteine cathepsin proteases

The cysteine cathepsin family is comprised of 11 members in humans (CTSB, C, F, H, K, L, O, S, V, W, and X/Z; Fig. 1.2)[14]. The majority of cysteine cathepsins possess endopeptidase activity, cleaving non-terminal peptide bonds within their protein substrates. Additionally, CTSB and CTSH possess carboxy- and amino-peptidase activity respectively. CTSC and CTSZ are the only two cysteine cathepsin family members that do not have endopeptidase activity. CTSC, also known as dipeptidyl peptidase I, is a strict dipeptidyl aminopeptidase, while CTSZ is a carboxymonopeptidase[15, 16]. CTSZ is unique in its possession of an integrin-binding Arg-Gly-Asp (RGD) motif located in the pro-domain[17]. The enzymatic active site of cysteine cathepsins is comprised of a cysteine, a histidine, and an asparagine residue forming a classic acid-base-nucleophile triad. The majority of these enzymes are further post-translationally modified by glycosylation, a process that is critical for engagement with mannose-6-phosphate receptors (M6PRs) and lysosomal trafficking[18]. M6PRs can both sort lysosomal hydrolases from the trans-golgi network as well as recover secreted enzymes through the endocytic pathway. Indeed the tumor suppressive activity of M6PR is thought to be due in part to its ability to suppress aberrant trafficking of lysosomal enzymes[19].

In the context of cancer, the dynamics of many of these processes are altered. In response to oncogenic signaling, lysosome localization transitions from a peri-nuclear to a membrane proximal position[20, 21]. Secretion of cathepsins into the extracellular space can result from these alterations in endo-lysosomal

trafficking[22, 23]. Infiltrating immune and stromal cells can be similarly activated to secrete cathepsin proteases into the extracellular space[10, 24], but the underlying mechanisms of this altered trafficking are poorly understood and remain an important and open question in the field.

As members of the C1 or papain family of proteases, cysteine cathepsins are synthesized as inactive zymogens, and cleavage of an inhibitory propeptide is required for full proteolytic activity. These propeptides are either auto-catalytically removed at low pH or cleaved by other proteases. Multiple mechanisms have been identified by which propeptides either lose structure upon protonation or are excluded by stabilization of occluding loops, ultimately resulting in exposure of the active site and subsequent autocatalytic cleavage[25, 26]. These soluble propeptide fragments can retain their ability to inhibit cathepsin function and thus represent a further level of complexity in the regulation of cathepsin activity[27, 28]. Cathepsin activity is optimized for the reducing environment and the low pH of the lysosome[29, 30], which helps restrict their activity to this compartment under normal physiological conditions. However, it has long been appreciated that acidification occurs in the extracellular environment of the tumor[31, 32]. This adaptation supports the extracellular activity of secreted cathepsins proteases and the low pH has also been shown to induce cathepsin secretion[33, 34]. Indeed, experimentally raising the pH of the microenvironment causes a reduction in invasion and metastasis in models of carcinogenesis[35].

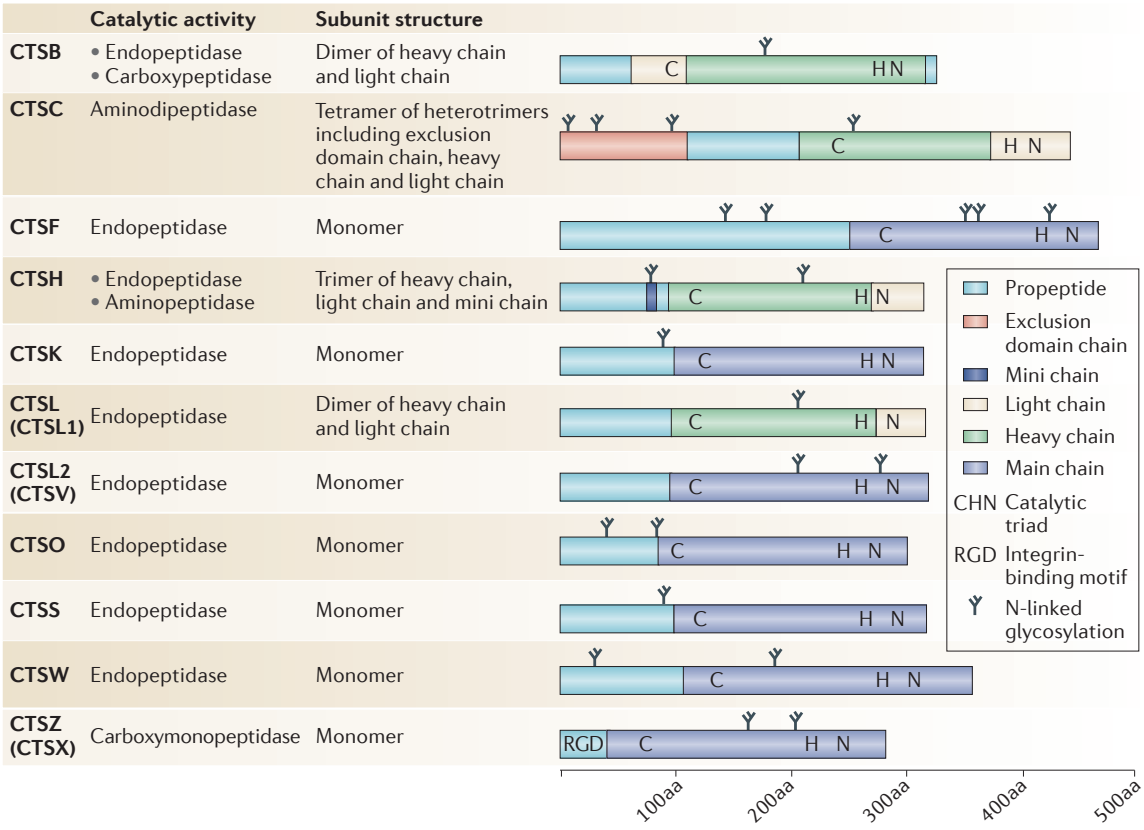

Figure 1.2: The cysteine cathepsin family of proteases. Within the protease family of cysteine cathepsins there is variation with regards to structure and activity. The catalytic activity and subunit structure of all 11 family members are shown here. Adapted from Olson O.C. and Joyce J.A. (2015). Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. *Nature Reviews Cancer* 15, 712–729.

Cathepsins and clinical prognosis

It has long been recognized that cathepsin expression is frequently increased in tumors in comparison with normal tissue, and this was a major motivation for investigators to begin studying cathepsins in cancer[36]. Increased cathepsin expression is significantly associated with poor prognosis in breast, lung, head and neck and colorectal cancer (CRC), melanoma and many other cancers[37, 38] (Table 1.1). Cathepsins are additionally predictive of response to anti-cancer therapy in several of these tumor types [39-41]. Expression of individual cathepsins can also discriminate between instances of venous *versus* lymphatic invasion[42], and correlates with site-specific metastasis[43].

While analysis of resected tissue has been invaluable in establishing the utility of expression of cathepsin family members as important prognostic indices, the measurement of circulating cathepsins in patient sera has also emerged as a non-invasive means to generate useful information on tumor malignancy. For instance, in CRC, high serum levels of cathepsin Z (CTSZ) were associated with shorter overall survival[44], and CTSL levels in the serum of patients with malignant ovarian cancer were elevated compared to benign tumors or healthy controls[45]. Interestingly, a large study of ~2000 individuals showed a significant correlation between elevated serum CTSS levels and increased mortality risk in older adults[46]. Further analysis of these cohorts revealed an association with both cancer mortality and cardiovascular mortality[46]. A summary of these different clinical studies is provided in Table 1.1.

Disease	Cathepsin expression	Clinical association	Ref.
Breast cancer	High CTSB and CTSL	High CTSB and CTSL levels in the primary tumour are indicative of poor prognosis for both disease-free survival and overall survival	39, 40, 43, 71, 152, 161-163
		High CTSB and CTSL levels correlate with reduced hormone receptor immunoreactivity	
	High CTSB	High CTSB is associated with lymph node metastases in patients with inflammatory breast cancer and is a powerful prognostic indicator of disease recurrence and overall survival in patients with lymph node-negative disease	
	High CTSL	High CTSL is predictive of poor response to systemic adjuvant endocrine therapy in patients with hormone receptor-positive cancer	
	High CTSB, CTSC, CTSL and CTSS	High CTSB, CTSC, CTSL and CTSS are predictive for brain-specific metastasis	
	CTSB, CTSC and CTSL	Only CTSB, CTSC and CTSL are predictive for lung-specific metastasis	
Colorectal cancer	High CTSB and CTSL	High CTSB and CTSL levels are significantly correlated with disease metastasis	41, 164-166
		High CTSB and CTSL activity is prognostic for poor overall survival in patients with colorectal cancer following curative resection	
	High CTSL	High levels of CTSL are associated with poor differentiation status	
	High CTSS	High CTSS expression is associated with reduced progression-free survival in patients treated with surgery alone. However, high CTSS expression was predictive for those patients who would derive benefit from adjuvant 5-fluorouracil and folinic acid treatment	
Lung cancer	High CTSB	High CTSB expression is prognostic of shorter overall survival in both squamous cell carcinomas and adenocarcinomas	167-171
	High CTSH	High CTSH levels are associated with decreased survival only for the smoker patient population	
	CTSS	CTSS is positively associated with increased overall survival	
Ovarian cancer	High CTSB	High CTSB expression is a negative predictor of tumour debulking success. High CTSB is negatively correlated with both progression-free survival and overall survival	172
Pancreatic adenocarcinoma	High CTSB and CTSL levels	In resectable pancreatic adenocarcinomas, high CTSB and CTSL levels are positively correlated with tumour grade and negatively correlated with overall survival following surgery	173
	High CTSB	High CTSB is correlated with increased lymphatic invasion. High CTSB is also prognostic for recurrence within 6 months of surgical resection	
Pancreatic neuroendocrine cancer	CTSB, CTSL and CTSZ	Increased levels of CTSB, CTSL and CTSZ are positively associated with increased tumour grade and invasion	9, 59
Osteosarcoma	High CTSK	High CTSK is positively associated with disease metastasis and poor overall survival	174

Table 1.1: Cathepsins as prognostic and predictive factors in human cancer. Illustrative examples of clinical findings for cathepsins as prognostic and predictive factors are provided here. The scope of this table has been restricted to malignancies that are discussed in the main text. CTS, cathepsin. Adapted from Olson O.C. and Joyce J.A. (2015). Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. *Nature Reviews Cancer* 15, 712–729.

Cathepsin activity-based probes

Given the myriad functions of cathepsins in cancer, as will be discussed in the following sections, it is important to critically assess the relevance of these correlative studies to the treatment of cancer patients. Specifically, it is essential to discriminate between the individual instances where cathepsin expression might provide important prognostic information, or additionally indicate a therapeutically targetable weakness, and those patients where it has no functional relevance. However, the assessment of aberrant cathepsin activity within the tumor is not always easily interpreted. The enzymatic activity of cathepsin proteases is tightly regulated at multiple levels including proteolytic activation, pH, redox potential, and stabilization via binding partners including macromolecules such as the glycosaminoglycans of the glycocalyx. Additionally, there is a class of reversible but tight binding endogenous inhibitors known as cystatins and serpins that provide a further level of control over proteolytic activity (Fig. 1.3)[47].

Moreover, cathepsin activity is additionally restricted at the level of expression in a cell type-specific manner, by the subcellular and extracellular trafficking of the enzymes, and their activation, stabilization and inactivation by their immediate environment. The complexity of cathepsin activity regulation has necessitated the development of molecular probes that can label active cathepsins *in situ*. These activity-based probes (ABPs; Fig. 1.4) employ thiol-reactive electrophiles to covalently label the active site cysteine in an enzymatic activity-dependent manner.

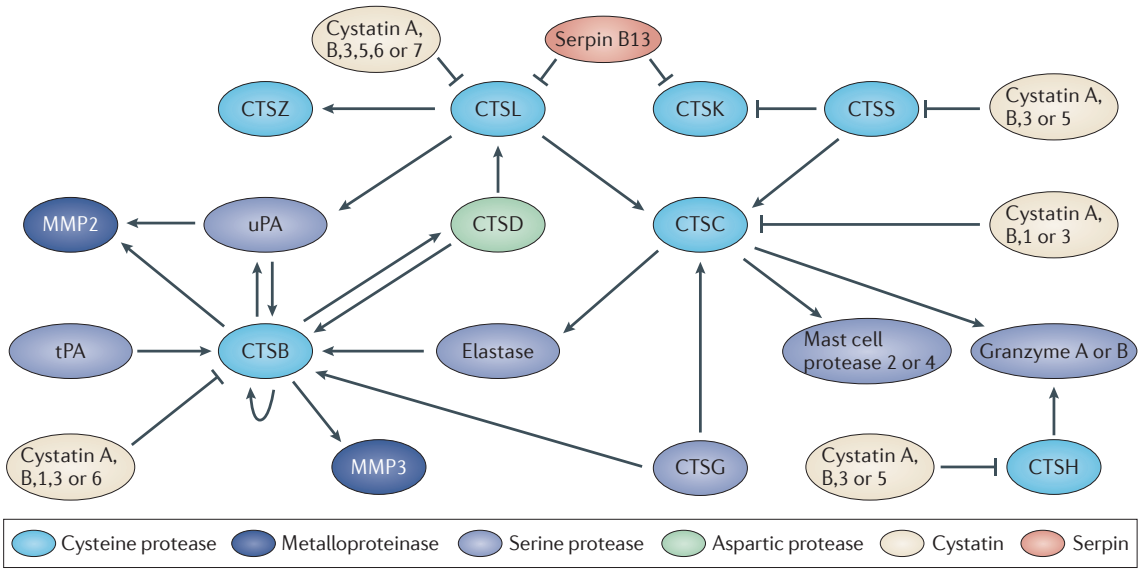

Figure 1.3: Integration of cysteine cathepsin proteases within a proteolytic network. Visualizing cathepsins within their local network of proteases and inhibitors underscores the complexity of proteolytic activity in a given tissue environment. Unlike classic proteolytic cascades, many of these interactions are bidirectional and the overall network is nonlinear, which enables positive and negative feedback and compensation. Within the network, certain proteases such as cathepsin B (CTSB) and CTSL represent highly integrated nodes whereas others, such as CTSH, CTSK or CTSZ, represent more discrete activities. Note that the endogenous cathepsin inhibitors, cystatins and serpins, show a considerable level of molecular promiscuity and overlap in their specificity. MMP, matrix metalloproteinase; tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator. Adapted from Olson O.C. and Joyce J.A. (2015). Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. *Nature Reviews Cancer* 15, 712–729.

ABPs are useful not only for investigation into the role of cathepsins in the tumor microenvironment[8,10], but also have applications in medical imaging and diagnostics[48-51], as well as surgical guidance in the context of tumor resection[52]. While ABPs can be very informative tools, interpretation of the data must always take into consideration the pharmacodynamics and pharmacokinetic properties of the ABPs. Cathepsins will only be labeled where the probes are bioavailable. Furthermore the fact that ABPs are also inhibitors of enzyme activity indicates that they are not necessarily biologically inert.

Cathepsins in the tumor microenvironment

Tumors represent complex tissue microenvironments[2], and in addition to cancer cells, many other cell types express cathepsins. These include fibroblasts, osteoclasts, neutrophils, mast cells, T cells, myoepithelial cells and endothelial cells, although none express cathepsins as robustly as tumor-associated macrophages (TAMs)[8, 10] (Fig. 1.4). Cathepsin expression is regulated in these different cell populations in a cell type-specific manner and their tumor-promoting or tumor-suppressive functions can similarly vary. Expression of cathepsins is directed by GC-motif transcription factors SP1 and ETS1[53] and is also part of a lysosomal biogenesis transcriptional network driven by the transcription factor TFEB[54]. In the context of the cancer cell, oncogene activation lies upstream of induced cathepsin expression in the majority of instances. For example, transformation of mammary epithelial cells with mutant HRAS up-regulates both CTSB and CTSL[22].

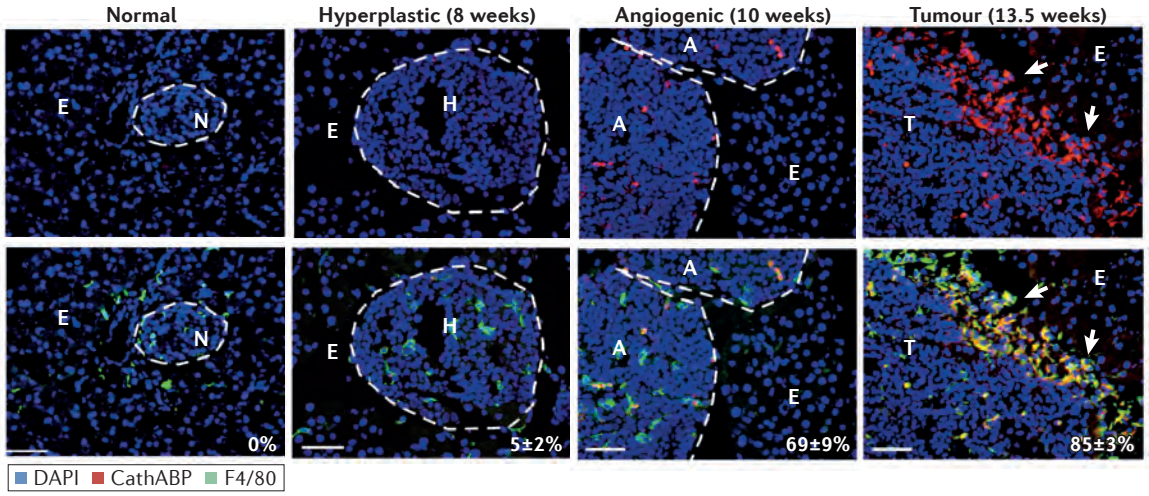

Figure 1.4: Cathepsin activity-based probes (ABPs) label tumor-associated macrophages in vivo. An example of the use of ABPs in the analysis of PanNET progression is shown here. The Cath-ABP demonstrates the accumulation of cathepsin activity-high cells (red) in the progression from normal islets (N) to hyperplastic islets (H), angiogenic islets (A) and finally invasive tumors (T). Co-staining with F4/80 (green) identifies these cells as macrophages. Intriguingly, the percentage of macrophages that are Cath-ABP+ (indicated on each image) increases with tumor progression and is highly enriched at the invasive front where the tumor invades into the surrounding normal exocrine (E) tissue. Adapted from Olson O.C. and Joyce J.A. (2015). Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. *Nature Reviews Cancer* 15, 712–729.

CTSB is also trafficked to the extracellular surface of the plasma membrane where it interacts with annexin 2 in caveolae[22, 55]. Similarly the *HER2* (also known as *ERBB2*) oncogene drives expression of *CTSB* through the transcription factor myeloid zinc finger 1 **(**MZF1), and CTSB is a functional driver of the invasive phenotype[20]. ETS transcription factors are also critical in regulating the expression of CTSB and CTSL in cancer cells[53, 56]. Finally interferon-γ has been shown to induce CTSS expression in lung cancer cells[57], supporting a role for tissue inflammation in regulating cathepsin activity within the tumor microenvironment.

The education of macrophages within the tumor microenvironment results in a pronounced upregulation of cathepsins. Interleukin-4 (IL-4) was identified as a key cytokine that induces cathepsin activity specifically in TAMs[10] (Fig. 1.4). While both cancer cells and TAMs can produce certain cathepsins, it has become clear that these respective cellular sources have a different impact on the tumor microenvironment. Bone marrow transplantation strategies in a RIP1-Tag2 pancreatic neuroendocrine tumor (PanNET) mouse model demonstrated that TAM-derived CTSB, CTSH and CTSS were either predominantly or exclusively responsible for the tumor-promoting functions of these distinct cathepsins[10, 58]. Conversely, CTSL promoted PanNET progression in a cancer cell-intrinsic manner[10], whereas CTSZ displayed tumor-promoting functions from both cancer cell-derived and the TAM-derived sources[59]. In an experimental CRC model, CTSS expression by both cancer cells and stromal cells was shown to be critical for the promotion of CRC growth and progression[60]. While these

examples illustrate that the tumor-promoting capacity of individual cathepsins can be cell type-specific, it remains an important and open question as to whether there are any post-translational modifications or biochemical differences in the proteases as a consequence of their cellular origin.

The roles of individual cathepsins can vary dramatically between different tumor types. Studies of CTSB and CTSC provide a particularly illuminating example of this disease-specific variability. While both cathepsins are upregulated during the course of tumorigenesis in multiple cancer types, the functional relevance of each is highly context-dependent[9, 61]. CTSB has potent tumor-promoting roles in the RIP1-Tag2 PanNET mouse model[9] and the MMTV-PyMT mouse model of mammary carcinoma[62-64] (Table 1.2). *CtsB*-deficient mice have significantly reduced tumor volume, in association with decreased cancer cell proliferation[62-64]. By contrast, *CtsB* deletion has no effect on the progression of the K14-HPV16 mouse model of squamous cell carcinoma (SCC), despite abundant CTSB expression in both the stromal and epithelial cell compartments[61].

Conversely, CTSC displays the opposite profile of functional importance in tumor progression. Deficiency of this protease does not impact either PanNET[9] or mammary cancer progression in mice[61], whereas in the context of SCC, *CtsC* deletion results in a potent block in disease progression[61]. Moreover, SCC cells implanted orthotopically in *CtsC*-null animals fail to grow, implicating host-derived CTSC as a critical regulator of disease progression.

Gene knockout	Cancer type	Genetically engineered mouse model	Phenotype	Refs
Cysteine cathepsins				
Ctsb$^{-/-}$	PanIN	LSL-*Kras*^{G12D}; PDX1-Cre	Delay in disease onset and 54% reduction in proliferation of PanINs; no difference in apoptosis	67
	PDA	LSL-*Kras*^{G12D}; LSL-*Trp53*^{R172H}; PDX1-Cre	Delay in disease onset and 23% reduction in proliferation of PDA; 30% reduction in incidence of liver metastasis	67
	PanNET	RIP1-Tag2	72% reduction in tumour burden; significant decrease in tumour invasion; 229% increase in apoptosis, 44% reduction in proliferation and 56% decrease in angiogenesis	9
	Mammary adenocarcinoma	MMTV-PyMT	40% reduction in tumour burden (by weight) and 50% reduction in lung metastasis; reduced cell proliferation	62, 63
	Squamous cell carcinoma	K14-HPV16	No effect on tumour progression or tumour grade	61
Ctsc$^{-/-}$	PanNET	RIP1-Tag2	No effect on tumour progression	9
	Mammary adenocarcinoma	MMTV-PyMT	No effect on tumour progression	61
	Squamous cell carcinoma	K14-HPV16	Decreased tumour progression and tumour grade	61
Ctsh$^{-/-}$	PanNET	RIP1-Tag2	40% reduction in tumour burden; 2-fold increase in apoptosis and 59% decrease in angiogenesis	58
Ctsl$^{-/-}$	Squamous cell carcinoma	K14-HPV16	Increased tumour progression with increased tumour grade; 180% increase in lymph node metastasis	74
	PanNET	RIP1-Tag2	88% reduction in tumour burden; 337% increase in apoptosis and 58% decrease in proliferation	9
Ctss$^{-/-}$	PanNET	RIP1-Tag2	47% reduction in tumour burden; 164% increase in apoptosis and 42% decrease in angiogenesis	9, 111
Ctsz$^{-/-}$	Mammary adenocarcinoma	MMTV-PyMT	No effect on tumour burden or proliferation; reduced tumour cell death	64
	PanNET	RIP1-Tag2	63% reduction in tumour burden; 1.8-fold increase in apoptosis and 86% decrease in proliferation; no effect on angiogenesis	59
Ctsb$^{-/-}$*Ctsz*$^{-/-}$	Mammary adenocarcinoma	MMTV-PyMT	45% reduction in tumour burden; no difference in cell proliferation	64
Ctsb$^{-/-}$*Ctss*$^{-/-}$	PanNET	RIP1-Tag2	51% reduction in tumour burden; 85% decrease in proliferation; no effect on angiogenesis or apoptosis	78
Ctsb$^{-/-}$*Ctss*$^{-/-}$ *Ctsz*$^{-/-}$	PanNET	RIP1-Tag2	58% reduction in tumour burden; 3.8-fold increase in apoptosis and 53% decrease in proliferation; 73% decrease in tumour vascularization	78
Cystatins				
Cstb$^{-/-}$	Mammary adenocarcinoma	MMTV-PyMT	23% reduction in primary tumour by weight; no effect on metastasis	175
Cst3$^{-/-}$	Squamous cell carcinoma	K14-HPV16	Increased tumour progression or angiogenesis	176
	PanNET	RIP1-Tag2	Increased tumour burden with increased tumour proliferation and increased angiogenesis	111

Table 1.2: Genetic studies of cathepsin and cystatin functions using genetically engineered mouse models of cancer. Cst, cystatin; Cts, cathepsin; HPV16, human papilloma virus type 16; K14, keratin 14 promoter; MMTV, mouse mammary tumor virus; PanIN, pancreatic intraepithelial neoplasia; PanNET, pancreatic neuroendocrine tumour; PDA, pancreatic ductal adenocarcinoma; PDX1, pancreas and duodenum homeobox 1; PyMT, polyoma middle T antigen; RIP1, rat insulin promoter; Tag2, SV40 large T antigen. Adapted from Olson O.C. and Joyce J.A. (2015). Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. *Nature Reviews Cancer* 15, 712–729.

Indeed, bone marrow transplantation experiments and add-back of CTSC-expressing cancer-associated fibroblasts demonstrated specific contributions of leukocyte- and fibroblast-derived CTSC in promoting angiogenesis and tumor growth. CTSC, which is a dipeptidyl aminopeptidase, is capable of activating multiple proteases including granzymes A and B, mast cell proteases 2 and 4, and neutrophil elastase[65, 66], suggestive of CTSC regulating cancer progression through an apical position within its local proteolytic network (Fig. 1.3). However, genetic deficiency for either mast cell protease 4 (*Mcpt4*) or neutrophil elastase (*Elane*) had no significant effect on SCC tumor progression[61], suggesting the possibility that only the simultaneous loss of multiple downstream protease targets will recapitulate the deficiency of *CtsC* alone.

