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Cellular Uptake of Neutrophil Elastase Links Inflammation to Adaptive Immunity

Elizabeth A. Mittendorf

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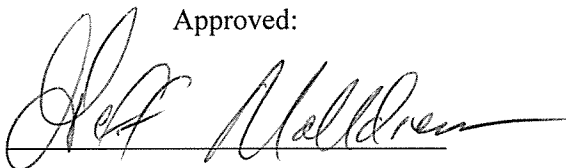
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CELLULAR UPTAKE OF NEUTROPHIL ELASTASE LINKS

INFLAMMATION TO ADAPTIVE IMMUNITY

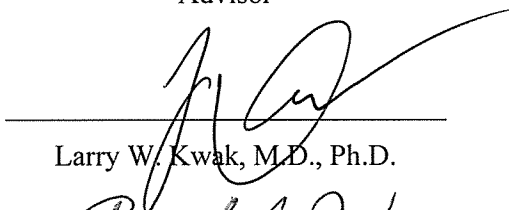
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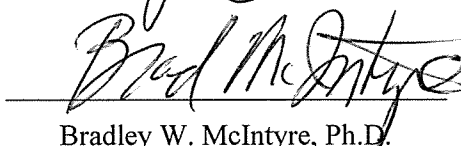


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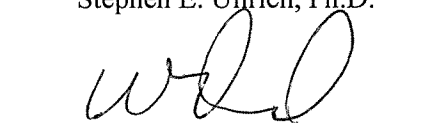
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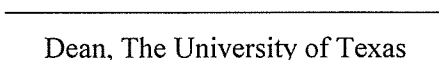


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CELLULAR UPTAKE OF NEUTROPHIL ELASTASE LINKS

INFLAMMATION TO ADAPTIVE IMMUNITY

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DOCTOR OF PHILOSOPHY

By

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ABSTRACT

Many tumors arise from sites of inflammation providing evidence that innate immunity is a critical component in the development and progression of cancer. Neutrophils are primary mediators of the innate immune response. Upon activation, an important function of neutrophils is release of an assortment of proteins from their granules including the serine protease neutrophil elastase (NE). The effect of NE on cancer has been attributed primarily to its ability to degrade the extracellular matrix thereby promoting invasion and metastasis. Recently, it was shown that NE could be taken up by lung cancer cells leading to degradation of insulin receptor substrate-1 thereby promoting hyperactivity of the phosphatidylinositol-3 kinase (PI3K) pathway and tumor cell proliferation. To our knowledge, nobody has investigated uptake of NE by other tumor types. In addition, NE has broad substrate specificity suggesting that uptake of NE by tumor cells could impact processes regulating tumorigenesis other than activation of the PI3K pathway.

Neutrophil elastase has been identified in breast cancer specimens where high levels of NE have prognostic significance. These studies have assessed NE levels in whole tumor lysates. ***Because the major source of NE is from activated neutrophils, we hypothesized that breast cancer cells do not have endogenous NE but may take up NE released by tumor associated neutrophils in the tumor microenvironment and that this could provide a link between the innate immune response to tumors and specific adaptive immune responses.*** In this thesis, we show that breast cancer cells lack endogenous NE expression and that they are able to take up NE resulting in increased generation of low molecular weight cyclin E (CCNE) and enhanced susceptibility to lysis by CCNE-specific cytotoxic T lymphocytes. We also show that after taking up NE and proteinase 3 (PR3), a second primary granule protease with significant homology to NE, breast cancer cells cross-present the NE- and PR3-derived peptide PR1 rendering them susceptible to PR1-targeted therapies. Taken together, our data support a role for NE uptake in modulating adaptive immune responses against breast cancer.