It remains unclear whether this differential tumor-promoting activity of individual cathepsins in distinct organs is generally due to tissue-specific substrates, as shown for the brain-enriched CTSS substrate junctional adhesion molecule B (JAM-B; also known as JAM2)[43], or to alternative mechanisms of tissue-specific proteolytic regulation. It is interesting to note that the expression of CTSB and CTSC increase with tumor progression, even in those models where they have no critical function[8, 9, 61]. To this end, experiments to distinguish between the specificity resulting from the cancer cell lineage of origin *versus* that derived from the tissue microenvironment have yet to be performed, and these will be critical to discriminate between these possible mechanistic explanations.

In support of the concept that specific cathepsins enhance tumorigenesis in certain tissue microenvironments is the example of the tumor-promoting role of

CTSB in both pancreatic ductal adenocarcinoma as well as PanNET. Pancreatic ductal adenocarcinoma is studied using the KPC mouse model, where p53 is deleted and mutant KRAS is expressed in a pancreatic epithelial cell specific manner. In both pancreatic epithelial cells and neuroendocrine cells, genetic loss of *CtsB* in the KPC mouse model[67] and RIP1-Tag2 mouse model[9], respectively, severely impacts tumorigenesis (Table 1.2). Despite the different cells of origin for each pancreatic cancer type, loss of *CtsB* results in decreased proliferation and reduced invasion and/or metastasis in both. Interestingly, CTSB is also critical for the induction of experimental pancreatitis, largely through its role in trypsinogen activation[68]. Thus, in these three pathological conditions of the pancreas, CTSB appears to be a critical deregulator of normal tissue homeostasis.

While many cathepsins have been shown to have either a tumor-promoting function or no effect in specific cancer types (Table 1.2), CTSL is unique in having the ability to either enhance or suppress carcinogenesis in a context-dependent manner. CTSL is often expressed at high levels in tumors and in some instances, particularly in breast and head and neck cancer, CTSL expression serves as a valuable biomarker with negative prognostic indication for time to relapse and overall survival in patients[69-71] (Table 1.1). In the context of the RIP1-Tag2 PanNET mouse model, CTSL has a potent tumor-promoting function, the most pronounced of any of the other cysteine cathepsins analyzed[9, 10, 58, 59] (Table 1.2). Moreover, the deletion of this protease in these mouse models is comparable to genetic ablation of insulin-like growth factor 2 (*Igf2*)[72] or

vascular endothelial growth factor A (*Vegfa)*[73], two critical growth factors required for the rate-limiting steps of pancreatic neuroendocrine hyperplasia and angiogenesis, respectively. The tumor-promoting function of CTSL is mediated entirely by the cancer cell source of this enzyme, as the knockout phenotype cannot be rescued by transplantation of these mouse models with wild-type donor bone marrow[10].

However, the opposite effect is observed in the skin microenvironment where loss of *CtsL* enhances carcinogenesis in both the genetic K14-HPV16 mouse model as well as the classical two-step chemically induced DMBA-TPA mouse model of skin carcinogenesis[74, 75]. In fact, *CtsL*-knockout mice develop epidermal abnormalities including keratinocyte hyperplasia[76], resulting from increased epidermal growth factor (EGF) recycling and deregulated autocrine mitogenic signaling[74, 77]. These examples thus identify opposing, context-dependent functions for CTSL in regulating cell proliferation as a mechanistic explanation for the striking organ-specific differences of this enzyme in cancer.

These genetic experiments have been instrumental in elucidating the regulatory functions of cathepsins in cancer, and in contributing to our understanding of the underlying molecular mechanisms, as is discussed further below. In addition, analyses in genetically engineered mouse models have revealed both the functional redundancy of individual cathepsins, and the potential for compensation from other family members when multiple nodes in the protease web are perturbed. Two illustrative examples of these phenomena were uncovered in the MMTV-PyMT and RIP1-Tag2 mouse models. Deletion of

CtsB in MMTV-PyMT mice resulted in increased CTSZ at the surface of cancer cells, which was proposed to mask phenotypes of CTSB deficiency in this mouse model[62, 64]. Indeed, combined ablation of *CtsB* and *CtsZ* (which had no effect when deleted alone) resulted in pronounced decreases in both primary tumor development and lung metastasis[64] (Table 1.2). In the RIP1-Tag2 mouse model, combined deletion of *CtsB* and *CtsS* resulted in an additive effect in reducing angiogenic switching in preneoplastic lesions[78]. However, later in tumor progression, CTSZ is specifically upregulated and associated with a reversal of several tumorigenic phenotypes, suggesting functional compensation by this family member. Consistent with this hypothesis, deletion of *CtsZ* in a $CtsB^{-/-}$; $CtsS^{-/-}$ mutant background resulted in a substantial block in tumorigenesis in RIP1-Tag2 mice[78] (Table 1.2). Insights from these genetic studies could also have important implications for therapeutically targeting cathepsins using pharmacological agents, and suggest that pan-cathepsin inhibitors may be more efficacious in the long-term than selective inhibitors of individual cathepsins, as discussed further below.

Integration of cathepsin functions into lysosome biology

Cathepsins and protein catabolism in the lysosome

One of the major functions of the lysosome, and the proteases it contains within its acidic lumen, is the catabolism of proteins and degradation into their amino acid components[79] (Fig. 1.5). Proteins can traffic to the lysosome via the endocytic pathway or they can be sequestered from the cytosol in a process

known as autophagy by which a double-membrane vesicle containing the protein cargo, the autophagosome, fuses with the lysosome. The protein cargo is then degraded by cysteine cathepsins and other lysosomal proteases in the newly formed autolysosome. *CtsL*-deficient mouse embryonic fibroblasts display enlarged autolysosomes, indicating impaired catabolism of the cargo and decreased autophagic flux[80]. Another study examining angiotensin II (ANGII)-induced autophagy in macrophages reported that *CtsS* deficiency resulted in autophagosome accumulation, supporting a role for CTSS in mediating fusion with the lysosome[81]. Thus, cathepsins can play roles as both effectors and regulators of autophagic catabolism.

In cancer initiation, autophagy is proposed to suppress transformation in response to oncogene activation[82], yet this can ultimately be over-ridden. The nutrient sensor mTOR is critically important for licensing cell growth driven by oncogenic PI3K-AKT signaling and suppressing autophagy. Thus, the lysosome and its role in amino acid recycling contribute towards enabling cancer proliferation. Indeed, mTOR is regulated by the Ragulator complex, which assesses free amino acid content within the lysosomal lumen by measuring the efflux of specific amino acids across the lysosomal membrane[83-85]. Moreover, pancreatic ductal adenocarcinoma cells have been shown to be nutrient-poor and dependent on macropinocytosis of extracellular protein as an amino acid source[86, 87]. Increased activity of the MiT/TFE family of transcription factors is responsible for metabolic reprogramming of pancreatic cancer through increased expression of lysosomal genes such as *CTSS*[88].

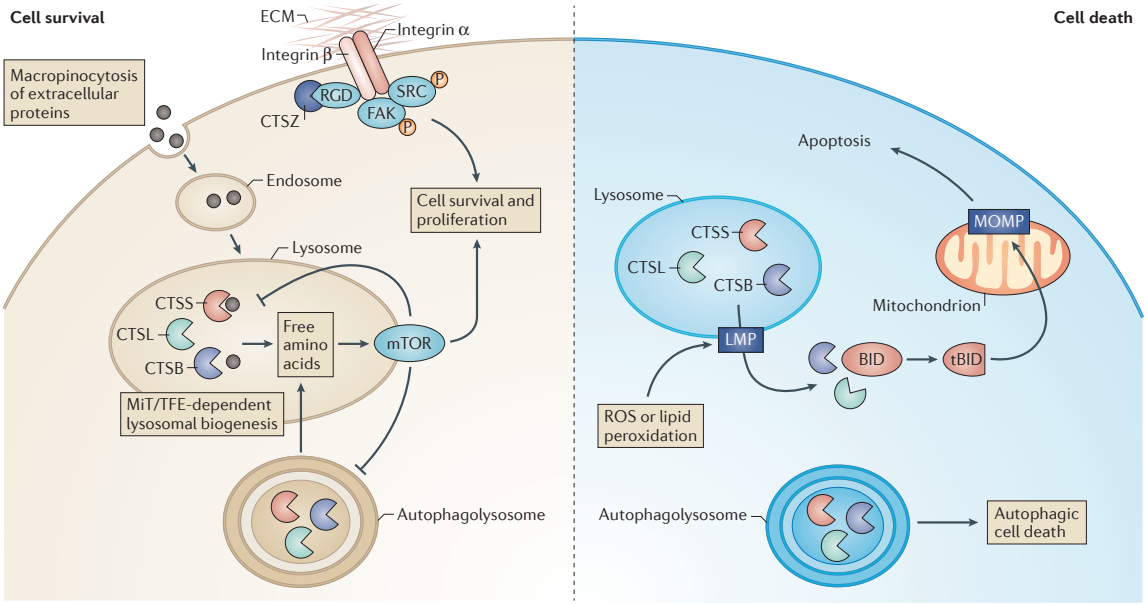

Figure 1.5: The yin and yang of cancer-associated lysosomal biogenesis. The increased lysosomal biogenesis and expression of cathepsins in cancer cells both supports and endangers their survival. Increased catabolic activity is critical for providing the necessary nutrients to license cell proliferation[90]. Additionally, cathepsin Z (CTSZ) has a critical non-catalytic function in promoting increased focal adhesion kinase (FAK) and SRC signaling and proliferation in a cancer cell-intrinsic manner[59]. In conditions of stress, however, lysosomal membrane integrity is compromised, either partially or completely, in a process termed lysosomal membrane permeabilization (LMP). Release of cathepsins into the cytosol can lead to mitochondrial outer membrane permeabilization (MOMP) and apoptosis through the cleavage of BID (producing tBID) by CTSB or CTSL[101]. ECM, extracellular matrix. Adapted from Olson O.C. and Joyce J.A. (2015). Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. *Nature Reviews Cancer* 15, 712–729.

This increased lysosomal and autophagic activity is critical for pancreatic tumorigenesis by allowing cancer cells to capitalize on alternative sources of amino acids. In conditions of essential amino acid scarcity, lysosomal degradation of internalized proteins re-activates mTOR, which in turn suppresses lysosomal catabolism and paradoxically cell proliferation[89]. Lysosomal protein catabolism is thus a necessary regulator of cancer cell proliferation and a critical survival pathway in the nutrient-stressed cell (Fig. 1.5).

Lysosomal membrane permeabilization and cathepsins as effectors of cell death

Transformed cells express higher levels of lysosomal proteins and it has been suggested that this reliance on lysosomal content could represent an “Achilles heel” by which cancer cells might be targeted to trigger their own death[90]. Indeed, transformed cells are sensitized to lysosomotropic agents capable of inducing lysosomal membrane permeabilization (LMP) and subsequent programmed cell death[91-93]. Conversely, the survival factor heat shock protein 70 (HSP70) supports the integrity of the lysosomal membrane and suppresses LMP, and has been shown to be critical for cancer cell survival[94, 95].

Permeabilization of the lysosomal membrane can occur in either a limited manner, allowing discrete amounts of cathepsins and other lysosomal hydrolases access to the cytosol, or in a more extensive manner leading to a committed lysosome-mediated cell death. Lysosomes are acute sensors of oxidative stress within the cell, and lipid peroxidation leads to impaired barrier function of the

lysosomal membrane resulting in LMP[96]. Dysregulation of the lipid composition of the lysosomal membrane can also lead to LMP. This can occur as the result of an increase in the enzymatic activities of phospholipase A2 and phospholipase C[97], or from aberrant acid sphingomyelinase activity[93]. An intriguing physiological example of lysosomal lipid dysregulation leading to LMP and cathepsin-dependent cell death occurs in the involuting mammary gland. Activation of signal transducer and activator of transcription 3 (STAT3) in mammary epithelial cells not only upregulates the expression of CTSB and CTSL and downregulates their endogenous inhibitor Serpin 2A (SPI2A), but also abrogates the secretory phenotype of these cells leading to the accumulation of milk fat globules in lysosomal vacuoles[98, 99]. STAT3 activation causes phagocytosis of secreted milk fat globules by mammary epithelial cells and the resultant increase in lysosomal free fatty acid results in LMP and cell death[99].

When the lysosomal membrane is permeablized in response to cellular stress, the release of active cathepsins initiates mitochondrial cell death programs[100] (Fig. 1.5). This can occur through the cleavage of BID by cathepsins, resulting in cytochrome *c* release and activation of the apoptosome[101] (Fig. 1.5). Cathepsins also cleave the mitochondrial-resident anti-apoptotic molecules BCL-2, BCL-XL, and myeloid cell leukemia 1 (MCL1) and thus promote apoptosis by reducing the threshold for mitochondrial membrane depolarization[102]. Not only does LMP and cathepsin release promote apoptosis, it also actively suppresses survival signaling and alternate forms of cell death. For example, CTSB released into the cytosol can cleave the lipid signaling enzyme

sphingosine kinase 1, which protects from sphingosine- and ceramide-induced cell death[103]. CTSB and CTSS have been shown to degrade receptor-interacting serine-threonine protein kinase 1 (RIPK1), thereby suppressing the necroptosis cell death pathway, and instead enforcing apoptosis[104]. In addition to depolarizing mitochondria, activated BAX and BAK are capable of permeabilizing the lysosomal membrane[105]. The role of LMP in amplifying apoptotic signaling is particularly important in cytokine-mediated cell death, and in *CtsB*-deficient hepatocytes the ability of tumor necrosis factor (TNF) to induce death is severely impaired[106]. Effective mitochondrial membrane depolarization and release of cytochrome *c* downstream of caspase 8 activation requires BID-dependent LMP[107]. LMP, however, also operates as an independent pathway as even in cells where apoptosis has been compromised through the loss of critical effectors such as BAX, BAD or caspases, LMP still leads to cell death[108, 109]. Indeed, while the apoptotic pathway is generally deregulated in cancer the obligate metabolic requirement for robust lysosomal activity described above indicates that the LMP pathway of programmed cell death is likely generally intact.

Functions of extra-lysosomal cathepsins

It is not always the case that extra-lysosomal cathepsins in the cytosol are the result of LMP and are indicative of impending cell death. While it remains unclear how these proteases access the cytosol, CTSZ and CTSH have been shown to modulate immune cell-ECM attachment via proteolytic modulation of cytoplasmic signaling pathways. Recent work has provided additional insight into the role of

CTSZ in promoting ECM adhesion and proliferation in cancer. *CtsZ*-deficient PanNET cancer cells show impaired proliferation and decreased adhesion to ECM proteins including fibronectin, collagen and the reconstituted ECM mixture Matrigel[59]. CTSZ is critical for active focal adhesion kinase (FAK) and SRC signaling within cancer cells in a catalytically-independent manner, and instead relies on the RGD motif uniquely found within the propeptide of CTSZ (Fig 1.5). Paradoxically, extracellular supplementation of full-length CTSZ is unable to rescue the proliferative defects of *CtsZ*-null cancer cells indicating that its growth-promoting activity appears to be entirely cytoplasmic. CTSZ has been reported to co-localize with integrins in membrane proximal vesicles[110] and it is likely in these vesicles, rather than on the cell surface, that the CTSZ RGD motif exerts its pro-proliferative function. The emergence of catalysis-independent roles for cathepsins in tumor progression represents an exciting advance in the field and it will be of interest to determine whether other cysteine cathepsins have similar non-proteolytic functions. Together, these findings demonstrate that important moonlighting roles for cathepsins exist independent of their typical lysosomal and extracellular locations.

Cathepsins in tumor biology

Cathepsins regulate angiogenesis

Many different studies have implicated cathepsins in regulating tumor angiogenesis[9, 10, 58, 60, 111] (Table 1.2). CTSS can stimulate angiogenesis directly by generating pro-angiogenic peptide fragments from the ECM protein laminin 5[111]. Conversely, CTSL and CTSS also generate the anti-angiogenic peptide endostatin from collagen XVIII[112], demonstrating the complex nature of these proteases in regulating angiogenesis in the tumor microenvironment. Cathepsins have also been shown to be downstream of VEGFA signaling, wherein VEGFA leads to an altered ratio of cathepsins to their endogenous inhibitors, resulting in an overall increase in cathepsin activity. Deletion or inhibition of CTSB demonstrated its importance in basement membrane degradation for the generation and expansion of new 'mother vessels' within tumors [113]. In addition to their roles as direct effectors of angiogenic signaling, cathepsins can also regulate pro-angiogenic proteolytic networks. Degradation of the endogenous tissue inhibitors of metalloproteases (TIMPs) by CTSB increases the pro-angiogenic functions of matrix metalloproteinases (MMPs) within the tumor microenvironment without the requirement for any change in MMP expression [114]. MMPs in turn degrade cystatin E, cystatin C and cystatin M, thereby increasing cathepsin activity[114, 115]. CTSL is critical for the activation of heparanase[116], which in turn can modulate angiogenesis and lymphangiogenesis[117] through the controlled release of heparan sulfate-bound growth factors. CTSL is also necessary for neovascularization by promoting the

invasion of endothelial progenitor cells into the stroma and their subsequent incorporation into blood vessels [118]. CTSK is upregulated in direct response to hypoxia and increases proteolytic activation of NOTCH1 in endothelial cells, thereby inducing neovascularization in response to ischemic injury [119]. Collectively, these studies demonstrate that cathepsins are not only critical regulators of the ECM remodeling required for angiogenesis and neovascularization, but are also involved in modulation of the signaling pathways driving these processes.

Cathepsins are involved in cell-cell junction disruption, cancer cell invasion and extravasation

The main focus to date of studies on the contribution of proteases to tumor invasion emphasizes their role in ECM degradation and the establishment of invasion tracks. However, it is becoming increasingly evident that proteases have important additional specialized functions including the cleavage of cell-cell adhesion molecules in order to free cancer cells from their neighbors, promoting a highly invasive phenotype[120]. E-cadherin is a cell adhesion molecule, and an important epithelial tumor suppressor both for its ability to maintain tissue organization and suppress intracellular signaling. It has been identified as a substrate for CTSB, CTSL and CTSS in RIP1-Tag2 tumors[10]. The activity of these proteases at the invasive edge of the tumor is associated with E-cadherin loss from the cell surface and disaggregation of the tumor.

Specific cleavage of cell-cell adhesion molecules has begun to emerge as an important mechanism of tumor promotion by cathepsins secreted into the extracellular space. Indeed, CTSS-mediated adhesion molecule shedding has recently been reported at a later step in the metastatic cascade. In the context of breast-to-brain metastasis, CTSS mediates cleavage of several junctional adhesion molecules (JAMs), most notably JAM-B, in order to allow cancer cells to breach the blood-brain barrier[43]. Critically, genetic or pharmacological perturbation of CTSS activity hindered the ability of metastatic cells to effectively seed and colonize the brain. It is important to note that among the cathepsin family members, CTSS is best able to retain its optimal proteolytic activity profile at neutral pH[29]. Outside the acidic pericellular environment of the tumor many of the other cathepsins likely display reduced activity, while CTSS retains its functionality even in a neutral pH environment such as the microvasculature of the brain. This further emphasizes the importance of tissue context and microenvironment with regard to the specificity and redundancy of individual cathepsins in their tumor-promoting functions. Recently, broader sheddase functions for CTSB, CTSL and CTSS have been reported, resulting in the cleavage of cell adhesion molecules, transmembrane proteins and other molecules from the cancer cell surface[121], thus potentially further contributing to tumor invasion.

Many of the pro-invasive functions of cathepsins necessitate their altered trafficking either to the cell surface or into the extracellular space[120]. Interestingly, JAMs are targeted to the lysosome and degraded by cathepsins during

transforming growth factor-β (TGFβ)-induced epithelial to mesenchymal transition (EMT) in breast cancer cells[122]. The same proteolytic cleavage event can occur by regulated trafficking of the substrate to the protease or alternatively by relocalizing the protease to the substrate. This inversion of the typical trafficking process demonstrates how inexorably linked the roles of intra- and extra-cellular cathepsin proteases can be. Another intriguing mechanism by which cathepsins can be mis-routed within the cell, and either secreted or recycled, is via lineage-specific wiring of the endo-lysosomal pathway in melanoma[23]. This occurs in a RAB7-dependent manner, with RAB7 knockdown leading to enhanced secretion of multiple lysosomal cathepsins and matrix proteins, and a consequent increase in melanoma invasion.

Cathepsins in immune cell function

In order to understand the specific functions of cysteine cathepsins in infiltrating immune cells, including in cancer, one must appreciate the critical role that lysosomal-mediated protein degradation plays in antigen presentation. The function of an antigen-presenting cell is to sample internalized proteins through the generation of peptide fragments by proteolytic cleavage and to subsequently display these antigens to cells of the adaptive immune system. Thus it is not surprising that any proteolytic processing event that occurs in the lysosome will rely heavily upon cathepsins. Intriguingly, not only are the antigens themselves cleaved into smaller peptides suitable for presentation by cathepsins[123], but the antigen machinery itself is activated, processed, and regulated by these very

same proteases. After MHC II complexes are transcribed and assembled in the ER, their molecular chaperone- the invariant chain (Ii), must be degraded prior to loading of peptide. In a mechanism that prevents premature loading of the complex, Ii is proteolytically processed in the lysosome by cathepsins. CTSL and CTSS have non-redundant roles in the degradation of Ii following initial processing by asparagine endopeptidase[124-127]. Interestingly, while thymic epithelial cells rely upon CTSL for Ii chain degradation, in macrophages CTSF plays an important role[128]. This can be viewed not necessarily as an example of redundant functions for cathepsins, but rather an instance of how the diversity in molecular and biochemical character of the different cathepsins is used to generate biological diversity across distinct cell types. Thus, differential endo/lysosomal proteolytic function yields unique characteristics in antigen presentation.

This concept of cathepsins as integral regulators of lysosomal function rather than simply degradative enzymes is further supported by the example of CD1d restricted antigen presentation. The efficient presentation of glycolipids by CD1d expressing thymocytes requires CTSL enzymatic activity[129]. While the CTSL substrate in this context remains to be identified, this demonstrates an integration of cathepsins in myriad lysosomal functions above and beyond simple protein catabolism. Further examination of the endo-lysosomal system in immune cells reveals that cathepsins also play critical roles in secretory granule function. NK cells depend on CTSC activity for activation of granzyme B and their cytolytic function[130], while other immune cells depend on CTSH to perform the same

function[131]. Similarly, CTSL is involved in the processing of perforin[132]. Intriguingly it has been demonstrated that during the process of lymphocyte degranulation, CTSB is released and attaches to the plasma membrane, thereby protecting the cell from released cytotoxic effector molecules[133].

Immune cells also rely on cathepsins for proteolytic degradation of matrix proteins in order to migrate and invade through tissue[134], and this mechanism can be co-opted to enhance cancer cell migration[10]. By contrast, CTSZ has emerged as a critical regulator of lymphocyte migration in a manner independent of ECM degradation. The carboxypeptidase has been found to localize in the cytosol of lymphocytes where it can process the cytoplasmic tails of integrins, promoting their activation and cellular migration[135, 136]. In a similar manner, CTSH can process the integrin and cytoskeletal integrator talin, leading to increased invasive behavior of cells[137]. Interestingly, macrophage-secreted CTSZ can be transferred to the surface of cancer cells where it enhances their motility in an integrin-dependent manner[59]. In sum, cathepsins are involved in immune cell function in myriad ways. In the context of cancer biology, it will be important to consider the possibility that the increased expression of cathepsins by cancer cells may represent a form of “leukocytic mimicry” by which they develop a more invasive and immuno-privileged behavior[43].

Tumor-associated macrophages in breast cancer

Colony stimulating factor-1 (CSF-1) is a critical growth factor for the development of macrophages promoting their survival, proliferation and differentiation from

bone marrow progenitors[138]. Indeed, mice lacking a functional copy of the *Csf-1* gene are highly deficient in macrophages[139]. In many cancers, tumor cells express high levels of CSF-1 and this is particularly true in breast cancer[140]. When the MMTV-PyMT transgenic mouse model of breast cancer was crossed with *Csf-1* deficient mice, the resulting tumors had a substantial reduction in TAMs, displayed impaired angiogenic switching and lacked the microvascular density characteristic of malignant lesions[141]. While there was no defect in tumor growth, a significant delay in invasion and lung metastasis was observed[142]. Indeed it was discovered that TAMs lead tumor cells when invading into the surrounding tissue through an CSF-1/EGF paracrine signaling loop[143]. In this same manner TAMs also assist tumor cells in intravasating into the vasculature and leaving the primary tumor[144].

There is increasing evidence that TAMs within breast cancer are heterogeneous, with different subsets promoting tumor progression and metastasis through distinct mechanisms[145-147]. Education of TAMs by IL-4 derived from CD4+ T cells has been shown to be critical to the development of the pro-metastatic TAM population[148]. Indeed, IL-4-induced polarization of macrophages induces upregulation of EGF and promotes tumor cell invasion[148]. Additionally, IL-4 has also been shown to promote expression of cathepsins and thus underlies the population of cathepsin-high pro-metastatic macrophages[10]. These macrophages have also been shown to suppress cytotoxic CD8+ T cell activation in an IL-10 dependent manner[149]. In sum, TAMs are critical regulators of breast cancer progression through their ability to promote angiogenesis, create

an immuno-suppressive environment, and drive tumor cell invasion and metastasis.

Chemotherapeutic treatment in breast cancer

Breast cancer is a heterogeneous disease and is largely classified by the expression of the hormone receptors for estrogen (ER) and progesterone (PR) as well as the human epidermal growth factor receptor 2 (HER2)[150]. Adjuvant chemotherapy, or post-operative chemotherapy, has greatly enhanced the survival of breast cancer patients[151]. Within these molecularly distinct subsets of breast cancer, however, there is significant variance in the benefit obtained from treatment with conventional chemotherapeutic agents. Hormone receptor-negative disease is generally more responsive to chemotherapy than hormone receptor-positive disease[151]. This does not mean, however, that hormone receptor-positive patients should not receive chemotherapy given that many do in fact benefit from this treatment. Indeed significant effort has been dedicated to identify predictors of patient risk and benefit for the selection of treatment regimens[39, 152]. When the primary tumor is locally advanced, neoadjuvant therapy or therapy delivered prior to surgery, has become the standard of care[153]. This allows for a reduction in tumor burden while simultaneously providing insight into the chemosensitivity of the disease prior to selection of the chemotherapeutic regimen[153]. For disease that is at high risk for recurrence, aggressive chemotherapeutic regimens are chosen that are generally based upon taxane and anthracycline chemotherapeutic agents[151](Table 1.3).

Chemotherapy	Class	Mechanism of action
Paclitaxel (Taxol)	Taxane	Microtubule stabilizer
Etoposide	Topoisomerase II inhibitor	Induces double strand breaks
Doxorubicin	Anthracycline antibiotic	DNA intercalator
Gemcitabine	Anti-metabolite	Inhibits ribonucleotide reductase (RNR), serves as a terminator nucleotide
Carboplatin	Platinum-based	Cross-links DNA

Table 1.3: Cytotoxic chemotherapeutic agents. This table lists the chemotherapeutic agents used in the studies described in this thesis. For each chemotherapy, the class and mechanism of action has also been listed.

Taxanes

Paclitaxel (Taxol) was first identified through an NCI initiative started in 1960 to identify compounds from the plant kingdom that had anti-tumor activity[154]. Taxol was isolated from the bark of the Pacific Yew and it was shown have significant activity in rodent models of leukemia and solid tumors[154]. While clinical development of the compound began in the 1960s, it was not until 1977 that the mechanism of action was uncovered when it was observed that Taxol bound and stabilized microtubules[155, 156]. Stabilization of tubulin results in activation of the spindle assembly checkpoint through failure to properly attach microtubules to pairs of sister chromatids[157]. In this manner Taxol has been shown to block cells in mitosis and induce cell death[158, 159]. Recent work, however, has questioned some fundamental assumptions about the mechanism by which Taxol kills cancer cells in vivo. When the treatment of cancer cells in culture was compared with the treatment of the same cancer cells grown as xenografts, significant differences were noted. While in culture, cancer cells treated with Taxol underwent prolonged mitotic arrest (~9 hours) and subsequently died in mitosis[160]. However when Taxol treatment kinetics were imaged in vivo using intravital microscopy techniques, shorter mitotic arrests were observed (~3-8 hours) and the cells exited out of mitosis without dividing in a process termed mitotic slippage[160]. These multinucleated and tetraploid cells persisted for variable lengths of time and often initiated cell death during interphase[160]. These findings demonstrate a clear difference in the tumor cell response to taxane therapy dependent upon their environment. Ultimately this provides significant

rationale to explore the impact of the tumor microenvironment on the mechanisms of Taxol-induced cell death, which is one of the major goals of this thesis.

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Chapter 2

Materials and methods

Animals and cell lines

All mice were housed according to institutional standards of the Research Animal Resource Center at Memorial Sloan-Kettering Cancer Center (MSKCC). MMTV-PyMT mice (FVB/n background) were obtained from K. Podsypanina and H. Varmus and have been previously described [1, 2]. Cathepsin $B^{-/-}$ [3] and Cathepsin $L^{-/-}$ [4] mice in the FVB/n background were obtained from T. Reinheckel. Cathepsin $C^{-/-}$ [5] and Cathepsin $S^{-/-}$ [6] mice were obtained in the BL/6 background from C. Pham and H. Chapman respectively, and were backcrossed for >10 generations into a pure FVB/n background in the Joyce laboratory. Transgenic CAG-EGFP FVB/n animals were purchased from The Jackson Laboratory and maintained within our facility. Wild-type FVB/n, Athy/Nu, or C57BL/6 animals were purchased from Charles River Laboratories and also bred within our animal facility.

Mice were anesthetized with Avertin and perfused with saline, and tissues were post-fixed in 10% formalin for one hour before sucrose incubation overnight and OCT (Tissue-Tek) embedding for frozen sectioning. For paraffin embedding, formalin-fixed tissues were processed through an ethanol series into paraffin and embedded. The TS1-TGL cell line was derived from a PyMT mammary tumor (FVB/n background), labeled with a triple-imaging TGL vector [7], and sorted twice for GFP expression and EpCAM (BioLegend) positivity on a BD FACS Aria. The

TS2 and TS6 cell lines were similarly generated in our laboratory from a PyMT primary tumor and lung metastasis respectively. Additional tumor cell lines used included Met-1, which was derived from a PyMT lung metastatic lesion (in the FVB/n background) [8]; AT- 3, a cell line derived from a PyMT primary tumor in the C57BL/6 background [9]; and the MDA-MB-231 patient cell line [10]. The mouse cancer associated-fibroblast (CAF) cell line used for conditioned media (CM) experiments was isolated as previously described [11]. Additional stromal cell lines used include the PyMT CAF line CAF2 [12], the preadipocyte line 3T3-L1[13], the hemangioma endothelial cell line EOMA [14], and the yolk sac endothelial cell line C166 [15]. For orthotopic implantations, both syngeneic and xenograft, 5 x 10^5 cells were implanted 1:1 in Matrigel (BD, growth factor-reduced) into the fourth mammary fat pads of wild-type FVB/n or Athy/Nu mice aged 6–9 weeks.

Bone marrow-derived macrophages (BMDMs)

Femurs and tibiae from either FVB/n or C57BL/6 mice were harvested under sterile conditions from both legs and marrow flushed out using a 25-gauge needle. The marrow was passed through a 40 μm strainer and cultured in 30 mL Teflon bags (PermaLife) with 10 ng/mL recombinant mouse Csf-1 (R&D Systems). Bone marrow cells were cultured in Teflon bags for 7 d, with fresh Csf-1-containing medium replacing old medium every other day to induce macrophage differentiation.

In vitro drug treatments

For Taxol treatment, cells were treated with 50 nM Taxol (dissolved in medium) or an equivalent amount of DMSO for controls (0.0005%). For cathepsin inhibition, 10 μM JPM-OEt was used, with an equivalent amount of DMSO used for controls (0.01%). For MEK1/2 inhibition, 500nM PD318088 was used, with an equivalent amount of DMSO for controls (0.0025%). Cells were treated with doxorubicin, etoposide, carboplatin and gemcitabine at concentrations of 300 nM, 20 μM, 50 μM, and 400 nM, respectively. The highest concentration of DMSO (0.08%) was used as a vehicle control for the panel of chemotherapies.

Annexin V/DAPI cell death co-culture experiments

Tumor cells and differentiated macrophages were mixed at a 1:1 cell ratio, 200,000 per each cell type, in medium (DME + 10% FBS) and plated onto 12-well plates 24 h prior to treatment. Met- 1 and AT-3 cells had a significantly increased proliferative rate compared with TS1 or TS2 cell lines and were therefore plated at a density of 100,000 per well. Tumor cells and macrophages were matched for genetic background; FVB/n BMDMs were used for the TS1, TS2, and Met-1 cell lines, while C57BL/6 BMDMs were used for the AT-3 cell co-culture. At the time of treatment, media was replaced with fresh medium containing Taxol (or other cytotoxic agent) or equivalent DMSO. For experiments involving cathepsin inhibition, JPM was added at the same time as the other agents to the appropriate samples. Forty-eight hours following treatment, cocultures were trypsinized, and all media were collected throughout the

experiment to prevent loss of dead cells. Samples were then washed once with FACS buffer (1% BSA in PBS), followed by Fc block for 15 min at 4°C and antibody labeling for 15 min at 4°C. Antibodies used for cell type identification were EpCAM-APC/Cy7 (1:500; BioLegend) and F4/80-PE (1:50; Serotec). Samples were then washed in Annexin V-binding buffer (AVBB; 0.1 M HEPES at pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$) and labeled for 10 min at room temperature with 1.5 mL of Annexin V-APC (BD Biosciences) in 50 mL of AVBB. Fifty microliters of AVBB with 1 ug/mL DAPI (Invitrogen) was then added to each sample, followed by acquisition on an LSR II flow cytometer (BD).

DNA content and intracellular signaling co-culture experiments

Tumor cells and differentiated EGFP+ macrophages were mixed at a 1:1 cell ratio, 100,000 per each cell type, in medium (DME + 10% FBS) and plated onto 12-well plates 24 h prior to treatment. The cell numbers have been reduced to accommodate the increased length of the assay (72 vs 48 hours). At various time points following treatment, co-cultures were trypsinized, and all media were collected throughout the experiment to prevent loss of dead cells. Samples were then washed once with PBS, and fixed and permeablized using a Transcription Factor/Permeabilization kit (eBioscience). Cells were stained with antibodies against yH2AX (Cell Signaling; 1:500) and phsohpo-p53.s15(Cell Signaling: 1:1000) diluted in permeabilization buffer and incubated at 4 degrees celcius overnight. Cells were washed and stained with anti-rabbit-APC (Jackson Immunoresearch; 1:300) for 3 hours at room temperature and then washed. Cells

were resuspended in FACS buffer containing 5 ug/mL DAPI (Invitrogen), followed by acquisition on an LSR II flow cytometer (BD).

Live imaging of mitotic arrest

Tumor cells were plated at 100K per well on glass bottom 12-well plates (In vitro Scientific) 24 hours prior to treatment. EGFP+ BMDMs or CellTracker Green CMFDA (Invitrogen) labeled stromal and tumor cell lines were plated at 50K per well. Three randomly selected fields of view were selected and imaged for 24 hours following 50nM Taxol treatment. Live imaging movies were acquired using a spinning-disc confocal microscope (Nikon). Duration of mitotic arrest was measured by DIC, using green fluorescence to discriminate between tumor cells and stromal cells. More than 30 mitotic arrests were measured for each experimental condition. Chemoprotective effect was confirmed by subsequent flow cytometry analysis at 48 hours post-treatment.

Conditioned media (CM) experiments

Experiments with CM were conducted as for the co-culture experiments, with CM used in place of macrophages throughout the course of the experiment. Medium that had been conditioned for 24 hr by wild-type BMDMs or CAFs was passed through 0.22 μm filters to remove cellular debris and then added 1:1 with fresh medium to tumor cell monocultures. Controls received fresh medium containing 10% serum. Where JPM was used, it was added to tumor cell cultures following the same timeline as for the co-culture experiments. For CM fractionation, 3000

MWCO Amicon Ultra-15 centrifugal filter units were loaded with CM and spun at 5,000 x G for 1-hr at 4 degrees celcius.

Bead phagocytosis assay

BMDMs were labeled with CellTracker Green CMFDA (Invitrogen) and seeded on coverslips 24 h before treatment with either 50 nM Taxol or DMSO control. FluoSpheres, 1-mm red fluorescent (580/605) carboxylate modified microspheres (Invitrogen), were coated overnight in PBS containing 1% (w/v) BSA. Twenty-four hours after treatment, microspheres were added at 50 x excess and incubated for 1 h at 37°C or 4°C. Cells were then washed four times with PBS, and the coverslips were mounted on slides for analysis. Images were visualized under an Axio Imager M1 microscope and acquired using Volocity image acquisition software.

Cathepsin activity-based probe (ABP) imaging

Mice were injected with 150 mL of a 200 μM Cy3B-conjugated cathepsin ABP [16, 17] (synthesized by the Organic Synthesis Facility at MSKCC) intravenously via the tail vein. The probe was allowed to circulate for 1 h before mice were sacrificed, and tissues were processed as previously described [17].

In vivo dosing of drugs

The maximum tolerated dose (MTD) of Taxol via intraperitoneal (i.p.) injection has been previously determined [18]. Accordingly, Taxol (Sigma) was administered i.p. at 50 mg/kg per dose and dissolved in a 1:1:2 cremophor EL:ethanol:PBS solution (Taxol stock maintained in 1:1 cremophor EL:ethanol, then mixed fresh 1:1 with PBS prior to injection) on day 0 and day 17 for the 5-wk trials. The broad-spectrum cathepsin inhibitor JPM-OEt was synthesized by the MSKCC Organic Synthesis Facility, prepared in a 25% DMSO solution, and administered twice daily i.p. for a final dose of 100 mg/kg per day. Vehicle controls in the combination drug trial received equivalent volumes of 1:1:2 cremophor EL:ethanol:PBS solution alone on day 0 and day 17 for the 5-wk trials, as well as 25% DMSO twice daily in volumes equivalent to JPM dosing. Etoposide was purchased as a 20 mg/mL injection solution (TOPOSAR, Teva) and diluted 1:10 in 0.9% sterile saline solution before being administered i.p. at a dose of 10 mg/kg. The etoposide injection solution vehicle consisted of 2 mg/mL citric acid anhydrous, 80 mg/mL polysorbate 80, 650 mg/mL polyethylene glycol 300, and 33.2% (w/v) dehydrated alcohol (pH 3.0–4.0). MMTV-PyMT mice were treated as follows: for Figure 3.1A: from 9–10 wk of age, following one dose of Taxol or vehicle; and for Figure 3.7C: 9–14 wk of age in the regression trial. Orthopically implanted mice were treated as follows: For Figure 3.1A, FVB/n mice orthopically implanted with TS1 cells were treated when tumors reached a total volume of 500 mm^3 with a single injection of Taxol (50 mg/kg) or vehicle, and sacrificed 1 wk later. For Figure 3.8F, mice were treated with 10 mg/kg etoposide on days 0,

7, and 14. Etoposide vehicle control was diluted, injected, and dosed in the same manner as the drug arm of the trial. JPM and the equivalent control were administered as described above. For Figure 3.7E, Athy/Nu mice orthotopically implanted with MDA-MB-231 cells were treated when tumors reached a total volume of 500 mm^3 at a determined Taxol MTD of 25 mg/kg. Taxol and vehicle controls were administered on days 0 and 7 as described above.

Protein isolation, labeling, and Western blotting

Samples were lysed in lysis buffer (5 mM Na-acetate at pH 5.5, 1 mM EDTA, 2 mM DTT, 0.1% Triton X-100), and protein was quantified using the BCA assay (Pierce). Protein lysates were loaded onto SDS-PAGE gels and transferred to PVDF membranes for immunoblotting. Membranes were probed with antibodies against the following:

Cathepsin B (R&D Systems; 1:2000),

Cathepsin C (R&D Systems; 1:1000),

Cathepsin H (R&D Systems; 1:1000)

Cathepsin L (R&D Systems; 1:1000)

Cathepsin S (R&D Systems; 1:1000)

Cathepsin X/Z (R&D Systems; 1:1000)

Actin (Sigma; 1:5000)

Cleaved caspase 3 (Cell Signaling; 1:500)

GAPDH (Cell Signaling; 1:2000)

Phoshpo-ATR.s428 (Cell Signaling; 1:1000)

ATR (Cell Signaling; 1:1000)

Phoshpo-ATM.s1981 (Abcam; 1:1000)

ATM (Cell Signaling; 1:1000)

Phospho-FAK.t397 (Cell Signaling; 1:1000)

FAK (Cell Signaling; 1:1000)

Phospho-PERK.t980 (Cell Signaling; 1:1000)

PERK (Cell Signaling; 1:1000)

Phospho-MEK1/2.s202/204 (Cell Signaling; 1:1000)

MEK1/2 (Cell Signaling; 1:1000)

Phospho-ERK1/2.t202/204 (Cell Signaling; 1:1000)

ERK1/2 (Cell Signaling; 1:1000)

Primary antibodies were detected using HRP-conjugated anti-goat or anti-rabbit antibodies (Jackson Immunoresearch) using chemiluminescence detection (Peirce). Bands from Western blots were quantified in the dynamic range using the Gel Analysis module in ImageJ software.

RNA isolation and qRT–PCR

RNA was prepared from samples using TRIzol reagent (Invitrogen) and subsequently DNase-treated. One microgram of RNA was used in cDNA synthesis reactions using the SuperScript III First-Strand system (Invitrogen). Real-time qRT–PCR was performed on cDNA samples using the ABI 7900HT Fast Real-Time PCR system. The following Taqman assays were purchased from Applied Biosystems:

Cathepsin B (Mm00514439_m1),

Cathepsin C (Mm00515580_m1),

Cathepsin F (Mm00490782_m1),

Cathepsin H (Mm00514455_m1),

Cathepsin K (Mm00484036_m1),

Cathepsin L (Mm00515597_m1),

Cathepsin O (Mm00617413_m1),

Cathepsin S (Mm00457902_m1),

Cathepsin W (Mm00515599_m1),

Cathepsin X/Z (Mm00517697_m1),

CD68 (Mm00839636_g1),

CD45 (Mm00448463_m1),

and Ubiquitin C (Mm01201237_m1).

Staining and analysis of tissue sections

For frozen tissues, 10-μm sections were fixed in acetone, preincubated with 1xPNB-blocking buffer (Perkin Elmer Life Sciences), and incubated with the primary antibody of interest overnight at 4°C. Appropriate Alexa dye-tagged secondary anti- bodies (Invitrogen) were used at a 1:500 dilution and incubated for 1 h at room temperature, followed by incubation in 2 mg/mL DAPI solution (Invitrogen) for 5 min. Species-matched immunoglobulins were used as negative controls. Slides were mounted in ProLong Gold Anti-fade Reagent (Invitrogen), and the tissue sections were visualized under a Carl Zeiss Axio Imager Z1

microscope equipped with an Apotome and AxioCam MRm camera, as well as an automated stage and PixelLink and PCO cameras (TissueGnostics). The primary antibodies used were as follows:

Rabbit anti- mouse Iba1 (1:1000; WAKO Chemicals),

Rat anti-mouse CD34 (1:100; Serotec),

Rat anti-mouse EpCAM (1:500; eBioscience),

Rabbit anti-mouse cleaved caspase 3 (1:200; Cell Signaling),

and rat anti-mouse CD45 (1:200; Serotec).

TissueQuest software (TissueGnostics) was used for colocalization analysis and absolute quantitation of immunofluorescent staining. The acquisition and analysis of images for all these analyses was performed in a blinded manner.

Patient samples

Paraffin-embedded samples from core biopsies at the time of diagnosis and excision samples after neoadjuvant chemotherapy at the time of surgical resection were obtained from Dr. Edi Brogi (MSKCC Breast Pathology service). All patient samples were obtained in compliance with the Institutional Review Board at MSKCC. Samples were stained with mouse anti-human CD68 antibody (Dako) at a 1:1 dilution, then further incubated with a biotin-conjugated goat anti-mouse secondary antibody (Vector BA-9200) and the Vectastain ABC kit (Vector Laboratories), followed by detection with SIGMAFAST DAB (Sigma). Cytokeratin staining was performed at the MSKCC Breast Pathology service. For the detection of CD68 in combination with antibodies against cathepsin B or

cathepsin S (1:1000 and 1:500, respectively; R&D Systems), a multiple antigen-labeling method was developed. Briefly, cathepsin B or cathepsin S was visualized using peroxidase substrate and NovaRed detection, followed by anti-CD68 incubation, alkaline phosphatase substrate, and NovaBlue detection. To enable clear visualization of the red/ brown (cathepsin B or cathepsin S) and blue (CD68) double staining, no counterstain was used for these experiments. All images were visualized under a Zeiss Axio Imager Z1 microscope and acquired using AxioVision image acquisition software.

Statistical analysis

Throughout this study, means ± SEM (standard error of the mean) are reported unless otherwise specified. For all two-way comparisons, with the exception of tumor volume curves, either unpaired t-tests or Mann-Whitney rank sum tests were used. For tumor volume curves, AUC analyses were performed for each mouse and averaged per treatment group, incorporating measurements from the entire 5 weeks.

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Chapter 3

Macrophages and Cathepsin Proteases Blunt Response to Chemotherapy[†]

Preface

It is now well-appreciated that cathepsin expression and activity are frequently increased in cancer as compared to normal tissue[1]. This is particularly true for breast cancer, where increased expression of cathepsins have been shown to be correlated with metastasis and indicative of poor prognosis. The validity of cathepsin proteases as a therapeutic target in breast cancer, however, remains an unresolved question[2-9]. Studies in a genetically engineered mouse model of breast cancer, MMTV-PyMT (PyMT), have yielded conflicting results. Once daily treatment of the pan-cathepsin inhibitor JPM-OEt (100mg/kg) had no significant outcome upon tumorigenesis[10]. These findings are in contrast to studies performed in a model of pancreatic neuroendocrine tumors (PanNETs) where this inhibitor was robustly effective as a monotherapy[11]. This data suggests that breast cancer, unlike PanNETs, is less critically dependent on aberrant cathepsin activity during tumorigenesis. However, treatment with the same pan-cathepsin inhibitor using a ferri-liposome drug delivery system was able to significantly reduce tumor volume[12]. This suggests that bioavailability of the inhibitor is of

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[†] Some of the results presented herein were published in the following citation: Shree T.*, Olson O.C.*, Elie B.T., Kester J.C., Garfall A.L., Simson K., Bell-McGuinn K.M., Zabor E.C., Brogi E., and Joyce J.A. (2011) Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. *Genes & Development* 25(23): 2465–2479. * These authors contributed equally to this work.

significant concern to response in breast cancer and that efficient and selective targeting to the tumor microenvironment is critical for treatment efficacy. Collectively the clinical and experimental data demonstrates that the tumor microenvironment of breast cancer is characterized by increased and aberrantly localized cathepsin activity, and yet there are conflicting results as to the extent of critical dependence on this proteolytic activity for disease maintenance and progression.

We hypothesized that in the steady-state of the breast tumor many of the pro-tumorigenic functions of cathepsins might be redundant with alternative biological pathways and activities. However if we disrupted the homeostasis of the tumor we might reveal specific functions for cathepsin proteases in reestablishing homeostasis. Moreover, while cathepsin proteases have been shown to contribute to many critical steps in baseline tumorigenesis[11 13], we have yet to explore the ability of these enzymes to buffer the tumor against stress and insult. Given the role of cathepsin proteases as critical effectors of lysosomal catabolism and autophagy [1], biological processes known to buffer cells against nutrient stress, it is of interest to examine whether these enzymes play this role broadly in the tumor microenvironment.

In order to investigate potential roles of cathepsin proteases in the response to therapeutic intervention we have chosen to focus on conventional chemotherapeutic agents rather than targeted therapies. In this manner we hope to focus on the functions of cathepsins in modulating cytotoxic stress generally rather than specifically on oncogenic signaling. To this end paclitaxel (Taxol) was

chosen as an investigational chemotherapeutic agent clinically relevant to the treatment of breast cancer[14].

Results

Taxol increases cathepsin activity in mammary tumors

In order to investigate the role of cathepsin proteases in the response to therapeutic treatment we initially treated a cohort of tumor-bearing PyMT mice with Taxol. Additionally we derived several mammary cancer cell lines from PyMT tumors for use in orthotopic implantation models and cell-based assays. Both transgenic and orthotopic PyMT models were treated with the maximum tolerated dose (MTD) of Taxol, 50mg/kg as has been previously reported[15]. MTD Taxol treatment suppressed tumor growth in both contexts, with notable differences in tumor volume apparent by 3 and 7 days post treatment (Fig. 3.1A).

We were interested in assessing cathepsin protease levels following Taxol treatment and found increased cathepsins B, C and L protein in whole tumor lysates from Taxol-treated tumors as compared to vehicle controls (Fig. 3.1B). Similarly we determined cathepsin mRNA transcript levels using quantitative RT-PCR (qRT-PCR) analysis and observed increases in transcript levels of cathepsins B and S (Fig. 3.1C). There was a trend towards an increase in cathepsin L transcripts in line with increased protein expression, however this was not significant. These findings indicate a relative upregulation of cathepsin proteases in Taxol treated tumors as compare to vehicle controls.

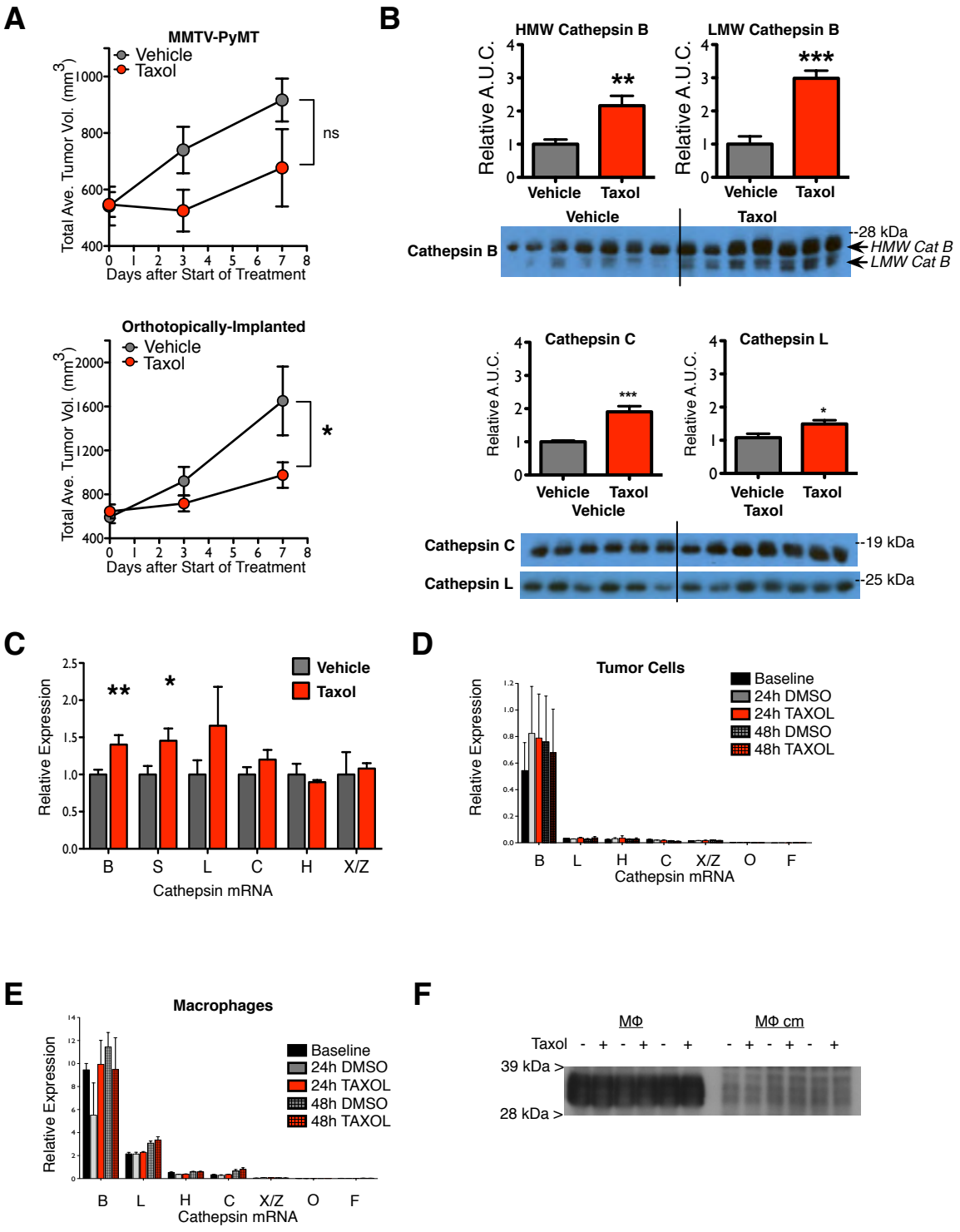


Figure 3.1: Cathepsin levels in mammary tumors increase following Taxol treatment.

Figure 3.1: Cathepsin levels in mammary tumors increase following Taxol treatment. (A) Tumor volume curves for PyMT transgenic mice (left) and orthotopically implanted mice (right) treated with one dose of either vehicle or Taxol (50 mg/kg) on day 0, as described in the Materials and Methods. n = 16–17 for PyMT mice; n = 5–8 for orthotopic model of TS1 PyMT cell line; (*) $P < 0.05$; (ns) not significant. (B) Taxol increased cathepsin levels at the tumor site. Implanted tumors were harvested from mice 7 d after vehicle or Taxol treatment, and whole-tumor lysates were assayed for cathepsin proteins. Quantitation of high-molecular-weight (HMW) and lower-molecular-weight (LMW) active cathepsin B (left) and cathepsin C and cathepsin L (right) are shown above the corresponding Western blots. n = 6–7 mice per group; (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.0001$. (C) Cathepsin transcripts also increased after Taxol treatment. qRT–PCR analysis for cathepsin genes in lysates from implanted tumors, relative to an endogenous control gene, Ubiquitin C, and normalized to vehicle. n = 7–8 mice per group; (*) $P < 0.05$, (**) $P < 0.01$ by the Mann-Whitney test. (D,E) qRT–PCR analysis of cathepsin transcripts in tumor cells (D) or macrophages (E) in culture following treatment with DMSO control or Taxol showed that expression does not change significantly. (F) Cathepsin activity levels in macrophages do not change following Taxol treatment. Macrophage (mϕ) cell lysates (left) or conditioned media (CM) (right) were labeled with the biotinylated cathepsin activity-based probe (ABP), DCG04 [19], showing there is no change in the levels of active cathepsins following treatment with Taxol versus vehicle. n = 3 independent experiments in each case. These data were generated in collaboration with Tanaya Shree.

However, the use of such “whole tumor” approaches does not allow us to discriminate between the possible cellular sources of this upregulation. Cathepsins are expressed both by tumor cells and multiple different stromal cell types within the tumor microenvironment, most notably tumor-associated macrophages (TAMs)[11]. Indeed, use of an *in vivo* cathepsin activity-based probe (ABP) has demonstrated that TAMs are the predominant cathepsin activity-high cell within the tumor microenvironment in the PyMT model[16]. Thus there are two formal possibilities for increased cathepsin protease levels within the tumor; either Taxol treatment induced cell-intrinsic upregulation of cathepsins in either tumor or stromal cells, or alternatively Taxol treatment led to an increase in the relative proportion of cells expressing high levels of cathepsins. There is precedent in the literature for both of these scenarios. Radiation has been shown to induce upregulation of cathepsin S in tumor cells[17], and chemotherapy has been shown to mobilize bone marrow-derived endothelial progenitor cells to the tumor [18].

In order to discriminate between these two competing hypotheses for increased cathepsin expression we began by assessing the ability of Taxol to induce upregulation of cathepsins in a cell intrinsic manner. The PyMT tumor cell line TS1 was treated with physiologically relevant concentrations of Taxol (50nM) and cathepsin transcript levels were analyzed by qRT-PCR. No significant differences in transcript levels were observed for any of the cathepsins between DMSO- or Taxol-treated tumor cells (Fig. 3.1D). In order to determine whether it was possible for Taxol to induce upregulation of cathepsins in macrophages we

generated bone marrow-derived macrophages (BMDMs) form wild-type (WT) mice and similarly treated them in culture. Again no cell-intrinsic induction of cathepsins was observed in the transcript levels of Taxol-treated BMDMs (Fig. 3.1E). Additionally we assessed cathepsin activity in treated BMDMs using the biotinylated ABP DCG04[19]. Congruent with the lack of changes at the mRNA transcript level no changes were observed in the cathepsin protease activity level in either the BMDM cell lysates or in the cathepsins secreted into the BMDM conditioned media (CM) (Fig. 3.1F). Collectively this data does not support direct upregulation of cathepsins in response to Taxol.

Taxol induces an influx of TAMs

As previously reported, cathepsin activity-high cells within the tumor microenvironment are predominantly macrophages[16]. We therefore reasoned that the increase of cathepsins in-Taxol treated PyMT tumors could possibly be due to a increase in TAM infiltration. Tumor tissue samples were stained for the macrophage specific marker Iba-1 by immunofluorescence and automated image analysis was used to quantify macrophage abundance (Fig. 3.2A). We observed a 53% increase in TAMs in the Taxol-treated tumors 7 days after therapy as compared to vehicle controls (Fig. 3.2B). This correlated with increased levels of the macrophage specific transcript CD68 and the pan-leukocyte transcript CD45 as measured by qRT-PCR (Fig. 3.2C,D). Immunofluorescencent quantification of CD45+/Iba1- infiltrating cells, corresponding to non-macrophage leukocytes, showed that this population actually trended towards a decrease (Fig. 3.2E).

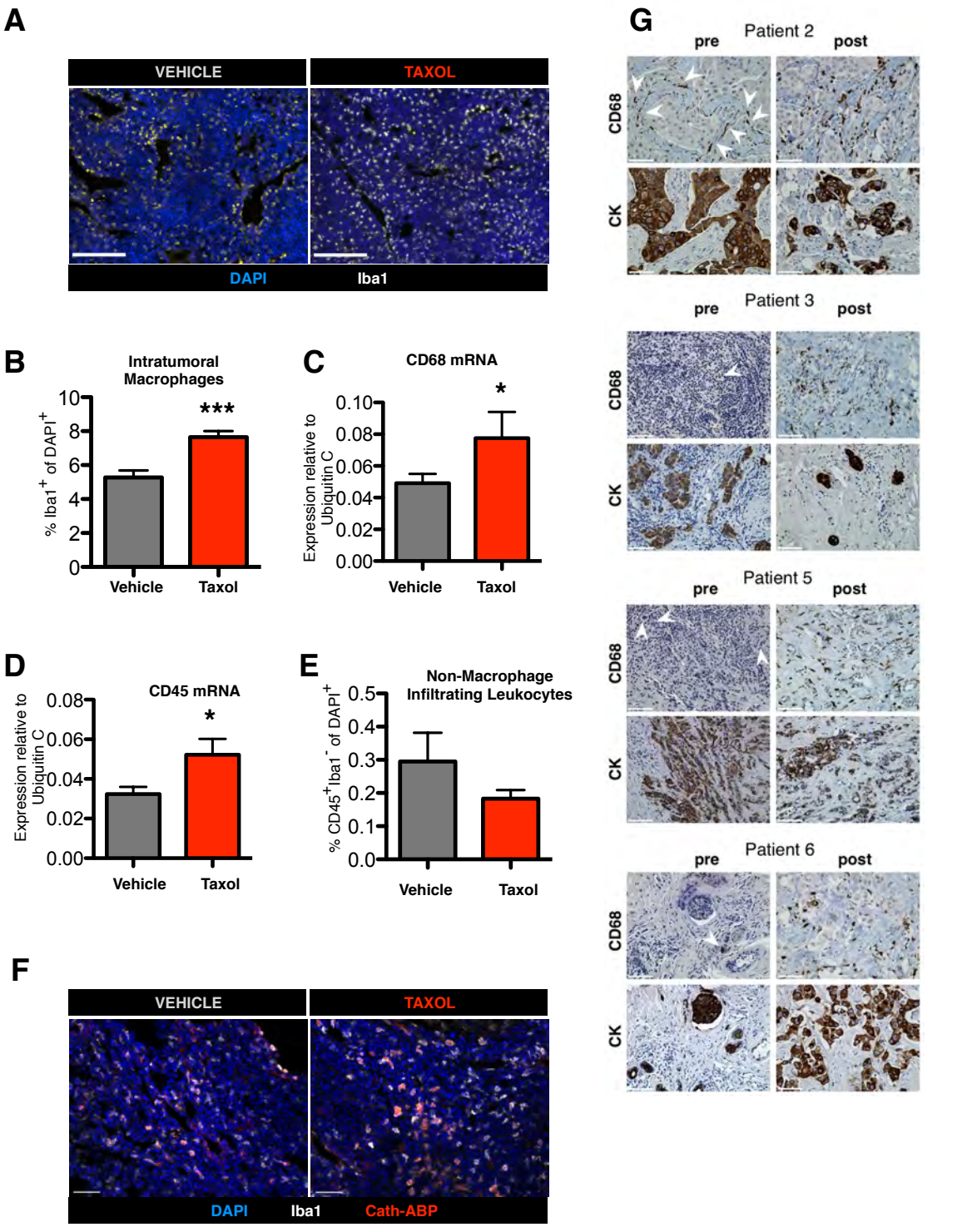


Figure 3.2: Chemotherapy induces a specific increase in macrophage infiltration in breast tumors.

Figure 3.2: Chemotherapy induces a specific increase in macrophage infiltration in breast tumors. (A) Macrophages accumulate in tumors after Taxol treatment. Representative images of orthotopic tumors stained for the macrophage marker Iba1 7 days after vehicle or Taxol treatment (Bars: 100 mm). (B) Quantitation of intra-tumoral Iba1+ cells in tumors of vehicle- and Taxol-treated mice via image analysis. n = 12 vehicle, 9 Taxol; (***) $P < 0.0001$. All data in B–F are from whole tumors isolated 7 d after vehicle or Taxol treatment. (C, D) qRT– PCR from whole-tumor lysates for transcripts of CD68 (macrophages) and CD45 (all leukocytes) 7 d after vehicle or Taxol treatment (n = 7–8 tumors); (*) $P < 0.05$. (E) Quantitation by image analysis of the percentage of CD45+Iba1- cells of total DAPI+ cells in tumors after treatment with vehicle or Taxol (n = 6 mice per group). (F) Representative images of tumor sections from mice injected with the cathepsin ABP after vehicle or Taxol treatment and co-stained with the macrophage marker Iba1. (G) Macrophage numbers increase in breast cancer patients following neoadjuvant chemotherapy. Representative images of matched samples pre- and post-treatment from patients 2, 3, 5, and 6 stained with CD68 or a pan-cytokeratin (CK) antibody to visualize tumor cells. Positively stained cells are labeled in brown. White arrowheads indicate rare CD68+ cells in the pretreatment biopsies. Patient information can be found in Table 3.1 (Bars: F,G, 50 mm). These data were generated in collaboration with Tanaya Shree.

TABLE 3.1. Information on patient samples used in this study.

Supplemental Table 1. Information on patient samples used in this study.

Patient No.	Tumor grade	ER Status	PR Status	HER2 Status	Chemotherapy	Clinical Response to Chemotherapy	Pathological Response to Chemotherapy	Survival Status	CD68(+) cells after treatment
1	II/III	Negative	Negative	Negative	DC-T	Partial	Partial	NED	↑
2	III/III	Negative	Negative	Negative	DC-T	Partial	Partial	NED	no change
3	III/III	Positive	Negative	Positive	DC-T, Trastuzumab	Minimal	Minimal	NED	↑
4	I/III	Positive	Positive	Negative	DC-T	Minimal	Minimal	NED	↑
5	III/III	Positive	Negative	Negative	DC-T	Partial	Partial	DOC	↑
6	III/III	Negative	Negative	Positive	DC-T	Partial	Partial	DOD	↑
7	III/III	Negative	Negative	Positive	EC-T	Partial	Partial	NED	↑

Table key:

Chemotherapy given: DC= Doxorubicin and Cyclophosphamide; T= Paclitaxel; EC= Epirubicin and Cyclophosphamide

NED= no evidence of disease; DOC= died of other cause; DOD= died of disease; ↑= increase

This indicates that the increase in abundance of macrophages is specific to this cell type and not general to all infiltrating immune cells. In order to confirm that macrophages remained the primary source of cathepsin activity within the tumor microenvironment following treatment we injected mice with a cathepsin ABP as previously described[16]. Upon co-staining these tissues with Iba1 we observed that as in the vehicle-treated tumors the majority of cathepsin activity co-localized with the macrophage marker in the Taxol-treated tumors as well.

To confirm our finding that Taxol treatment results in increased infiltration of cathepsin-expressing TAMs in human disease we were able to obtain matched samples from breast cancer patients. These consisted of a biopsy taken at the time of diagnosis and a second tumor sample taken at the time of resection following intervening neo-adjuvant chemotherapy. Indeed, we observed striking increases in macrophages in the majority of patient samples analyzed (Fig. 3.2G, patient information summarized in Table 3.1). Moreover, we observed a very low abundance of TAMs in the patient samples pre-treatment, which is in contrast to the profusion of macrophages observed in the PyMT tumors at baseline. Thus not only do macrophages increase in abundance in the breast tumors of patients treated with Taxol, the magnitude of this increase appears to be much greater than what we observe in the mouse model.

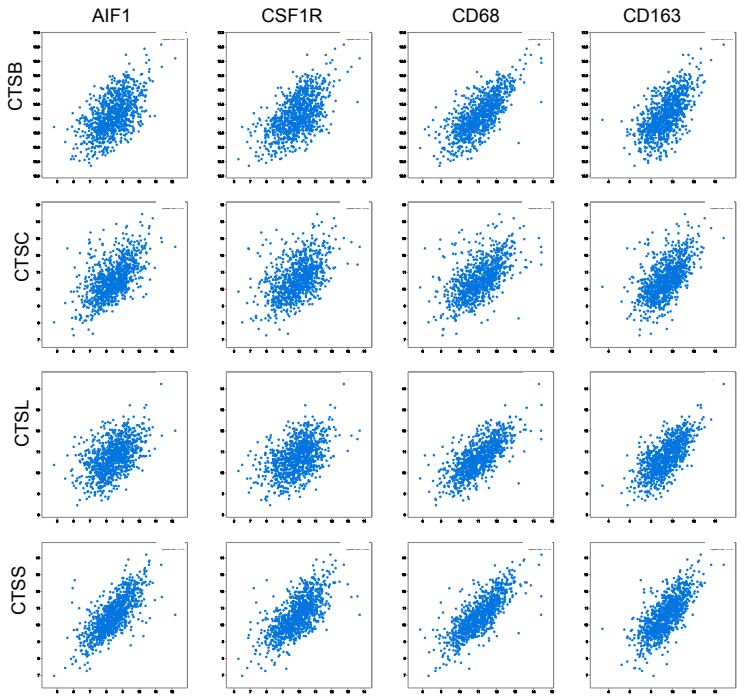
To validate this correlation between macrophages and cathepsin activity within a set human invasive breast carcinoma patients we analyzed the dataset of 1105 patients reported by the TCGA[20]. We observed significant positive correlations between the transcript levels of CTSB, CTSC, CTSL, and CTSS and

the macrophage markers AIF1, CSF1R, and CD68 (Fig. 3.3A). Furthermore there was a positive correlation between cathepsins and the macrophage M2 polarization marker CD163. These correlations strongly support the PyMT experimental data and suggest that in human disease the level of macrophage infiltration indicates the abundance of cathepsin proteases. To corroborate these correlations we performed multicolor immunohistochemistry on primary breast cancer patient samples and indeed observed colocalization of cathepsin B and S with the macrophage marker CD68 (Fig. 3.3B). While we additionally found cathepsin immuno-reactivity in endothelial cells it is clear that macrophages within breast cancer tissues abundantly express these proteases.

Macrophage-derived cathepsins prevent Taxol-induced tumor cell death

We next investigated the functional consequences of an increased abundance of macrophages within the tumor microenvironment. Specifically, we asked whether macrophages have any impact on Taxol-induced tumor cell death and whether the increase in macrophages has a significant role in treatment outcome. We established a co-culture assay to model interactions between breast cancer cells and macrophages using the PyMT cell line TS1 and syngeneic BMDMs (all in the FVB/n genetic background). TS1 cells and macrophages were plated in equal numbers and cultured together for 24 hours prior to Taxol treatment. At 24 and 48 hours following treatment cultures were harvested and the viability of cells was assessed by flow cytometry (Fig. 3.4A).

A



Spearman's rank correlation coefficients

	AIF1	CSF1R	CD68	CD163
CTSB	0.53	0.55	0.72	0.61
CTSC	0.57	0.5	0.58	0.6
CTSL	0.5	0.46	0.72	0.68
CTSS	0.74	0.69	0.78	0.72

B

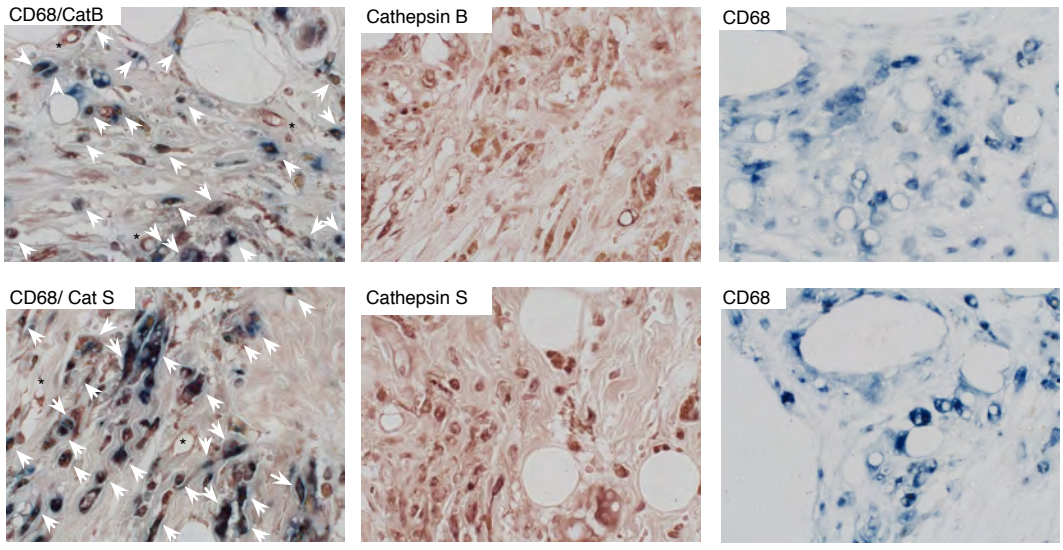


Figure 3.3: Tumor-associated macrophages express cathepsins B and S in human breast cancer patient samples.

Figure 3.3: Tumor-associated macrophages express cathepsins B and S in human breast cancer patient samples. (A) Co-expression analysis was performed on the TCGA dataset comprised of RNA-seq on 1105 breast cancer patient samples [20]. Transcript levels for cathepsins B, C, L and S positively correlate with the macrophage markers AIF1, CSF1R, and CD68 as well as the marker of M2 macrophage polarization CD163. Spearman's rank correlation coefficients are presented in the corresponding table. $P < 0.0001$ for all correlations shown. (B) Representative images of breast tumor patient samples stained with a CD68 antibody (blue), and antibodies against either cathepsin B or S (red/brown color) to visualize tumor cells. White arrows indicate macrophages showing co-staining with cathepsin B or S. Cathepsin positive endothelial cells are indicated with asterisks. Images captured using a Zeiss Z1 Axioimager and 40x objective lens. These data were generated in collaboration with Tanaya Shree.

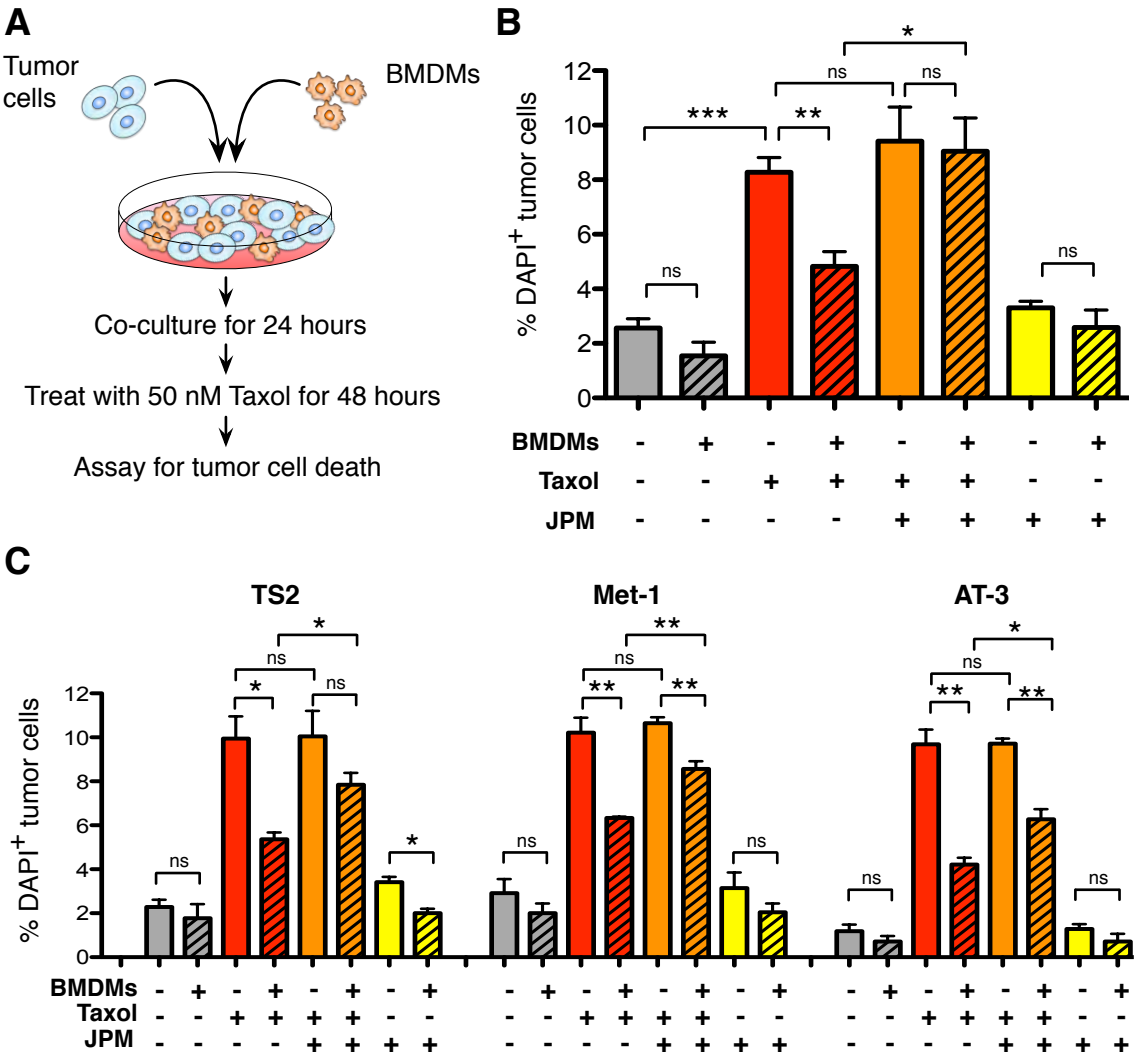


Figure 3.4: Macrophages protect tumor cells from cell death in a cathepsin activity dependent manner. (A) Schematic of co-culture assay. (B) Percentage of DAPI+ (dead) tumor cells in mono- or co-culture of the TS1 cell line with wild-type (WT) BMDMs 48 h after Taxol (50nM) or DMSO treatment. Addition of the pan-cathepsin inhibitor JPM (10μM) abrogated the protective effect of BMDMs following Taxol treatment. (C) BMDMs also protected from Taxol-induced cell death in additional PyMT-derived tumor cell lines, TS2, Met- 1, and AT-3, with experimental conditions as in B. For all graphs, data are from three independent experiments, each done in triplicate. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (ns) not significant.

Macrophages and breast cancer cells were distinguished by the cell type-specific markers CD11b and EpCAM respectively. Cell death was measured by Annexin V, which binds to phosphatidylserine exposed on the plasma membrane of an apoptotic cell, as well as by membrane permeabilization as measured by the DNA immuno-fluorescent dye 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI).

We observed that upon Taxol treatment tumor cell death increased 3.2-fold (p=0.0001) (Fig. 3.4B). By contrast, co-culture of tumor cells with macrophages reduced tumor cell death in response to Taxol by 42% (p=0.0041) (Fig. 3.4B). Interestingly, Taxol did not significantly increase the levels of macrophage death in our co-culture system (data not shown). We next wanted to know whether the cathepsin protease activity of macrophages was functionally involved in the macrophage-mediated chemoprotection observed. The pan-cathepsin inhibitor JPM-OEt was incorporated into the co-culture system and surprisingly we observed a complete abrogation of the macrophage-mediated chemoprotection (p=0.017) (Fig. 3.4B). JPM-OEt had no effect on cell viability of tumor cells or macrophages treated in mono-culture, with or without Taxol (Fig. 3.4B). This indicates that the macrophage-mediated chemoprotection of tumor cells against Taxol is dependent upon cathepsin activity.

In order to assess the generalizability of this finding, multiple different PyMT cell lines were tested. These included TS2, another PyMT cell line derived in our lab[21]; Met-1, a cell line derived from a lung metastasis[22]; and AT-3, a cell line derived from a PyMT tumor on the C57BL/6 rather than the conventional

FVB/n background [23]. Macrophage-mediated chemoprotection was observed for all three of these cell lines in response to Taxol (Fig. 3.4C). Furthermore the chemoprotection observed in all instances was at least partially dependent on cathepsin activity and sensitive to inhibition by JPM-OEt. However, inclusion of JPM-OEt in co-cultures with Met-1 and AT-3 cell lines did not restore Taxol-induced cell death to the level observed in the mono-cultures. This data is therefore suggestive that macrophages are capable of suppressing cell death in response to chemotherapy through additional mechanisms independent of cathepsin activity.

We next sought to determine whether specific cathepsin family members were responsible for mediating this suppression in Taxol-induced tumor cell death. While inhibitors with relative selectivity for individual cathepsins have been reported, we chose to take a more specific genetic approach to determining the specific contributions of individual cathepsins in mediating chemoprotection. BMDMs were derived from WT, CTSB-/-, CTSC-/-, CTSL-/-, and CTSS-/- mice. These represent the four cathepsins observed to be upregulated in Taxol-treated whole tumors at either the protein or the mRNA transcript level. In order to first determine whether the deficiency of any of these cathepsins would impair macrophage development in our BMDM differentiation protocol, differentiation markers CD11b and F4/80 were assessed. No noticeable difference in BMDM differentiation was observed (Fig. 3.5A). Given the biologically important role of macrophages as phagocytes we also wanted to test whether cathepsin deficiency would impair the phagocytic ability of BMDMs.

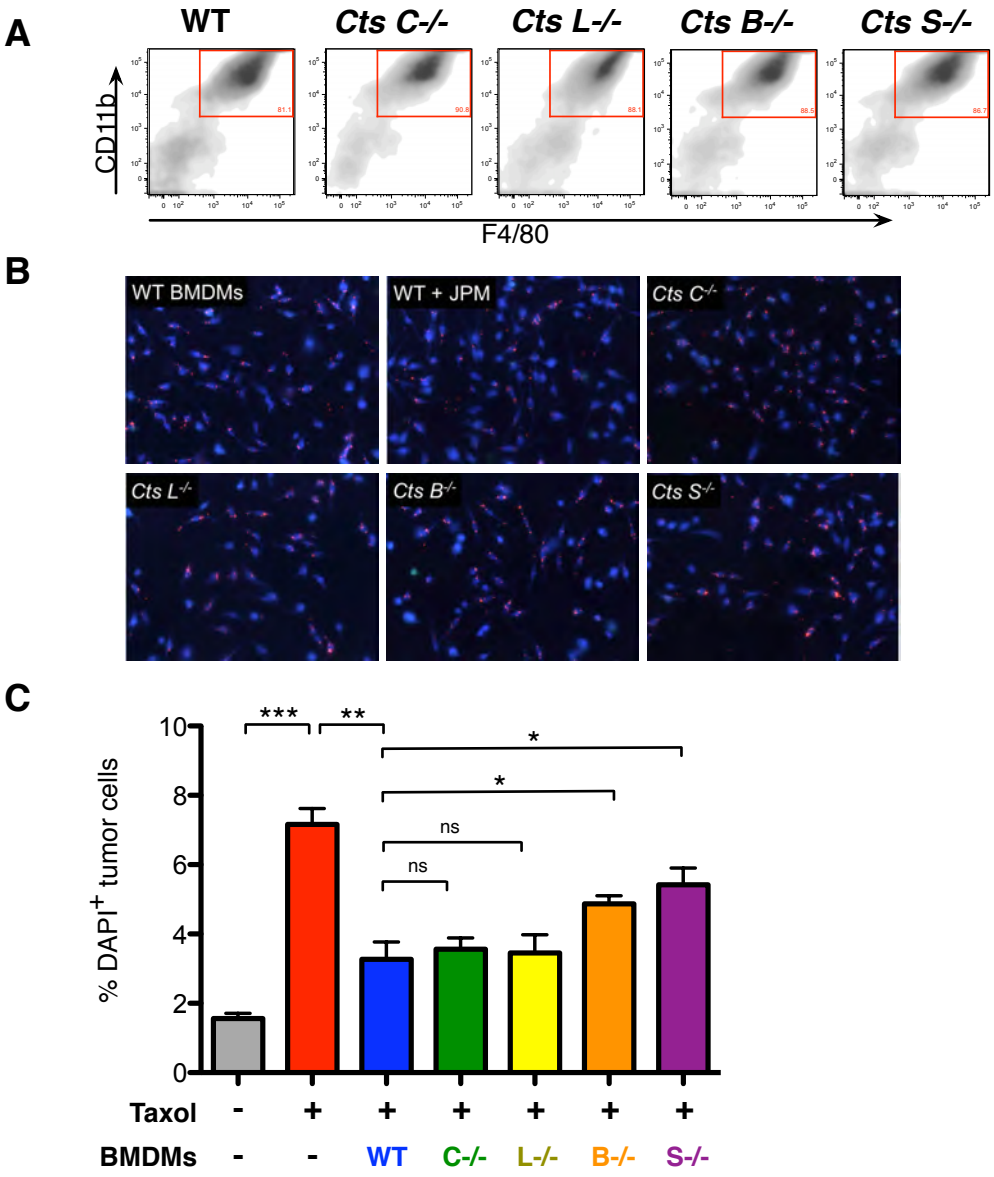


Figure 3.5: Macrophage-mediated protection depends on cathepsins B and S. (A) Percentage of TS1 tumor cell death in monoculture and in co-cultures with wild-type or cathepsin B-, cathepsin S-, cathepsin C-, or cathepsin L-null BMDMs 48 h after treatment. Deletion of cathepsin B or cathepsin S significantly reduced macrophage-conferred protection. For all graphs, data are from three independent experiments, each done in triplicate. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (ns) not significant. (B) Representative flow cytometry plots showing no major differences in differentiation of WT versus *cathepsin*-deficient BMDMs in response to colony stimulating factor-1 (Csf-1) in the macrophage derivation protocol. (C) Representative images of bead phagocytosis assay of WT versus *cathepsin*- deficient BMDMs, showing no differences in FluoSphere bead uptake in the absence of individual cathepsins or following incubation with the cathepsin inhibitor JPM. FluoSphere beads are labeled in red. All images were taken with an x20 objective lens on a Zeiss Axioimager M1 microscope.

Phagocytic ability was assessed by uptake of fluorescently labeled dextran beads, and neither JPM-OEt treatment nor deficiency for any specific cathepsin had an effect (Fig. 3.5B). These cathepsin-deficient macrophages were then incorporated into our co-culture system and evaluated individually for their ability to suppress Taxol-induced tumor cell death. CTSC-/- and CTSL-/- macrophages were not different in activity from WT macrophages indicating that the activity of either of these cathepsins is dispensable for the observed protection (Fig. 3.5C). Macrophages lacking either cathepsins B or S, however, demonstrated impaired protective ability ($p=0.0448$ and $p=0.0370$, respectively) (Fig. 3.5C). Interestingly, the loss of protection observed with either cathepsin B or cathepsin S null macrophages was not as great as that observed with JPM-OEt treatment, suggesting that the effect is at least partially redundant among cathepsin proteases. Combined deletion of both cathepsin B and S did not have an additive effect (data not shown), suggesting that additional cathepsin proteases are involved. Indeed, recent genetic studies have demonstrated compensation among cathepsin family members for specific aspects of tumorigenesis[24].

Finally, we investigated whether the cathepsin dependent macrophage-mediated chemoprotection relied upon cell-cell contact between macrophages and cancer cells or whether this activity was mediated through macrophage-secreted factors. Macrophage-secreted factors were collected by generating conditioned media (CM) for 48 hrs (Fig. 3.6A). Similarly, CM was collected from a cancer-associated fibroblast (CAF) cell line[25], representing another common stromal cell type within the tumor microenvironment.

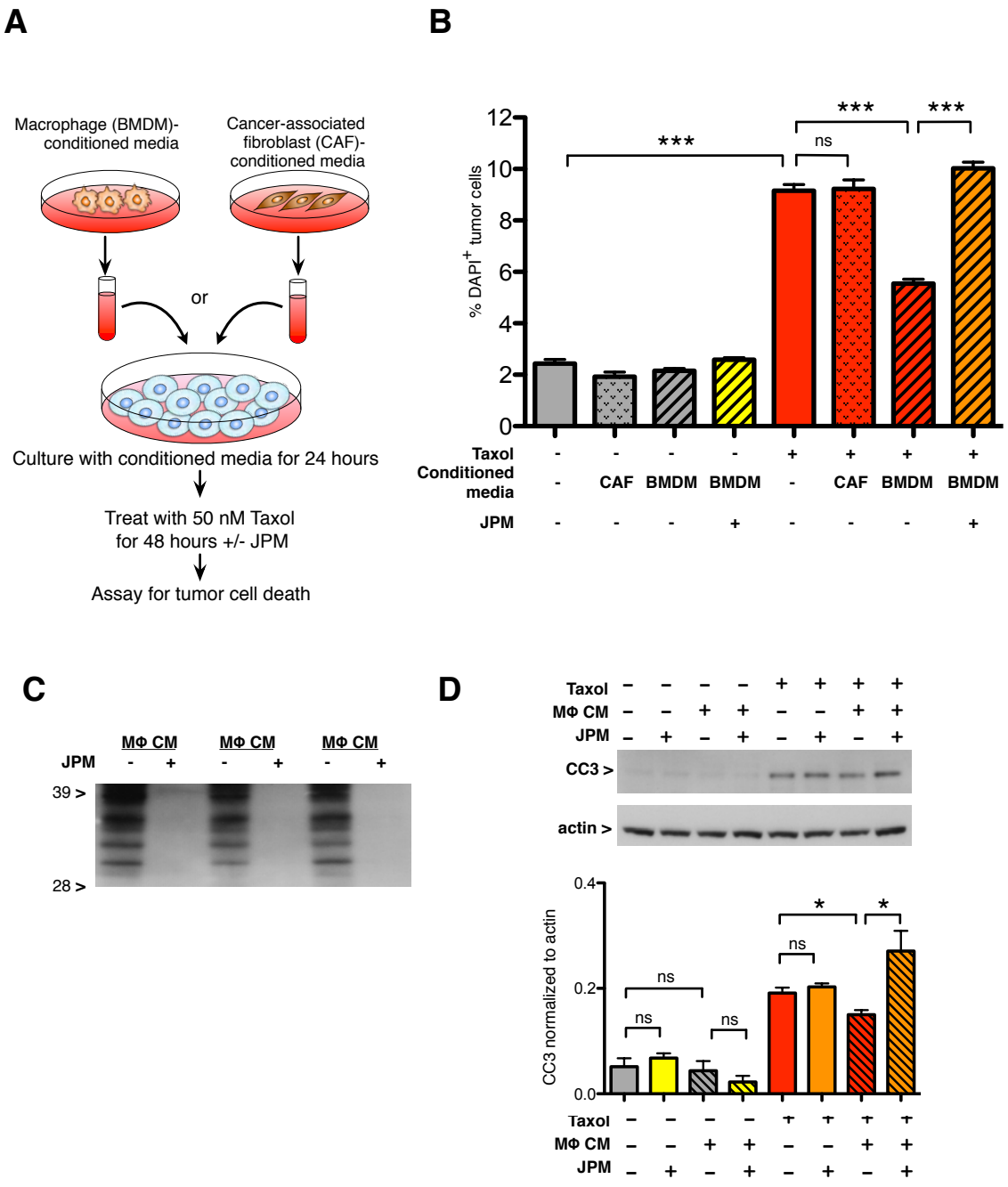


Figure 3.6: Macrophage-mediated protection is not dependent on cell-cell contact and can be recapitulated with macrophage-conditioned media.

Figure 3.6: Macrophage-mediated protection is not dependent on cell-cell contact and can be recapitulated with macrophage-conditioned media.(A) Schematic of experimental design for conditioned media (CM) experiments. (B) Macrophage-conferred protection is largely contact-independent. Cell death in TS1 tumor cells cultured alone or in the presence of wild-type bone marrow-derived macrophage (BMDM)-CM or cancer-associated fibroblast (CAF)-CM 48 h after treatment with Taxol or DMSO control. Addition of JPM (10μM) to the CM abrogated the BMDM-conferred protection. (C) BMDM conditioned media, treated with DMSO or JPM (10μM), was concentrated then labeled with the biotinylated cathepsin ABP, DCG04. There is a clear reduction in active cathepsin species following JPM treatment. (D) Tumor cell death in the BMDM conditioned media experiments was confirmed by western blot analysis of cleaved caspase 3 (CC3) at 24 hours after Taxol treatment. A representative western blot is shown on the left, with quantification of three independent experiments graphed on the right, * $P<0.05$, ns= not significant.

This comparison was introduced in order to evaluate the cell type-specificity of any activity found in macrophage CM. TS1 cells were plated with CM for 24 hrs prior to Taxol treatment and cell death was subsequently assessed as described above. Cell-cell contact was found not to be required for macrophage-mediated chemoprotection, as macrophage CM largely recapitulated the effects of co-culture in reducing Taxol-induced cancer cell death ($p<0.0001$) (Fig. 3.6B). In contrast, CAF CM did not mediate similar protection, demonstrating that macrophage CM specifically and not stromal CM generally was responsible for the protective effect (Fig. 3.6B).

Furthermore, the protective activity of macrophage CM remained sensitive to cathepsin inhibition ($p=0.001$) (Fig. 3.6B). Using the biotinylated ABP DCG04 we were able to demonstrate that JPM-OEt (10uM) was able to robustly inhibit the activity of cathepsin proteases within the macrophage CM (Fig. 3.6C). This data collectively suggests that the macrophage-secreted cathepsins represent the protective activity within macrophage CM. Lastly, we assessed the effect of macrophage CM on Taxol-induced apoptotic signaling. By western blot we observed decreased cleavage and activation of caspase 3, an executioner caspase (Fig. 3.6D). Cathepsin inhibition abrogated the ability of macrophages to suppress apoptotic signaling and restored levels of caspase 3 cleavage.

Cathepsin Inhibition sensitizes mammary tumors to chemotherapy

Given our findings that Taxol increases macrophage accumulation and cathepsin activity within the tumor microenvironment and that macrophages are able to suppress Taxol-induced tumor cell death in a cathepsin dependent manner, we hypothesized that this represented a mechanism of microenvironmental-acquired chemoresistance. To test whether macrophages and cathepsin proteases were indeed limiting the tumor response to Taxol treatment we next sought to combine Taxol treatment and cathepsin inhibition *in vivo*. We chose a regression trial design whereby transgenic PyMT mice with established tumors were treated with MTD Taxol every 2.5 weeks (Fig. 3.7A), and tumor volume was measured by external palpation using calipers. Given the poor intra-tumoral cathepsin inhibition reported upon use of this inhibitor previously in a 100/mg/kg once daily dosing regimen[10], we chose to treat twice-daily in order to achieve consistent bioavailability of the cathepsin inhibitor within the breast tumor microenvironment. Application of this twice-daily dosing schedule resulted in significant inhibition of cathepsin protease *in vivo* as measured in whole tumor lysates using the biotinylated probe DCG04 (Fig. 3.7B).

As a monotherapy, Taxol substantially slowed the outgrowth of tumors as compared to the vehicle treated control group ($p<0.01$, area-under-the curve [AUC] comparison) (Fig. 3.7C). Despite the improved intra-tumoral inhibition achieved with the twice-daily JPM-OEt dosing regimen, our results were consistent with the previously reported trial demonstrating a lack of efficacy for JPM-OEt as a monotherapy (Fig. 3.7C)[10].

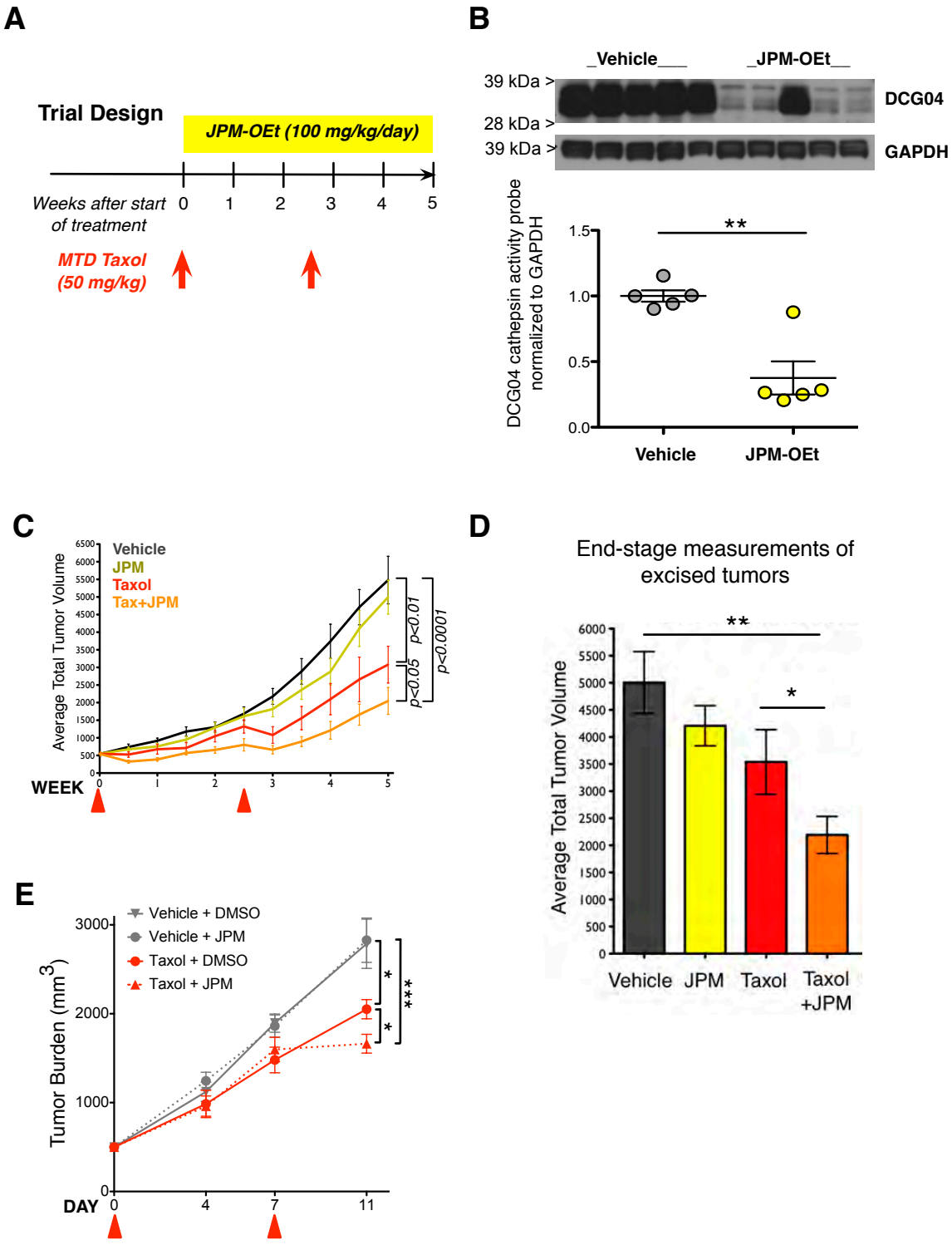


Figure 3.7: Cathepsin inhibition sensitizes tumors to Taxol treatment.

Figure 3.7: Cathepsin inhibition sensitizes tumors to Taxol treatment. (A) Trial schematic showing dosing of maximum tolerated dose (MTD) Taxol and JPM in a 5-wk regression trial (from 9–14 wk of age). (B) Whole tumor lysates from vehicle or JPM-treated PyMT tumors (representative tumors, n=5 each) were labeled with the biotinylated cathepsin ABP, DCG04. Quantification of active cathepsin bands relative to the loading control GAPDH shows there is a significant decrease in the levels of active cathepsins following JPM treatment administered twice daily at 100 mg/kg/day (P=0.0016). (C) Tumor volume curves for PyMT transgenic mice treated with Taxol, JPM, and Taxol+JPM, compared with vehicle, based on twice-weekly external palpation caliper measurements. n = 17 vehicle, 16 JPM, 16 Taxol, 16 Taxol+JPM. P-values in B are derived from area-under-the-curve (AUC) analyses. (D) End-stage tumor volumes based on measurements of excised tumors. (*) $P < 0.05$, (**) $P < 0.01$, unpaired t-tests. n = 14–15 per treatment group. (E) Athy/Nu mice orthotopically implanted with MDA-231 cells were treated with MTD Taxol (25mg/ kg per week) or vehicle control. Addition of JPM (100 mg/kg per day) significantly improved response to Taxol in this preclinical xenograft model. (*) $P < 0.05$ compared with Taxol alone; (***) $P < 0.001$ compared with vehicle; n = 9–12 mice per group. Triangles below the graphs in C and E indicate the time points at which Taxol was administered. These data were generated in collaboration with Tanaya Shree.

Addition of JPM-OEt to the Taxol treatment regimen, however, significantly enhanced response and had the greatest ability to restrain PyMT tumor growth ($p<0.05$, AUC comparison) (Fig. 3.7C). Additionally, at the end of the regression trial the tumors were excised and *ex vivo* volume measurements were taken. These measurements confirmed the data generated by external palpation and showed that the Taxol + JPM treated mice had tumors significantly smaller than those treated with Taxol alone ($p<0.05$) (Fig. 3.7D). These findings are congruent with the chemoprotective effects of macrophage-derived cathepsins that we identified *in vitro*, and demonstrate the specific benefit of cathepsin inhibition in the context of Taxol treatment.

Finally we sought to determine whether we would observe the same benefit of combined cathepsin inhibition and Taxol treatment in breast cancer models using patient-derived cancer cells. We used a human xenograft orthotopic implantation model by injecting MDA-MB-231 cells into the mammary fat pad of athymic nude mice, an immuno-compromised strain[26]. Due to the increased frailty of the athymic nude mice the MTD of Taxol was 25mg/kg rather than the 50mg/kg tolerated by the FVB/n mouse strain. Thus the previous regression treatment regimen was modified to weekly administration of 25mg/kg Taxol, with no alterations to the twice-daily dosing schedule of JPM-OEt. In accord with our findings in the transgenic PyMT breast cancer model, treatment of MDA-MB-231 xenografts with JPM-OEt had no effect on tumor growth. Taxol treatment was able to modestly slow xenograft growth ($p<0.05$), and the addition

of cathepsin inhibition to Taxol treatment significantly improved this response ($p<0.05$).

Macrophage co-culture protects tumor cells from death induced by additional chemotherapeutics

We next sought to determine whether the macrophage-mediated chemoprotection that we observed for the anti-mitotic agent Taxol was relevant to other conventional chemotherapeutic agents. In order to test this we constructed a small panel of chemotherapeutic agents with distinct mechanisms of action including the topoisomerase II inhibitor etoposide, the DNA intercalating agent doxorubicin, the nucleoside analog gemcitabine, and the DNA crosslinking agent carboplatin. We treated the TS1 cell line with these four chemotherapeutic agents in the co-culture cell death assay and observed macrophage-mediated chemoprotection in the response to etoposide and doxorubicin ($p<0.01$ and $p<0.001$, respectively) (Fig. 3.8A). The cancer cell response to gemcitabine and carboplatin, however, was unaffected by the presence of macrophages in the culture. Similar to the protective effects observed for Taxol treatment, macrophage-mediated chemoprotection was abrogated by cathepsin inhibition either largely in the case of doxorubicin treatment ($p<0.01$), or entirely as observed with etoposide ($p<0.01$) (Fig. 3.8A).

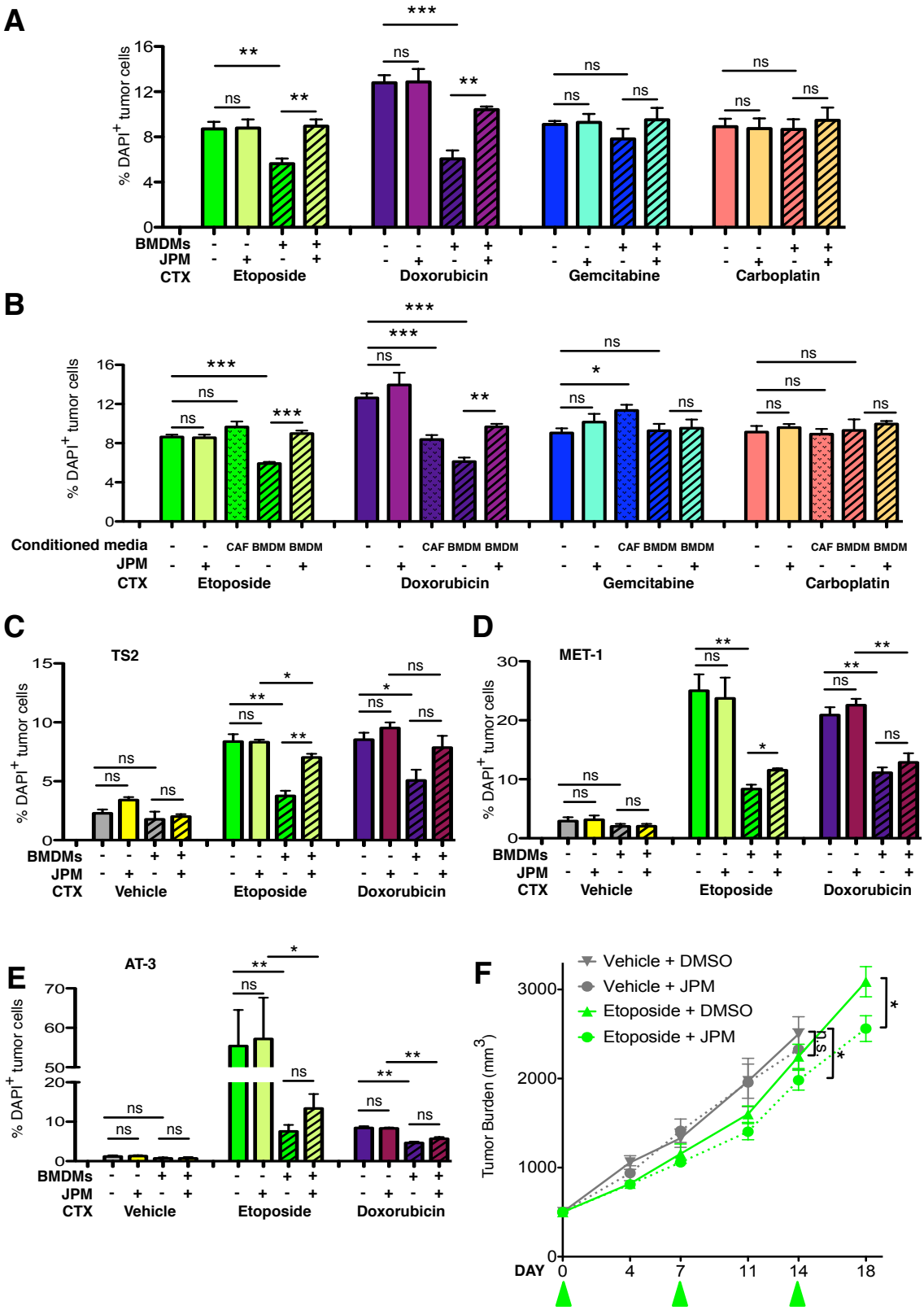


Figure 3.8: Cathepsin-mediated chemo-protection is relevant to additional chemotherapies.

Figure 3.8: Cathepsin-mediated chemo-protection is relevant to additional chemotherapies. (A) Percentage of DAPI+ (dead) tumor cells in mono- or co-culture of the TS1 cell line with BMDMs 48 hours after etoposide (20 μM), doxorubicin (300 nM), gemcitabine (400 nM), carboplatin (50 μM), or DMSO treatment. Co-culture with BMDMs was protective against treatment with etoposide and doxorubicin, but not gemcitabine or carboplatin. As with Taxol, the protective effect observed in BMDM co-culture was significantly abrogated by the cathepsin inhibitor JPM (10 μM). (B) Cell death in tumor cells cultured alone or in the presence of wild-type (WT) BMDM-CM or CAF-CM 48 h after treatment with etoposide, doxorubicin, gemcitabine, carboplatin, or DMSO control. For chemotherapies where macrophage-mediated protection was observed, etoposide and doxorubicin, this effect was assessed with additional murine breast tumor cell lines: TS2 (C), Met-1 (D), and AT-3 (E). Co-culture with BMDMs significantly reduced cell death in response to both drugs in all lines tested, with different degrees of cathepsin dependence. For all graphs, data are from three independent experiments, each performed in triplicate. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (ns) not significant.

Macrophage CM was similarly tested, and as observed with Taxol, macrophage CM was able to recapitulate the protective effects observed in the treatment of etoposide and doxorubicin, again in a cathepsin activity-dependent manner (Fig. 3.8B). Intriguingly, CAF CM did demonstrate some protective activity in the response to doxorubicin and conversely sensitized the cancer cells to treatment with gemcitabine ($p<0.001$ and $p<0.01$, respectively) (Fig. 3.8B). Testing the effect of macrophage co-culture on the response to etoposide and doxorubicin in other PyMT cell lines uncovered a considerable degree of variability (Fig. 3.8C,D, E). While all three cell lines demonstrated macrophage-mediated chemoprotection, the dependence of this activity upon cathepsin proteases was variable. The cell lines TS2 and Met-1 responded similarly to TS1 when treated with etoposide, but when treated with doxorubicin the abrogation of the protective effect by cathepsin inhibition failed to reach significance (Fig. 3.8C, E). For the AT-3 cell line, cathepsin inhibition failed to abrogate the macrophage-mediated protection observed for either drug. Thus we observed that while macrophage-mediated protection against specific drugs is broadly relevant across different PyMT cell lines, the dependence of this protection upon cathepsins is variable.

Finally we sought to determine whether the role of macrophages in mediating protection in our co-culture system was similarly relevant to treatment in the intact tumor microenvironment. Etoposide was selected for *in vivo* validation given the greater extent to which cathepsin inhibition reversed the protective effect for this drug (Fig. 3.8A). TS1 cells were orthotopically implanted

in the mammary fat pads of syngeneic FVB/n mice. Tumor-bearing mice were treated with etoposide weekly and the effect of including the cathepsin inhibitor JPM-OEt was assessed. Our treatment regimen of etoposide demonstrated a poor ability to suppress tumor growth and was not significantly different than vehicle treated animals at the 14-day time point (Fig. 3.8F). However, by 18 days the tumor burden of animals treated with combined etoposide and JPM was significantly less than in those animals treated with etoposide alone.

In summary, our findings of cathepsin dependent macrophage-mediated chemoprotection has been shown to be relevant to multiple, but not all, chemotherapeutic agents, and the addition of cathepsin inhibitors is beneficial in multiple mouse models of therapeutic intervention.

Chapter 3 references

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Chapter 4

Macrophages Alter the Activity of Antimitotic Agents

Preface

It has become increasingly appreciated that stromal cells provide growth factors and other pro-survival signal to tumor cells in their microenvironment[1]. Consistent with this it has been generally proposed that the stroma creates a chemo-resistant niche by increasing the tumor cell threshold for cell death[2]. Additionally the immune response has been shown to play a critical role in the response to cytotoxic chemotherapy, and the stroma can promote resistance by suppressing this immune response[3]. Finally the stroma has also been shown to promote resistance to chemotherapy simply by limiting the ability of the drug to reach the tumor cells[4]. Limited research, however, has been conducted into how the stroma affects the mechanisms by which cytotoxic chemotherapy kills tumor cells and this concept has been largely overlooked. Evidence has begun to accumulate, however, that certain chemotherapies behave differently in the physiological *in vivo* setting than in the culture dish. It has recently been shown through intra-vital imaging experiments that anti-mitotic agents in particular have different phenotypes in an *in vivo* setting than what is observed on plastic[5]. Given our continued interest in the role of the tumor microenvironment we sought to interrogate the effects of macrophages and other stromal cells on the mechanisms by which Taxol interfered with the cell cycle and induced genotoxic stress.

Results

Taxol induces multi-nucleation and genotoxic stress following prolonged mitotic arrest

Tumor-associated macrophages (TAMs) supply numerous survival factors to tumor cells, increasing their resistance to programmed cell death. We were interested in evaluating whether TAMs also altered the mechanism of action of chemotherapeutic agents and suppressed their ability to induce DNA damage and cellular stress. To address these questions, we continued to employ Taxol as our primary investigational chemotherapy and first sought to determine the mechanism by which Taxol induces genotoxic stress and results in cell death. Given that the basic mechanism of Taxol treatment involves microtubule stabilization[6, 7] and impairment of mitosis[8], we began our characterization by assessing the cell cycle of TS1 PyMT cells in response to treatment. We observed that after 24 hours of Taxol treatment there was a loss of the 2C peak, representing G1 diploid cells (Fig. 4.1A). This correlated with a subsequent increase in the 4C peak, representing G2/M diploid cells and/or G1 tetraploid cells. Furthermore, we observed the emergence of an 8C peak, representing G2/M tetraploid and/or G1 octoploid cells. Throughout the course of the assay we observed an accumulation of sub-2C DNA content cells, representing dead and dying cells with fragmented nuclei. The loss of the 2C peak suggests that at the 50nM concentration of Taxol cells are unable to successfully complete mitotic division and either die in mitosis or slip out of mitosis into a tetraploid state.

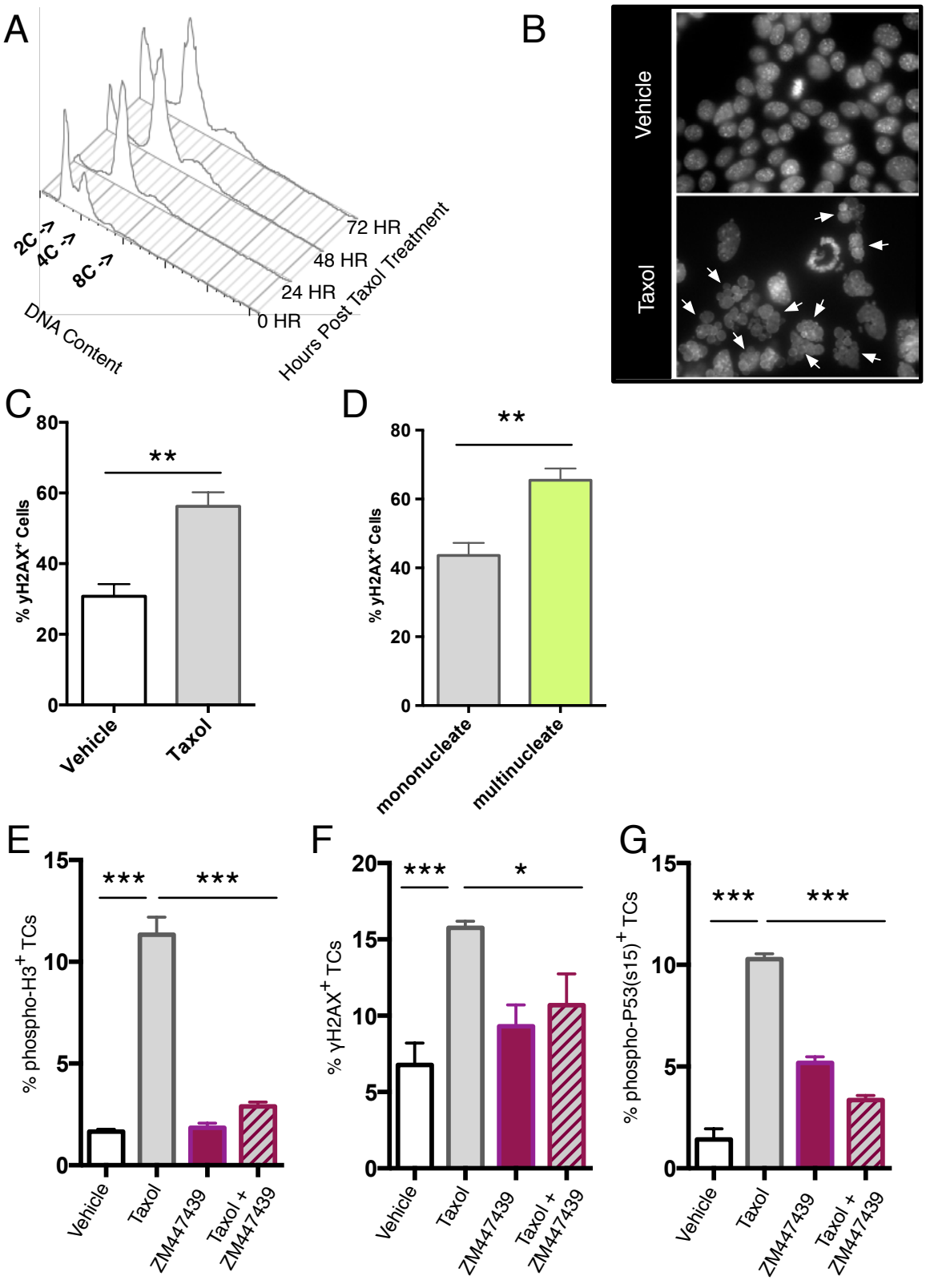

Figure 4.1: Prolonged mitotic arrest and subsequent mitotic slippage following Taxol treatment lead to tumor cell death.

Figure 4.1: Prolonged mitotic arrest and subsequent mitotic slippage following Taxol treatment lead to tumor cell death. (A) DNA content analysis of TS1 PyMT tumor cells treated with 50nM Taxol over a 72-hour time course. Following Taxol treatment cells accumulate in a 4C DNA content peak indicating cells arrested in mitosis or in a tetraploid G1 state. Some tetraploid cells additionally re-enter the cell cycle as represented by the 8C peak. At 48 and 72 hour time points, dead cells accumulate as a sub-2C peak. (B) Nuclear morphology of TS1 cells was assessed at 24 hours post-Taxol treatment. Taxol treatment resulted in many cells displaying a multinucleated state, likely as a result of mitotic slippage. Multinucleated cells are indicated using white arrows. (C) Cells with an active DNA damage response were measured by γH2AX staining 48 hours after Taxol treatment. Taxol induced a significant increase in γH2AX-positive cells. (D) Stratification of Taxol-associated γH2AX positive cells revealed increased γH2AX positivity in multinucleated cells. Multinucleated cells were not observed under Vehicle-treated conditions. For all graphs in C and D, data are from three independent experiments. (**) $P < 0.01$ by the Students t-test. These data were generated in collaboration with Hyunjung Kim. Abrogation of Taxol-induced mitotic arrest with the aurora A/B kinase inhibitor ZM447439 (2.5 uM) suppresses the genotoxic stress response. (E) TS1 tumor cells (TCs) were stained for the mitosis marker phospho-Histone H3 and measured by flow cytometry. Taxol-induced mitotic arrest increased the relative proportion of mitotic cells at 24 hours of treatment. Addition of the aurora kinase inhibitor ZM447439 suppressed the accumulation of mitotically arrested cells in response to Taxol. (F) Abrogation of the Taxol-induced mitotic arrest decreased the levels of γH2AX-positive cells as measured by flow cytometry. (G) Similarly, aurora kinase inhibition was able to impair suppress the Taxol-induced phosphorylation of p53. For all graphs in E, F and G, data are from three independent experiments, each performed in triplicate. (*) $P < 0.05$; (***) $P < 0.001$ by the Students t-test.

The emergence of the 8C peak suggests that at least some of these newly tetraploid cells are proficient to re-enter the cell cycle. Furthermore death during mitotic arrest seems inconsistent with this data given that the majority of sub-2C cells appear at later time points (48 and 72 hours). By studying the nuclear morphology of tumor cells at 24 hours-post Taxol treatment we observed extensive multi-nucleation, consistent with mitotic slippage as a predominant outcome of Taxol-induced mitotic arrest (Fig. 4.1B). To determine whether Taxol-induced mitotic arrest and slippage was resulting in genotoxic stress we stained against γH2AX, a marker of DNA damage and repair[9, 10]. We observed that Taxol indeed increased the proportion of γH2AX-positive cells by 25% (Fig. 4.1C). Moreover, when we stratified this data by examining the nuclear morphology of the Taxol treated cells we observed a significantly higher proportion of γH2AX-positive cells in the multinucleated population (Fig. 4.1D). Collectively this data demonstrates that Taxol-induced mitotic slippage and multi-nucleation correlates with increased γH2AX positivity.

Prolonged mitotic arrest has been proposed as a critical driver of response to anti-mitotic agents[11-14]. Indeed it has been demonstrated that when the duration of mitotic arrest is modulated with inhibitors of the spindle assembly checkpoint, the length of mitotic arrest dictates the fate of the cell following mitotic exit[15]. However, single cell analysis of the response to anti-mitotic agents by live cell imaging has provided conflicting results, indicating profound inter- and intra-line variation in the response, and no apparent correlation with time spent in

mitosis[16]. These experiments also demonstrated that prolonging mitotic arrest by overexpression of cyclin B1 did affect cell fate following mitotic exit.

For our purposes, we sought to determine whether the mitotic arrest was necessary for the induction of genotoxic stress by Taxol. To address this question we used the Aurora kinase A/B inhibitor ZM447439 to bypass the spindle assembly checkpoint and suppress mitotic arrest in response to Taxol treatment. Twenty-four hours after treatment TS1 cells were collected, fixed, and stained for the mitotic marker phospho-Histone H3. Treatment with Taxol alone caused a 6.9 fold increase in mitotic cells (Fig. 4.1E; $P < 0.001$) , consistent with enhanced arrest. Correspondingly, addition of ZM447439 to Taxol treatment significantly suppressed the enrichment of mitotic cells (Fig. 4.1E; $P < 0.001$) indicating a relative loss of mitotic arrest. We assessed γH2AX immuno-reactivity in these samples and similarly observed increased γH2AX in Taxol-treated samples compared to Vehicle, and addition of ZM447439 to Taxol treatment significantly reduced this induction (Fig. 4.1F; $P < 0.001$ and $P=0.028$, respectively). It is important to note that while addition of the aurora kinase inhibitor suppressed 87% of the Taxol-induced increase in mitotic cells, it only suppressed 56% of the Taxol-induced increase in γH2AX positive cells (Fig. 4.1E, F). Examining activation of the tumor suppressor p53 as an additional indicator of genotoxic stress, we again observed a similar pattern of increased p53 phosphorylation in Taxol treatment alone (Fig. 4.1F; $P <0.001$). Addition of ZM447439 suppressed 78% of the Taxol-induced increase in p53-activated cells

(Fig. 4.1G; P < 0.001). Thus the correlation between abrogation of mitotic arrest and p53 activation appears more pronounced than with γH2AX reactivity.

Tumor-associated macrophages suppress chemotherapy-induced γH2AX

We next introduced bone marrow-derived macrophages (BMDMs) into this cell culture assay to assess their effect on tumor cell cycle changes and genotoxic stress in response to Taxol. Consistent with our earlier findings where we measured cell death by Annexin V staining and DAPI exclusion, measurement of cell death by sub-2C DNA content reveals robust protection from Taxol at 48 hours with a 51% reduction in cell death (Fig. 4.2A; P < 0.01). Moreover, by observing extended time points in this experimental design, we observed a greater differential in tumor cell death at 72 hours, where the presence of BMDMs reduces Taxol-induced cell death by 69% (Fig. 4.2A; P <0.001). Intriguingly, there was increased persistence of 2C cells at 24 and 48 hours, while no significant difference remained by 72 hours (Fig. 4.2A; P < 0.01 and P = 0.026, respectively). Most notably, higher proportions of 4C and 8C tumor cells were present at 72 hours in the co-cultures (Fig. 4.2A; P < 0.01 and P < 0.001, respectively). Assessment of mitotic index in response to Taxol revealed an enrichment specifically at 24 hours following treatment, which returned to basal levels at 48 and 72 hours (Fig. 4.2B). BMDMs did not grossly impair the ability of Taxol to increase the mitotic index of tumor cells. However, macrophage co-culture did suppress the levels of genotoxic stress observed in response to Taxol (Fig. 4.2C,D).

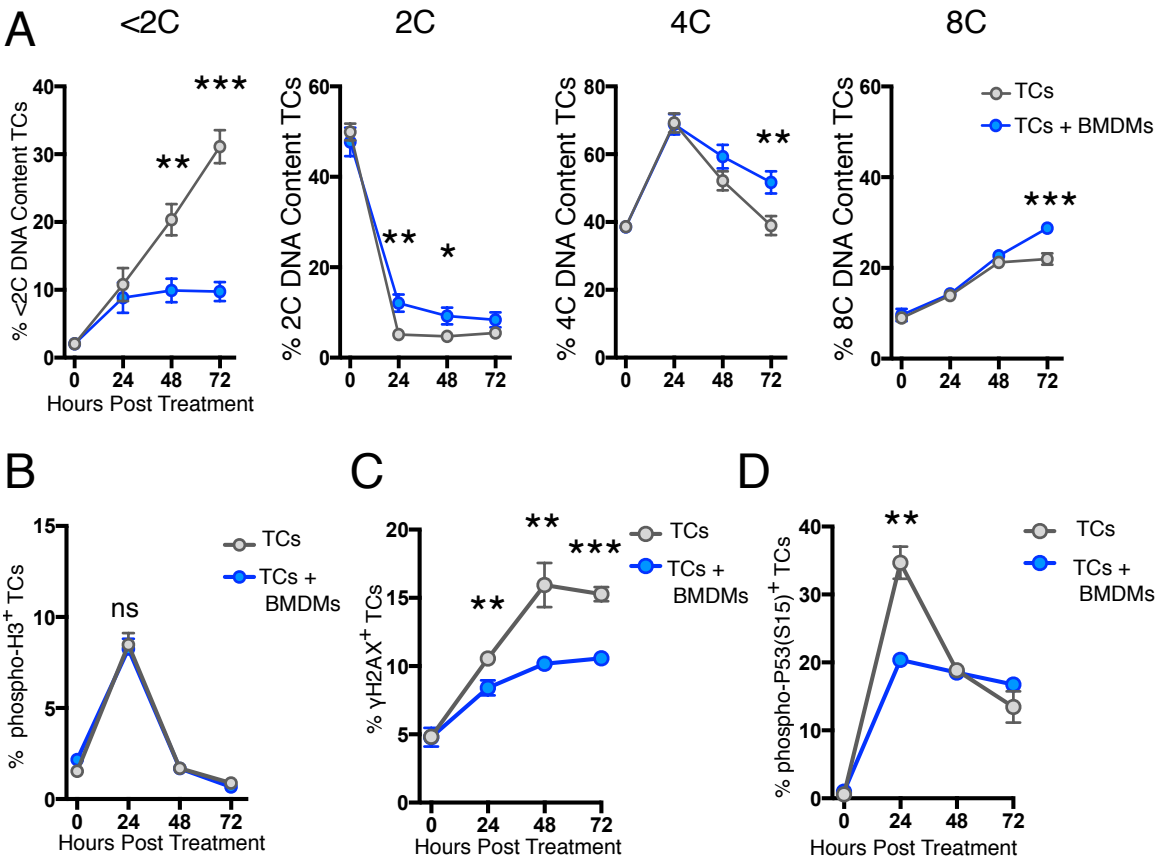

Figure 4.2: Macrophage co-culture suppresses genotoxic stress in response to Taxol treatment. (A) Co-culture with bone marrow-derived macrophages (BMDMs) modulates the cell cycle changes in TS1 tumor cells (TCs) upon treatment with Taxol. Co-culture suppresses the increase of the sub-2C population at 48 and 72 hours. Additionally BMDM co-culture increases the proportion of 4C and 8C populations at 72 hours. (B) Assessment of mitotic index by measurement of phospho-H3 by flow cytometry reveals that BMDM co-culture does not alter the ability of Taxol to provoke a mitotic arrest. (C) BMDM co-culture reduces the levels of γH2AX positive cells at all time points following Taxol treatment. (D) BMDM co-culture additionally suppresses p53 phosphorylation at its peak at 24 hours post-Taxol treatment. For all graphs in A, B, C, and D, data are from three independent experiments, each performed in triplicate. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ by the Students t-test.

BMDMs reduced the level of γH2AX-positive cells at all time points following treatment (Fig. 4.2C). BMDMs also reduced phosphorylation of p53 specifically at 24 hours (Fig. 4.2D; $P < 0.01$). Taken together this data demonstrates that the presence of macrophages reduces multiple markers of genotoxic stress in tumor cells and this correlates with reduced tumor cell death and increased persistence of tumor cells with high DNA content.

In order to determine whether the macrophage-mediated reduction in γH2AX levels was specific to Taxol treatment or general to other chemotherapeutic agents, we returned to the chemotherapeutic panel used previously [17] and ran these treatments in our adapted flow cytometric assay. We observed that macrophage co-culture generally reduced the levels of γH2AX-positive cells in response to all chemotherapeutic agents (Fig. 4.3A). This effect was least robust with Carboplatin, where γH2AX-positive cells were only significantly reduced at 24 hours post-treatment (Fig. 4.3A; $P = 0.011$). However, in response to etoposide, doxorubicin and gemcitabine, BMDMs significantly reduced the levels of γH2AX-positive cells at all time points following treatment (Fig. 4.3A). The effect of this reduction in genotoxic stress in response to this panel of chemotherapeutic agents is less clear and the correlation with tumor cell response is variable (Fig. 4.3B). Measurements of sub-2C DNA content populations revealed macrophage-mediated reductions in cell death of 80% and 67% in the response to etoposide and doxorubicin respectively (Fig. 4.3B; $P < 0.001$).

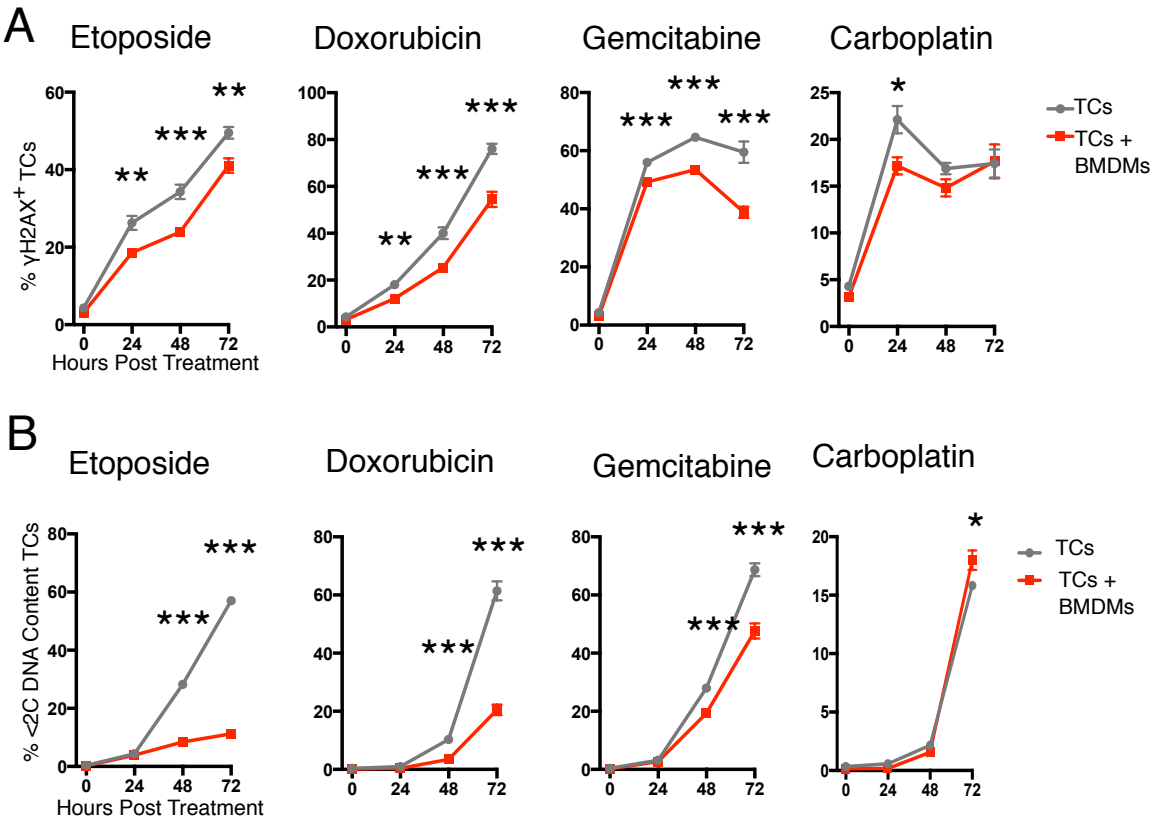

Figure 4.3: Macrophage co-culture suppresses γH2AX generally in response to distinct cytotoxic chemotherapies with variable impact on tumor cell death. (A) Measurement of γH2AX positivity by flow cytometry in response to etoposide (20μM), doxorubicin (300nM), gemcitabine (400nM), and carboplatin (50μM). Co-culture with BMDMs generally reduces the percentage of γH2AX-positive cells. (B) Assessment of chemotherapy-induced tumor cell death by measurement of the sub-2C population reveals variable modulation by BMDMs. For all graphs in A and B, data are from three independent experiments, each performed in triplicate. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ by the Students t-test.

For gemcitabine treatment the macrophage-mediated reduction in tumor cell death was a more modest 31% decrease, and surprisingly with carboplatin treatment we actually observed a 14% increase in tumor cell death at 72 hours (Fig. 4.3B).

We next sought to determine whether the ability of macrophages to suppress chemotherapy-induced genotoxic stress was dependent on cell-cell contact, or mediated by secreted factors (Fig. 4.4A). Tumor cells were plated with either BMDMs or conditioned media (CM) collected from BMDMs as previously[17]. After 24 hours the cultures were treated with either Taxol or Taxol plus BMDM-CM and γH2AX signal induction was measured over a 72-hour time course by flow cytometry. After 24 hours of treatment both BMDMs and BMDM-CM significantly reduced the percentage of γH2AX+ cells relative to cells treated with Taxol alone (Fig. 4.4A; P= 0.0017 and 0.0004, respectively). These effects persisted at 48 hours, but by 72 hours only direct co-culture with BMDMs significantly reduced tumor cell death as compared to tumor cells alone (Fig. 4.4A; P= 0.0025). It is possible that the protective activity present in the CM is consumed by this timepoint, given that the CM is not refreshed after Taxol treatment. The effect of CM on the sub-2C dead cell population was more robust, but displayed similar trends to the γH2AX data. At 72 hours co-culture reduced the population of sub-2C cells from 37% to 6% of the total, and CM reduced the population to 21% (Fig. 4.4B; P<0.001 for both comparisons).

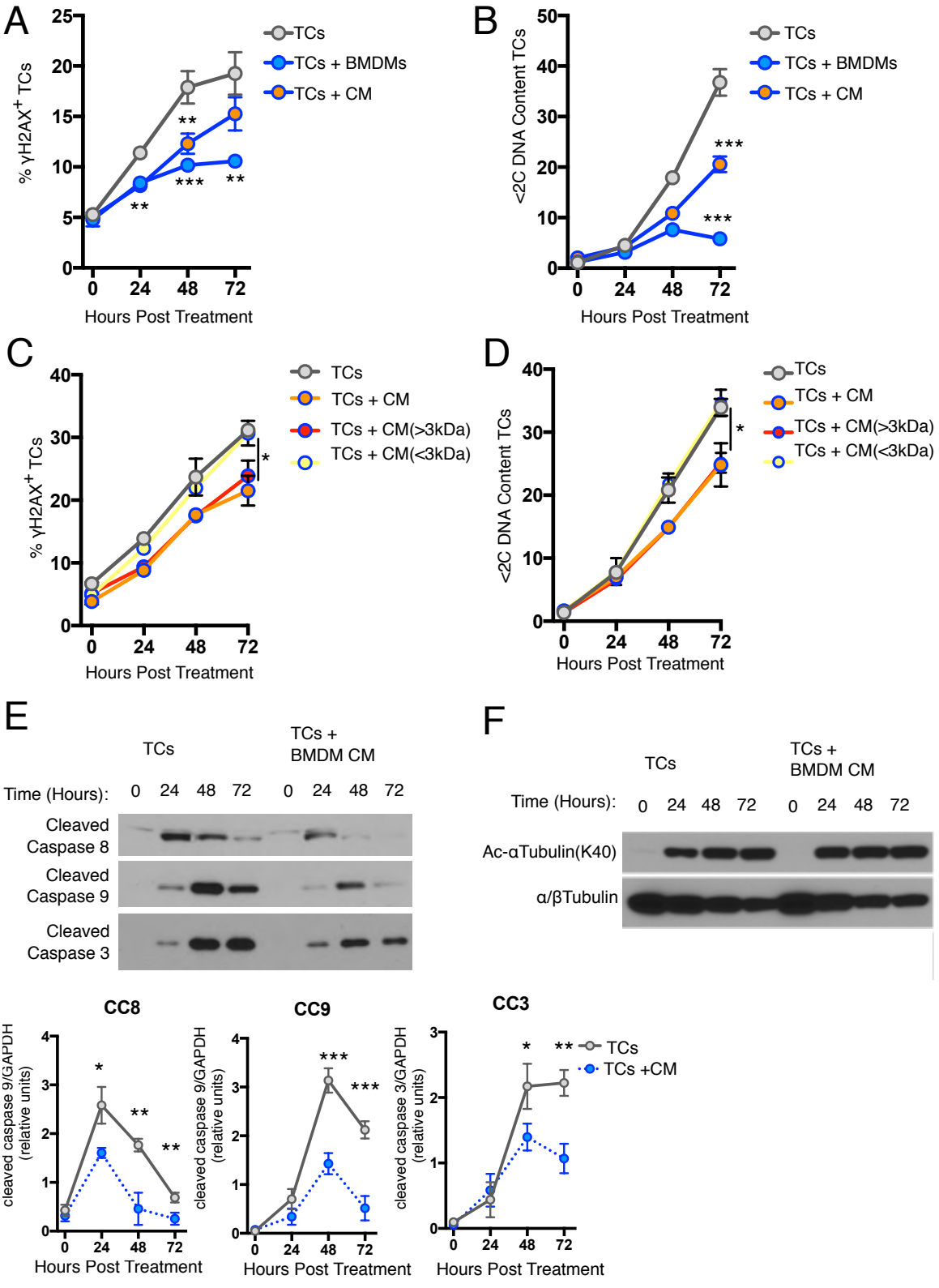

Figure 4.4: Macrophage-secreted factors suppress genotoxic stress and tumor cell death.

Figure 4.4: Macrophage-secreted factors suppress genotoxic stress and tumor cell death.(A) Measurement of γH2AX positivity by flow cytometry in response to Taxol reveals that BMDM-conditioned media (CM) suppresses the induction of genotoxic stress by Taxol treatment. (B) Assessment of chemotherapy-induced tumor cell death by measurement of the sub-2C population reveals that cell-cell contact is dispensable for macrophage-mediated chemoprotection. (C) Fractionation of BMDM CM indicates that the macrophage-secreted factor(s) responsible for suppressing γH2AX induction is >3kDa in size. (D) The suppression of tumor cell death by macrophage CM correlates with the reduction in γH2AX and is similarly mediated by the >3kDa fraction. (E) Western blot analysis reveals that BMDM CM suppresses activation of both the extrinsic caspase 8 apoptotic pathway and the intrinsic caspase 9 apoptotic pathway in response to a Taxol time course. For western blots a representative image is shown above, with quantification of three independent experiments graphed below. (F) Taxol-induced tubulin acetylation was measured and is unaffected by BMDM CM treatment. For all graphs in A-E, data are from three independent experiments, each performed in triplicate. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ by the Students t-test.

We therefore sought to determine whether the genotoxic stress reducing activity in macrophage-CM was mediated by metabolites and small signaling molecules or by larger secreted proteins. We fractionated the BMDM CM based on size using 3 kDa molecular weight cut-off spin columns to separate proteins from the media. Treatment of tumor cells with the protein fraction (the >3kDa fraction) recapitulated the effects observed with the complete CM, and both significantly reduced the proportion of γH2AX+ cells at 48 hours post-Taxol treatment compared to control media (Fig. 4.4C; P=0.0245 and 0.0234, respectively). The flow-through, containing all metabolites and small molecules below 3kDa in size, had no effect on γH2AX positivity and was not significantly different from control media-treated cells at any time point (Fig. 4.4C). Again, similar results were observed when we measured the sub-2C dead cell population, further supporting the connection between genotoxic stress and cell death (Fig. 4.4D).

Analysis of apoptotic signaling pathways revealed that BMDM-CM suppresses the early activation of the extrinsic caspase 8-dependent pathway, which peaks at 24 hours (Fig. 4.4E). This is particularly intriguing given recent work demonstrating the importance of the tumor cell-extrinsic caspase 8 dependent pathway in controlling response to cytotoxic chemotherapy[18]. Additionally we observed a reduction in activation of the cell intrinsic caspase 9-dependent apoptotic pathway, where there was greatest activation at 48 hours in the Taxol-alone condition (Fig. 4.4E). While it is possible that macrophage-CM suppresses activation of each pathway individually, the reduction in both

pathways is consistent with decreased genotoxic stress signaling upstream resulting in diminished apoptosis.

Given the alterations in downstream DNA damage and apoptotic signaling observed in BMDM CM-treated cells we sought to determine whether the activity of the drug was being directly impaired. Taxol binds to polymerized microtubules resulting in their stabilization and acetylation[19, 20]. Assessment of alpha-tubulin acetylation by western blot indicates no effect of BMDM CM on the ability of Taxol to induce microtubule stabilization (Fig. 4.4F). Collectively this data indicates that macrophage-CM is capable of suppressing Taxol activity downstream of its direct mechanism of action. Furthermore this is consistent with my observation that macrophages suppress genotoxic stress induced by multiple chemotherapeutic agents with distinct mechanisms of action (Fig. 4.3).

Stromal cells reduce the duration of mitotic arrest

Moving beyond microtubule stabilization, we next sought to determine precisely when macrophages are able to modulate the ability of Taxol to arrest cells in mitosis. We therefore adapted our co-culture assay for live-cell imaging to measure the duration of Taxol-induced mitotic arrest on a single cell level (Fig. 4.5A). Mitotic arrest was measured as the time between nuclear envelope breakdown and re-attachment of the cell to the plate. The duration of mitosis in untreated cells was ~25 minutes (data not shown), while in response to 50nM Taxol treatment the duration of mitosis increased to ~200 minutes (Fig. 4.5B).

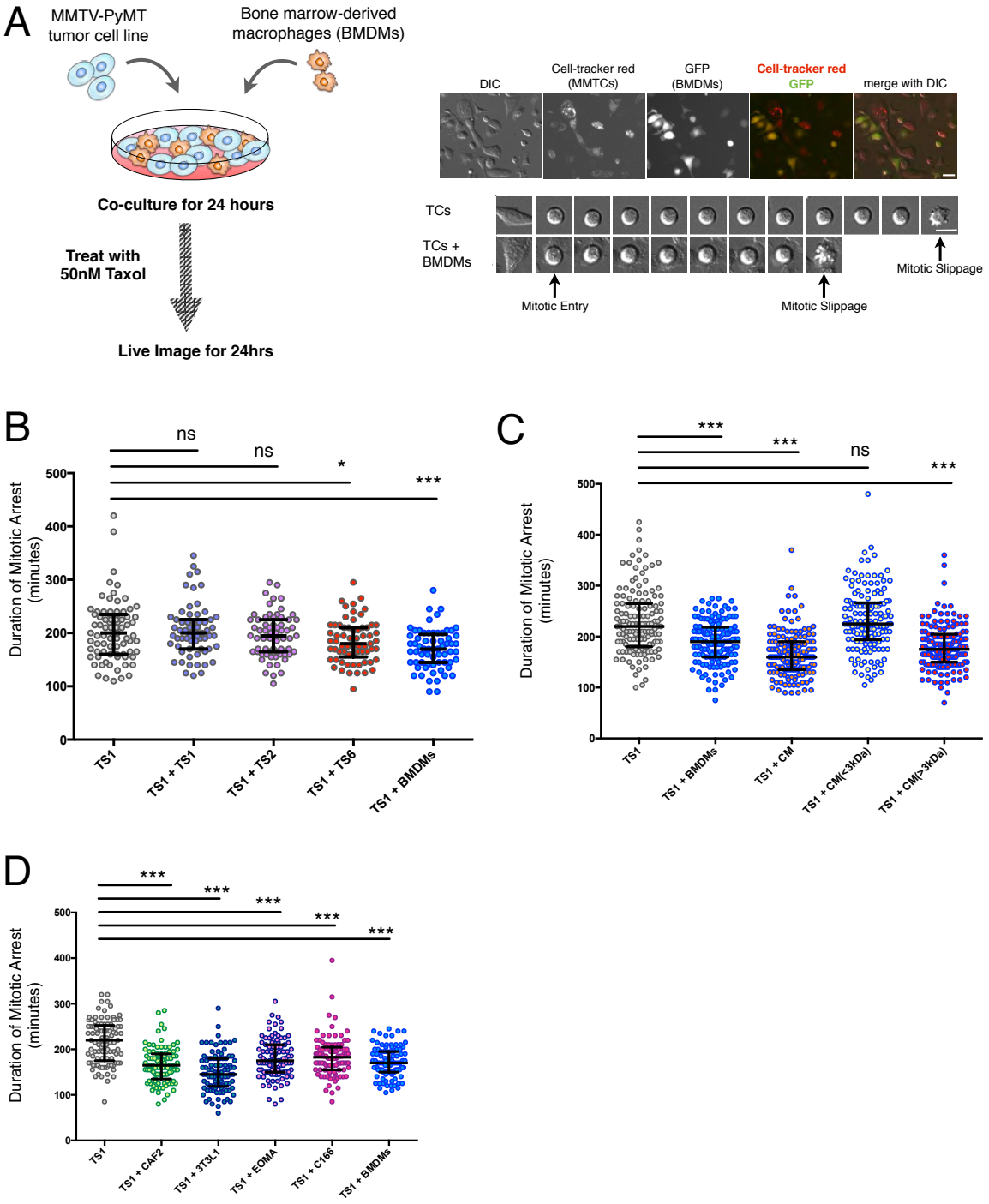

Figure 4.5: Macrophage-conditioned media reduces the duration of Taxol-induced mitotic arrest.

Figure 4.5: Macrophage-conditioned media reduces the duration of Taxol-induced mitotic arrest. (A) Experimental design for measuring tumor cell-mitotic arrest in co-culture. Tumor cells and admixed stromal cells are differentially fluorescently labeled to distinguish between the populations. The entry into mitosis is determined by the nuclear envelope breakdown, and mitotic exit is defined as the time of re-attachment. (B) Duration of Taxol-induced mitotic arrest of TS1 cells alone or in co-culture with other breast cancer cells or BMDMs. Neither of the epithelial PyMT cell lines, TS1 or TS2, had any effect on the duration of mitotic arrest. Co-culture with the mesenchymal: PyMT cell line TS6, or BMDMs, significantly reduced the duration of mitotic arrest. (C) Macrophage CM reduced Taxol-induced mitotic arrest to a similar extent as direct macrophage-co-culture. This ability was retained by the >3kDa CM fraction. (D) Co-culture of TS1 cells with other stromal cell lines similarly reduced the duration of mitotic arrest following Taxol treatment. For all graphs in B, C, and D, data are from three independent experiments. (ns) not significant; (*) $P < 0.05$; (***) $P < 0.001$ by the Students t-test . These data were generated in collaboration with Hyunjung Kim.

We first determined whether cell density on its own altered the duration of mitotic arrest by plating the TS1 cells with additional MMTV-PyMT cell lines. These included the epithelial lines, TS1 and TS2, as well as the mesenchymal PyMT cell line TS6 derived from a lung metastasis[17]. Addition of either epithelial cell line had no effect on the duration of mitotic arrest, however inclusion of the mesenchymal line TS6 resulted in a modest 9% reduction in the duration of the average mitotic arrest (Fig. 4.5B; P= 0.024). Inclusion of BMDMs had a more pronounced effect with a 17% reduction in mitotic arrest duration (Fig. 4.5B; P<0.001). These findings indicate that increased cell density alone is insufficient to modulate Taxol-induced mitotic arrest, but inclusion of macrophages, or to a lesser extent mesenchymal breast cancer cells, has the effect of reducing the duration of mitotic arrest. Furthermore, consistent with recent intra-vital imaging studies[5, 21] Taxol treatment results in mitotic slippage and failed division (Fig. 4.5A). This suggests that macrophages do not enhance the ability of the tumor cell to satisfy the spindle assembly checkpoint under conditions of increased microtubule stability, but rather decrease the ability of the tumor cell to maintain a prolonged mitotic arrest.

Given the link between duration of mitotic arrest and cell fate we next wanted to determine whether these results were consistent with our earlier findings of reduced genotoxic stress and cell death. Thus we set out to determine whether macrophage-CM, and more specifically the >3kDa fraction of the CM, was sufficient to mediate this reduction in mitotic arrest. Indeed treatment of tumor cells with BMDM-CM resulted in a 27% reduction in the duration of mitotic

arrest (Fig. 4.5C; P<0.001). Moreover the capacity to reduce mitotic arrest duration was present in the >3kDa fraction consistent with our previous findings (Fig. 4.5C; P < 0.001).

In order to determine whether these effects were generally relevant to infiltrating stromal cells we assessed the duration of mitotic arrest in co-culture with a panel of different stromal cell types relevant to the breast tumor microenvironment. These included cancer associated fibroblasts (CAF2), pre-adipocytes(3T3-L1), hemangioma endothelial cells (EOMA), and the yolk sac endothelial cells (C166), which were all were able to reduce the duration of Taxol-induced mitotic arrest in the TS1 cells (Fig. 4.5D; P< 0.001 for all comparisons). Recent findings have demonstrated that *in vivo* Taxol fails to induce prolonged mitotic arrest and results in extensive mitotic slippage of mitotic tumor cells[5, 21]. Our findings that stromal cells can reduce the duration of tumor cell mitotic arrest in a cell-extrinsic manner provide a potential explanation for this phenomenon.

Macrophage-conditioned media buffers tumor cells against chemotherapy-induced stress signaling

Finally, we sought to gain greater insight into how macrophage-secreted factors are capable of suppressing prolonged mitotic arrest and promoting cell survival and thus integrated the effect of BMDM-CM on tumor cell signaling pathways. We first examined phosphorylation of the key DNA damage recognition and signaling molecules ATR and ATM. Interestingly we did not observer marked

increases in activation of either pathway in response to Taxol treatment (Fig. 4.6A). However, macrophage-CM suppressed phosphorylation of ATR even before Taxol administration (Fig. 4.6A; P= 0.0196). This suggests that even prior to Taxol-induced mitotic arrest, conditioned media already impacts the level of genotoxic stress experienced by the tumor cell.

Subsequently examining canonical breast cancer cell signaling pathways we made some intriguing findings. In response to Taxol treatment we observed a progressive loss of FAK activation in accordance with loss of the total protein (Fig. 4.6B). When cells were treated with BMDM-CM, however, total FAK protein levels rebounded by 72 hours (Fig. 4.6B; P= 0.0012). Attempts to assess the role of FAK signaling on Taxol-induced cell death were unfortunately stymied by cross-reactivity of an available FAK inhibitor with the cell cycle deregulator CDK1 (data not shown).

We also found that the ER stress pathway was activated in response to Taxol treatment as indicated by the increased levels of phospho-PERK (Fig. 4.6B). This is not surprising given that tetraploidization has been previously reported to induce ER stress[22]. Consistent with our findings on the role of macrophage-secreted factors in buffering tumor cells against stress, treatment with BMDM-CM results in a resolution of the Taxol-induced ER stress by the 48 hour time point (Fig. 4.6B; P= 0.0018). To assess the role of PERK signaling in driving Taxol-induced cell death, a PERK inhibitor was used in our cell death assay but it had no impact cell fate (data not shown).

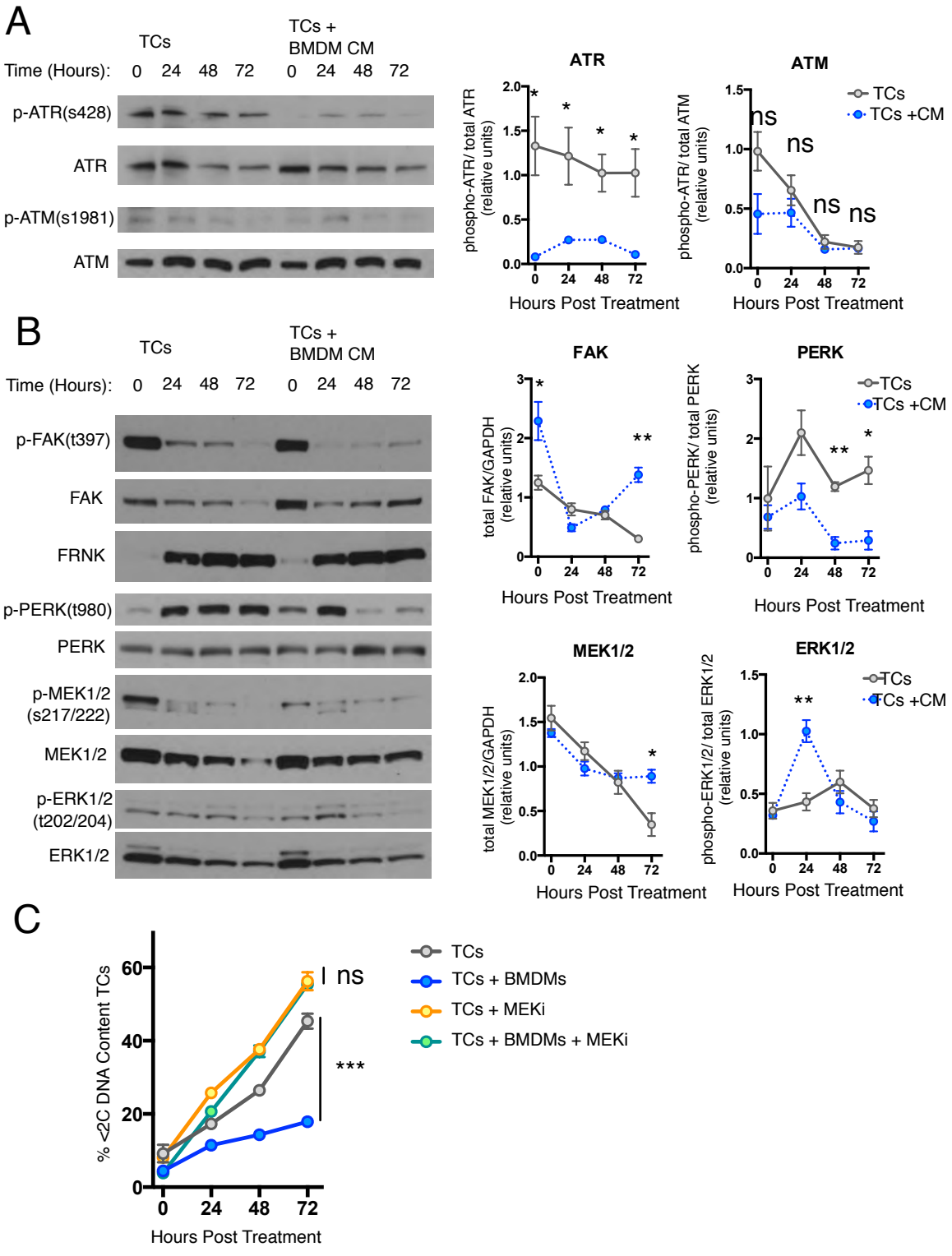

Figure 4.6: Macrophage-conditioned media buffers tumor cells against chemotherapy-induced stress signaling.

Figure 4.6: Macrophage-conditioned media buffers tumor cells against chemotherapy-induced stress signaling.(A) Activation of ATR and ATM, key DNA damage signaling nodes, was assessed in response to Taxol treatment. Conditioned media suppressed phosphorylation of ATR. (B) Signaling activity of cell adhesion, ER stress, and the MAPK pathway was assessed. BMDM-conditioned media suppressed Taxol-induced loss of FAK and MEK protein levels as well as the activation of PERK. For western blots a representative image is shown on the left, with quantification of three independent experiments graphed on the right. (C) Use of the MEK inhibitor PD0325901 (500nM) in the co-culture assay abrogated the protective effect of macrophages. For the graphs in A-C data are from three independent experiments. (ns) not significant; (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ by the Students t-test .

Turning our attention to the MAPK pathway we observed trends towards increased levels of MEK1 and MEK2 similar to what was observed for FAK. Taxol treatment resulted in a progressive reduction in the levels of MEK1 and 2, but treatment with macrophage-conditioned media prevented this loss of protein (Fig. 4.6B; P= 0.0214). Indeed examining downstream pathways, we observed increased phospho-ERK1/2 levels 24 hours after treatment in the CM-treated cells (Fig. 4.6B; P= 0.0073).

In order to assess the importance of the MAPK pathway in macrophage-mediated resistance to Taxol-induced cell death we included a MEK1/2 inhibitor in our cell death assay. Concurrent treatment with the MEK inhibitor and Taxol completely abrogated the protective effects of macrophage co-culture (Fig. 4.6B). Interestingly, this effect was specific to disruption of the MAPK pathway as the use of inhibitors targeting the canonical PI3K/AKT survival pathway had no impact on Taxol-induced cell death or macrophage-mediated chemoprotection (Data not shown). Collectively our data suggest that macrophage-secreted factors are able to suppress Taxol-induced mitotic arrest and tumor cell death and that this is dependent upon tumor cell MAPK activity.

Chapter 4 references

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Chapter 5

Discussion

Cancer by its very definition is a disease of disrupted tissue homeostasis. Neoplastic transformation, whereby strict control of cell number and tissue composition is lost, is suggestive of a process where concepts of equilibrium are no longer relevant. In fact our findings suggest the opposite is true, that many of the processes occurring during cancer development are reactive to disequilibrium of the physiological system. This is particularly true in the context of therapeutic treatment, where the insult to the tumor initiates many reactive processes which work to blunt tumor cell death and effect repair of the tumor microenvironment. Here we see engagement of the body's wound healing response to resolve the tissue damage and restore homeostasis. This process reveals a fundamental truth about cancer as a disease. While the cancer cells themselves display a loss in proliferation control and ability to equilibrate in an autonomous manner, the processes of physiological homeostasis remain intact in the host. The non-transformed cells within the tumor, the stroma and the immune infiltrate, respond to the environmental cues provided by the tumor cells and attempt to restore tissue homeostasis, each in a manner in accordance with their biological functions. These stromal responses can either enhance or hinder disease progression, but it is fundamentally a process by which non-transformed cells are responding to changes in the environment and altering their behavior in predictable and stereotyped manner. Thus in any consideration of a treatment

modality we must consider the reaction and response of the tumor cells as well as the reaction of the tumor microenvironment. Anti-cancer therapy will be unsuccessful where the microenvironment re-equilibrates to a pro-tumorigenic state. Successful treatment modalities, however, must alter the tumor microenvironment such that the new equilibrium is one that is antagonistic to cancer cell persistence and regrowth.

Infiltrating leukocytes in the response to chemotherapy

We have demonstrated that in response to chemotherapeutic treatment macrophage numbers increase within the tumor, thereby providing increased levels of pro-tumorigenic effector molecules, including active cathepsin proteases[1]. It is not surprising that in the context of significant tumor cell death we see an influx of macrophages given their canonical role as professional phagocytes. The body is responding to the presence of dead and dying cancer cells and these phagocytes are being recruited to remove them.

Other studies have reported increased secretion of myeloid cell-recruiting factors by tumor cells in response to therapeutic treatment, including CSF-1, CXCL1, CXCL2 and/ or CXCL12[2-4]. In our own studies we did not observe specific upregulation of any myeloid cell-recruiting cytokines or chemokines, suggesting a broader and more redundant mechanism. Indeed it was subsequently shown that injection of necrotic tumor cell debris alone is sufficient to recruit macrophages to the local environment[5].

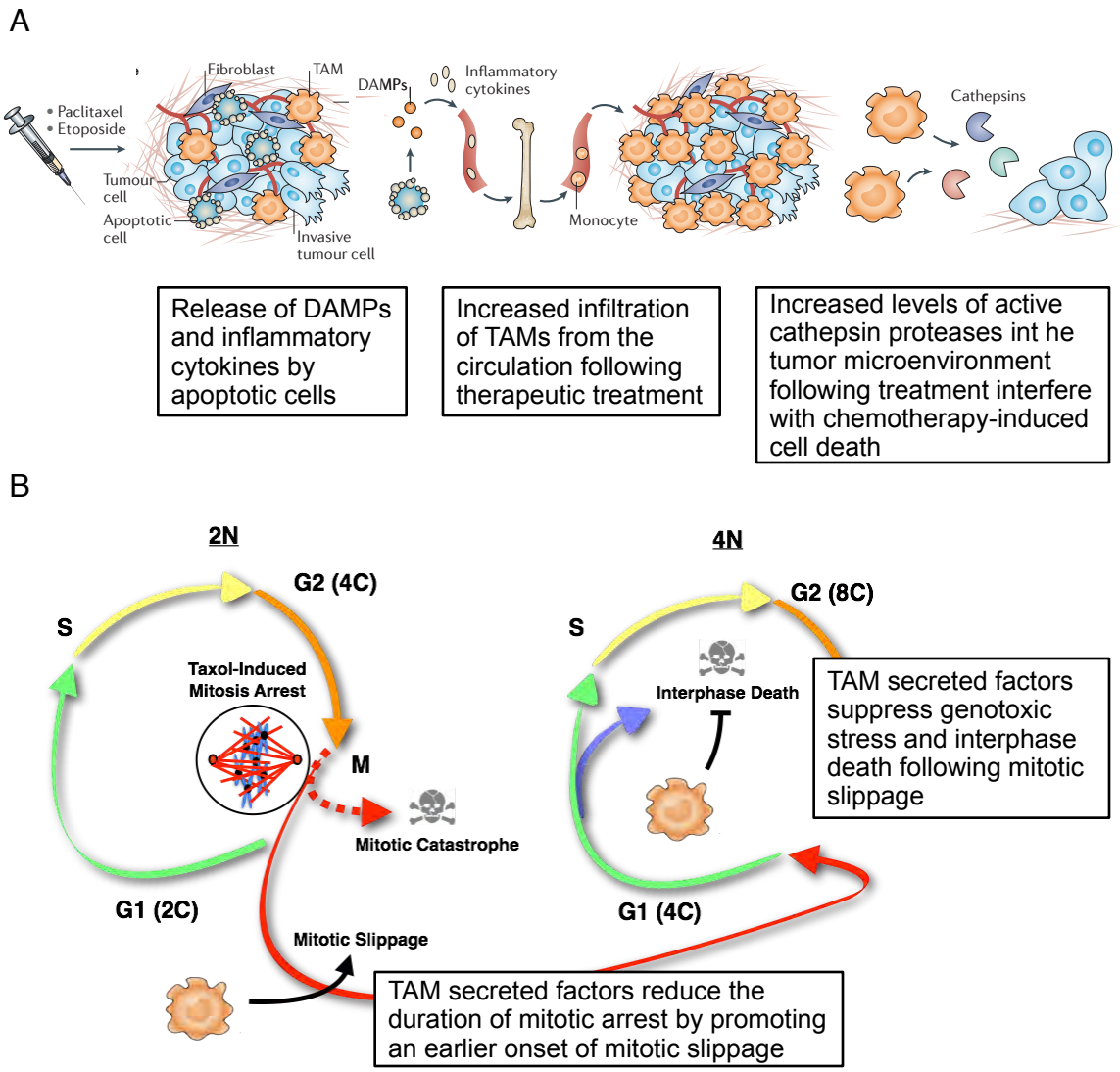

Figure 5.1 Tumor-associated macrophages (TAMs) promote tumor cell survival and suppress the activity of chemotherapeutic agents in breast cancer. (A) The adaptive upregulation of cathepsins occurs through the increased recruitment of cathepsin-high TAMs in response to chemotherapeutic agents such as paclitaxel or etoposide. These adaptive increases in intratumoral cathepsin activity levels blunt therapeutic efficacy, which can accordingly be improved by cathepsin inhibition. (B) Macrophage-secreted factors reduce the duration of mitotic arrest induced by antimitotic agents by promoting earlier mitotic slippage. Following mitotic slippage, tumor cells co-cultured with macrophages or treated with macrophage-conditioned media (CM) have reduced levels of γH2AX and p53 phosphorylation and undergo lower levels of interphase cell death. DAMPs, danger-associated molecular patterns. Part (A) adapted from Olson O.C. and Joyce J.A. (2015) Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. *Nature Reviews Cancer* 15, 712–729.

Thus it is not critical for the apoptotic tumor cells themselves to secrete “come find me” signals in order to recruit macrophages. Nonetheless, inhibitors targeting these specific cytokine/receptor pairs in combination with either chemo- or radiotherapy have shown efficacy in preclinical models[3-8]. It remains unclear, however, whether upregulation of these cytokines or chemokines is necessary for the efficacy of these combination treatment strategies. Indeed these inhibitors have been shown to suppress TAM accumulation independently of therapeutic treatment [9, 10]. Thus it is likely that these inhibitors are acting by generally suppressing myeloid cell recruitment and may not need to be tailored to the specific chemotherapy-associated recruitment signals.

We observed that the enrichment of TAMs following chemotherapeutic treatment was specific for this innate immune population, as we did not observe a similar increase in the bulk leukocyte population. Not only did we detect an increase in cathepsin activity-high TAMs in mouse models of breast cancer, but also in patient samples. Additional research studies independently confirmed these findings in neoadjuvant breast cancer patient samples and also revealed alterations to the leukocyte complexity of the tumor[2, 11]. Chemotherapeutic treatment resulted in an increase in the ratio of CD8+ cytotoxic T cells to CD4+ T helper cells[11]. CD8+ cytotoxic T cells have been shown to drive an anti-tumor immune response and correlate with improved patient prognosis[2]. However, TAMs have been shown to be capable of suppressing CD8+ cytotoxic T cell activation within the tumor[12]. Thus in response to chemotherapeutic treatment we observe an increase in immunosuppressive TAMs in conflict with a T cell shift

towards increased cytotoxic T cells. Indeed patients with high numbers of cytotoxic T cells and low levels of TAMs had the best survival[2]. Moreover it has recently been shown that in the post-treatment setting with increased TAMs and CD8+ T cells this immunosuppressive microenvironment is more sensitive to immunomodulatory checkpoint inhibitors[13]. Collectively the immune infiltrate reacts to chemotherapy-induced cell death in a complex manner with the capacity to redefine the equilibrium of the tumor following treatment. Macrophages play a critical part in defining the immunosuppressive environment and suppressing chemotherapy-induced adaptive immune activation.

Tumor-associated macrophages as regulators of tumor cell death

In addition to their role as phagocytes and important components of the patient's immune system, macrophages also have key roles in development and management of normal tissue homeostasis[14]. Our understanding of the macrophage as a key regulator of mammary epithelial cell homeostasis is informed by several studies into their role in mammary development, estrous cycle alterations, and post-lactation mammary gland involution[15-17]. In mice lacking CSF-1, a factor critical for macrophage development, the mammary gland is unable to develop fully during pregnancy and these mothers display a lactation defect[15]. During the estrous cycle the mammary gland goes through a process of expansion and retraction correlating with increased macrophage presence and proximity. Depletion of macrophages is sufficient to prevent both gland expansion as well as gland retraction[16]. Thus macrophages can be viewed as critical

positive and negative regulators of the mammary epithelium under normal cycling conditions. In post-lactation involution of the mammary gland, macrophage recruitment is critical for the effective initiation and execution of programmed cell death in mammary epithelial cells[17]. If macrophages are depleted, mammary epithelial cells remain viable even in the presence of static milk and STAT3 activation, the known drivers of involution[17]. Collectively the literature supports a view of macrophages as integral parts of the functioning mammary gland with regulatory roles in normal proliferation and apoptosis of mammary epithelial cells. Considered from an evolutionary and a multicellular organismal perspective there is logic to macrophages possessing this regulatory role. Given the dynamic nature of the mammary gland and its need to expand and involute according to the natural history of the organism, mammary epithelial cells are highly susceptible to neoplastic transformation. Endocrine regulation of the mammary gland represents a systemic level of control, while macrophages within the mammary gland represent an additional level of local regulation.

Given the potency of macrophages as effector cells and their ability to provide myriad factors to the tumor microenvironment, it becomes a difficult task to unravel the critical mechanisms of TAM-mediated tumor progression. Our studies have shed light into specific contributions of TAMs in the therapeutic response, namely their ability to secrete high levels of cathepsin proteases into the tumor microenvironment. While we observed no significant impact of cathepsin inhibitor monotherapy in breast cancer models we discovered that this activity becomes critical during the chemotherapeutic response. This suggests

that in breast cancer the functions of cathepsin proteases under normal proliferative conditions are non-limiting, but under conditions of stress they provide a critical signal for tumor cell survival and regrowth. While we do indeed see an increase in TAMs and cathepsin activity following chemotherapeutic treatment, we have not observed any apparent differences in the localization or secretion of these proteases. Nor have we observed any differences in which specific cathepsin family members are expressed in the tumor microenvironment. Thus our data suggests that cathepsins proteases are behaving similarly in the untreated and treated context, however that function is only critical in the latter situation.

Cathepsin proteases in the response to chemotherapy

The underlying mechanism of how cathepsin proteases provide survival signals to tumor cells during chemotherapeutic treatment remains an area of active investigation. Cathepsin proteases, mainly cathepsins B and S, are secreted by macrophages into the conditioned media and their activity is critical to macrophage-mediated chemoprotection. Given that cathepsin inhibition at the time of conditioned-media application to the tumor cells is sufficient to abrogate the protective effect of macrophages, the data indicates that the critical substrates are tumor cell-derived. It is possible these proteases act on extracellular substrates, either in the extracellular matrix or on the outer surface of the tumor cell membranes. Indeed cathepsin S is able to maintain its specific proteolytic activity even at neutral pH [18, 19]. Alternatively, cells can recover

secreted proform cathepsin through the cation-independent mannose-6-phosphate receptor, IGF2R [20]. Indeed it has been reported that CTSL-/- thymocytes can gain cathepsin L from the wild-type thymic stroma [21]. Thus it is not inconceivable that macrophage-derived cathepsins are trafficked to the lysosomes of tumor cells where they could alter tumor cell lysosomal function and autophagy.

While the increase in cathepsins proteases that we observed following chemotherapy was caused by increased numbers of TAMs, other examples of tumor cell-intrinsic upregulation of cathepsin proteases have also been reported. Indeed ionizing radiation has been shown to upregulate cathepsin S mRNA levels in breast cancer cells in an Irf1-dependent manner[22]. Increased protein levels of cathepsin S resulted in decreased response to subsequent radiation. This demonstrates a role for this protease in tumor cell-intrinsic acquired therapeutic resistance. This is particularly interesting given that cathepsin S is usually poorly expressed in epithelial tumor cells, however it has also recently been shown that it contributes to site-specific metastasis[23]. There is evidence for both tumor cell intrinsic and extrinsic upregulation of cathepsin S as a mechanism of acquired resistance and this must be taken into consideration when designing strategies to simultaneously treat the tumor and the reactive microenvironment. If macrophages are depleted, it is possible that tumor cells will compensate and acquire the increased cathepsin expression usually associated with TAMs. In this instance it seems preferable to target the cathepsins directly as our data indicates. Additional independent studies have also identified

colorectal cancer as well as pancreatic ductal adenocarcinoma as malignancies where cathepsin inhibition improves response to therapy [24, 25]. In accordance with our own findings, in both instances cathepsin inhibition as a monotherapy had no efficacy[24, 25].

In addition to tumor cell-intrinsic cathepsin expression and TAM-secreted cathepsin proteases, cathepsin activity within myeloid cells has also been shown to be an important driver of therapeutic resistance. Myeloid-derived suppressor cells are sensitive to treatment with 5-fluorouracil and gemcitabine and undergo limited lysosomal membrane permeabilization, releasing some lysosomal cathepsins into the cytosol. This relocalization of intracellular cathepsin activity results in the activation of the NLRP3 inflammasome in a cathepsin B-dependent manner[26]. The subsequent IL-1β production drives an IL-17 mediated tumor regrowth. Cathepsin B inhibition suppresses this reactive IL-1β driven inflammation by myeloid-derived suppressor cells [26]. Collectively when we consider our own data as well as other studies we can conclude that chemotherapy is often associated with altered expression and localization of cathepsin proteases within the tumor microenvironment and this consistently contributes to acquired chemoresistance.

From a translational perspective, the integration of cathepsin-targeting strategies into established chemotherapeutic regimens appears to hold substantial promise. The dual nature of cathepsins as cell death effector molecules and survival factors [27], however, indicates the caution with which this strategy should be approached. While there is considerable potential for

decreasing the variability of response and suppressing adaptive resistance by inhibiting cathepsins, it is important to determine the consequences of such an approach. It will be critical to fully characterize which chemotherapeutic agents are sensitive to modulation by cathepsins *versus* those that depend on cathepsins to mediate their cytotoxic functions. Furthermore as the role of the innate and adaptive immune system in therapeutic response becomes increasingly appreciated we must consider how cathepsin inhibition might affect this process. Do cathepsins contribute to a more immunogenic or an immunologically silent cancer cell death? Will cathepsin inhibitors alter antigen presentation in a manner that suppresses the immunological response? These are questions for which answers must be found in order to effectively target these proteases for improved chemotherapeutic benefit. An alternative strategy has also been proposed by which cathepsin activity and the lysosome can be exploited as a weakness for the tumor [28]. Lysosomal destabilizing agents have the potential to form a new class of anticancer agents with good selectivity over normal tissue. Similarly, pro-drugs have been designed to exploit the high cathepsin proteolytic activity within the tumor and restrict drug activation to these regions [29 30].

Fundamentally, the dysregulation of cathepsins appears to be a critical component of neoplastic transformation and tumorigenesis. It is the inherent nature of proteases to degrade or irreversibly alter their target substrates. In this manner cathepsins exhibit both of these properties by recycling proteins for cancer cell survival and proliferation, as well as irreversibly modifying the tumor

microenvironment leading to growth, invasion, and disease progression. Cathepsins also act as a "biological ratchet", progressively driving the microenvironment to malignancy. Our understanding of the specific roles of individual cathepsins in different cancer types, microenvironments, and stages in malignant progression has expanded substantially in the last decade. In the next decade we hope to achieve an integrated understanding of cathepsin activity in the tumor microenvironment and determine the specific instances where the reliance of cancers upon cathepsins represents a fatal weakness that can be therapeutically exploited.

The role of the tumor microenvironment in the response to antimitotic agents

Intravital microscopy has been a major technological advance that has begun to provide significant insight into the complexities of the tumor microenvironment. This technique helped elucidate vascular features that changed with tumor development and modulated sensitivity to doxorubicin in a tumor cell-extrinsic manner [5]. More recently this technique has been used to visualize the response to anti-mitotic agents *in vivo*, revealing striking differences in the mechanism of action from that observed in vitro [31]. In vitro treatment of human cancer cell lines with Taxol resulted in prolonged mitotic arrest resulting in mitotic catastrophe, whereby the cells initiated programmed cell death. However when these same cancer cell lines where implanted *in vivo*, where they established tumors, Taxol treatment resulted in substantially shorter mitotic arrests which resulted in mitotic

slippage rather than mitotic catastrophe[31]. While in vitro cancer cell death occurred generally within the first 24 hours, concurrent with the prolonged mitotic arrest, *in vivo* cell death occurred at later time points following mitotic slippage. Similar *in vivo* imaging studies have demonstrated a critical role for the tumor microenvironment in regulating drug delivery to the cancer cells [32 5]. In response to antimitotic agents, however, a clear dose-dependent response was observed with increasing concentrations of Taxol leading to chromosome mis-segregation, multipolar spindles, and ultimately prolonged mitotic arrest and slippage [31]. Thus stromal limitations on drug delivery are unlikely the cause of the differences between *in vivo* and *in vitro* experiments*,* as the differences observed occur at saturating pharmacodynamics [31]. Given that the concentration of Taxol is significantly enriched in tumor cells [33], it is also possible that the tumor microenvironment does not limit drug delivery but instead promotes drug efflux. The hypothesis that drug efflux pumps such as MDR1 mediate clinical resistance to taxanes has been extensively studied, however, without producing compelling supporting evidence [34].

Interestingly these intravital studies are consistent with older clinical studies. Seeking to understand the response to Taxol in breast cancer patients, clinicians had taken serial fine-needle aspirations from patient tumors undergoing treatment[35]. This study observed that the apoptotic index was always higher than the mitotic index, which would be unexpected if apoptosis was a direct outcome of Taxol-induced mitotic arrest[35]. Furthermore they also observed that in non-responder patients apoptosis peaked at 24 hours-post treatment, while in

responder patients apoptosis continued through the 96 hours of observation[35]. Believing that Taxol killed tumor cells by mitotic catastrophe at the time it was hypothesized that *in vivo* some “field effect” propagated tumor cell death from those cells that underwent a Taxol-induced mitotic arrest to those cells that never underwent mitotic arrest. Assessing this data in light of the recent intravital imaging studies, however, supports a model where Taxol only induces a transient mitotic arrest *in vivo* followed by mitotic slippage with apoptosis occurring in the subsequent interphase. This model explains why mitotic index is lower than apoptotic index and how apoptosis can persist at time points where the mitotic index has returned to basal levels. Ultimately these studies demonstrate that Taxol appears to have a different mechanism of action, one based on shorter mitotic arrests and mitotic slippage, and it is possible that the tumor microenvironment is responsible for this difference.

Investigating the effect of macrophages on Taxol-induced mitotic arrest, we observed that macrophages are capable of significantly reducing the duration of cancer cell mitotic arrest. This represents the first example of mitotic arrest being regulated in a tumor cell-extrinsic manner. Similar effects were observed using STLC, an inhibitor of the mitotic kinesin EG5, which induces mitotic arrest independent of perturbations of microtubules. Thus macrophages are able to reduce the duration of tumor cell mitotic arrest independent of the mechanism by which arrest is induced. Given that we observe that mitotic arrest results in mitotic slippage regardless of the presence of macrophages, this suggests that the breast cancer cells are exiting mitosis in the presence of an active spindle

assembly checkpoint through the degradation of cyclin B1[36]. This basal degradation of cyclin B1 has been shown to occur through targeted proteasomal degradation, presumably through incomplete inhibition of the anaphase-promoting complex (APC) [36]. Thus macrophage co-culture may enhance the rate of cyclin B1 degradation through one of several possible mechanisms. Macrophage co-culture could alter the ratio of components of the mitotic checkpoint complex (MCC) and those of the APC, such that residual activity of the APC increases the basal rate of cyclin B1 degradation. Indeed it has been demonstrated that inhibition of the APC by the spindle assembly checkpoint (SAC) is not a binary cell state identity, but rather a dynamic process [37]. Alternatively, there are several factors that cause MCC turnover and drive mitotic exit. These proteins include p31^{Comet} and TRIP13, which are believed to function in concert to inactivate the MCC component MAD2, and their overexpression has been shown to drive early mitotic exit [38-40]. Increased expression of these MCC inhibitors as a consequence of macrophage co-culture could similarly cause an earlier mitotic exit. Either mechanism results in a shorter mitotic arrest, which has been shown to correlate with a decreased degree of multinucleation and DNA damage signaling in the subsequent interphase[41]. Indeed it has been shown that an active spindle assembly checkpoint is required for Taxol-induced apoptosis and it is believed that the duration of this signaling output controls cell fate [42]. Our own studies are consistent with these observations as inhibition of the spindle assembly checkpoint using aurora kinase inhibitors impairs the ability of Taxol to induce phosphorylation of the tumor suppressor p53 or the histone H2AX.

Consistent with the reduction in mitotic arrest duration observed with macrophage co-culture we also observe a decrease in these same markers of genotoxic stress in tumor cells. Furthermore, macrophages increase the survival of these multinucleated and newly tetraploid cells.

The mechanisms by which macrophages modulate tumor cell mitotic arrest and subsequent cell fate are not yet fully defined although we have been able to gain some insight into this process. Cathepsin protease inhibition does not affect the ability of macrophages to reduce the duration of tumor cell mitotic arrest or the subsequent genotoxic stress signaling. This suggests that macrophage-derived cathepsin proteases are operating by an independent pro-survival mechanism, without affecting the manner in which Taxol induces cell damage and stress. The mitotic arrest modulatory activity, however, is similarly secreted by macrophages and their conditioned-media can replace direct co-culture. Moreover, use of molecular weight cut-off concentration spin columns has demonstrated that the activity is >3 kDa is size and likely a secreted protein. Studies are ongoing to further characterize and identify the secreted factor(s).

Treatment of tumor cells with macrophage-conditioned media also had a significant effect on tumor cell signaling during the response to Taxol. Interestingly we observed an upregulation of the dominant-negative endogenous inhibitor of FAK, FAK related non-kinase (FRNK), following Taxol treatment with a concordant loss of total and phosphorylated FAK. While the tumor cells remain adherent this suggests that the Taxol-treated state shares some signaling similarities with anoikis. In tumor cells treated with macrophage-conditioned

media we observe some recovery of FAK following the upregulation of FRNK. Additionally we find that macrophage-conditioned media causes a slight increase in ERK1/2 phosphorylation at 24 hours after treatment. Addition of a MEK1/2 inhibitor to the Taxol treatment enhanced tumor cell death and abrogated the ability of macrophages to modulate the response to Taxol. However it remains unclear whether the ability of macrophages to modulate the response to Taxol involves the MAPK pathway directly, or whether MAPK inhibition simply makes the tumor cells insensitive to the cell-extrinsic influence. Furthermore, it is possible that the MEK1/2 inhibitor alters the macrophage secretome and further experiments need to be performed using macrophage-conditioned media.

Intriguingly, other stromal cell types of the breast cancer microenvironment including cancer associated fibroblasts, pre-adipocytes, and endothelial cells were similarly able to reduce the duration of Taxol-induced mitotic arrest in tumor cells in co-culture experiments. However, in contrast to macrophages, conditioned media from these cell types was insufficient to replicate the reductions in tumor cell genotoxic stress and cell death. It is possible that other stromal cell types modulate the duration of mitotic arrest through mechanisms involving cell-cell contact rather than through secreted factors. Cell contact inhibition has been shown to suppress critical signaling pathways, such as EGFR, in an NF2/merlin dependent manner [43]. Thus infiltrating stromal cells that are not competent to form adhesion junctions with epithelial tumor cells will induce tumor cell signaling through the disruption of contact inhibition. While our data indicate that macrophages suppress the

duration of mitotic arrest through a secreted ligand, it is possible that other stromal cells achieve the same result simply through disruption of the epithelial organization and signaling derepression. Alternatively it is also possible that macrophages secrete these factors at baseline while other stromal cells require education by the tumor cells. Other epithelial breast cancer cells had no effect on mitotic arrest, while breast cancer cells that had undergone EMT and displayed a mesenchymal morphology displayed some capacity to reduce mitotic arrest. This is especially intriguing as EMT has been shown to be important for chemoresistance in a cell-intrinsic manner[44], but my findings indicate the possibility that this chemoresistance phenotype can also be regulated by the local microenvironment. Collectively these findings support the hypothesis that tumor cell mitotic arrest *in vivo* is strongly modulated by the presence of stromal cells and this should be incorporated into any investigations into the mechanisms of antimitotic agents.

Conclusions and perspectives

The tumor microenvironment adds significant complexity onto the rudimentary perspective of cancer as a clonal genetic disease. This is no less true when we consider the therapeutic strategies by which we attempt to target and treat cancer. The role of the stroma in treatment response is generally one of reaction, of buffering against stress in the microenvironment and reestablishing conditions promoting growth and disease progression. Indeed optimized Taxol treatment schedules, which prioritize time to progression over objective

response, indicate the increased efficacy of initial high doses to suppress growth followed by tapering to lower maintenance doses to control disease[45]. Thus by managing the reaction in a stage-dependent manner, acquired resistance is suppressed and lower doses can be used. Intriguingly one of the main effects of this approach is the reduction in intratumoral necrosis, thus likely decreasing macrophage recruitment and TAM-mediated disease promotion.

The use of chemotherapy to prime the tumor and sensitize to immunotherapeutic treatment[13] represents another variation on this approach, where the reaction to the chemotherapeutic treatment is not blunted but instead its momentum is redirected to establish an anti-tumorigenic state. Identifying the proper microenvironmental targets to add to chemotherapeutic regimens will require extensive preclinical testing as strategies like macrophage depletion have been successful in certain disease types, while in others compensation has been observed by other infiltrating immune cells[46, 47]. In such instances targeting effector molecules, such as cathepsin proteases, may prove more advantageous.

However, a certain amount of caution must also be kept in mind given some high-profile failures in microenvironmental targeting including broad-spectrum MMP inhibitors for breast cancer and smoothened inhibitors for pancreatic cancer. Both examples resulted from instances where the preclinical models used were insufficient to capture the complex and unintended consequences of modulating these microenvironmental forces. However our studies and others emphasize the critical importance of considering the microenvironmental contribution to therapeutic response and how it can effect

the action of the drug at every level. We are therefore optimistic for the future insights gained in this field given that we have finally developed the technological advances and the intellectual framework to fully understand the underlying mechanisms driving patient responses to anticancer therapy.

Chapter 5 references

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