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CHAPTER 1: INTRODUCTION

IMMUNITY AND INFLAMMATION IN CANCER

Immunosurveillance

The immune system is complex and its role in resisting or eradicating formation and progression of tumors is unresolved. In the early 1900s, Paul Ehrlich proposed that the immune system has a role in protecting the host from cancer.¹ Subsequent to that, in 1957, Burnet stated:

It is by no means inconceivable that small accumulations of tumor cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumor and no clinical hint of its existence.²

Around the same time, Thomas suggested that protection from neoplastic disease was the primary function of cellular immunity.³ Burnet and Thomas both speculated that lymphocytes could recognize and eliminate transformed cells and these observations led to the development of the immunosurveillance hypothesis. This hypothesis proposed that the immune system can recognize tumor-specific antigens that are found on a developing tumor and can interfere with its progression by eliminating nascent tumor cells.⁴

Initial attempts to prove the validity of the immunosurveillance hypothesis experimentally were difficult due to the complexity of required models. However, by the mid-1990s, improved mouse models allowed researchers to provide evidence that the immune system was involved in controlling tumor development. There were two key findings. The first was demonstration that endogenous interferon γ (IFN- γ) could protect against tumor formation and growth. In a tumor transplant model, Dighe et al. found that chemically-induced Meth A fibrosarcomas grew faster and more efficiently in syngeneic BALB/c mice treated with a neutralizing monoclonal antibody specific for murine IFN- γ .⁵ In addition, by stably overexpressing a dominant-negative mutant of the IFN- γ receptor alpha chain in Meth A sarcomas, they were able to ablate tumor sensitivity to IFN- γ . When these tumors were transplanted into naïve syngeneic hosts, they had enhanced tumorigenicity and reduced

immunogenicity. From these data, they concluded that IFN- γ has a role in tumor cell recognition and elimination. The second key finding involved perforin, a component of the cytolytic granules of cytotoxic T cells (CTL) and natural killer (NK) cells that mediates target cell killing. Using C57BL/6 mice deficient in perforin, van den Broek et al. showed that tumors induced by multiple methodologies, including injection of syngeneic tumor cell lines as well as viral and chemical carcinogenesis, were eliminated better by wild-type mice than the perforin^{-/-} mice.⁶ These results were corroborated by other investigators suggesting an important role for perforin-dependent cytotoxicity by CTL and NK cells in immune control of tumor development.⁷ A more definitive study supporting immunosurveillance and the importance of IFN- γ and lymphocytes used RAG-2^{-/-} mice that lack NK, T and B cells.⁸ After injection of methycholanthrene (MCA), 129 /SvEv RAG-2^{-/-} mice developed sarcomas with greater frequency and more rapidly than wild-type controls. In addition, RAG-2^{-/-} mice were more likely than wild-type controls to spontaneously develop epithelial tumors. When MCA-induced sarcomas isolated from wild-type or RAG-2^{-/-} mice were transplanted into RAG-2^{-/-} recipient mice, their growth rates were similar suggesting that tumors arising in the presence or absence of an intact immune system were not inherently different. In transplantation experiments, 17 of 17 sarcomas from wild-type mice became established and grew in naïve immunocompetent mice while 8 of 20 sarcomas generated in RAG-2^{-/-} mice were rejected when transplanted into immunocompetent hosts. These experiments suggested a role for the immune system in suppressing tumor growth. The data also suggested a process of immunoselection whereby tumors formed in the absence of an intact immune system are more immunogenic than those formed in immunocompetent hosts.⁸ The broader implication of these findings is that immune selection pressure may favor the development of less immunogenic tumors. This in part explains how tumors may develop in an immunocompetent host. Based on these findings, the group led by Old and Schreiber proposed the concept of cancer immunoediting; a refinement of the immunosurveillance hypothesis.⁹

Cancer Immunoediting

Cancer immunoediting takes a broader view of the interaction between the immune system and the developing tumor by acknowledging both host-protecting and tumor-sculpting activities. It is comprised of three phases: elimination, equilibrium and escape.⁹ The elimination phase can be seen as a more contemporary view of the original immunosurveillance hypothesis in which the innate and adaptive immune systems both play a role in the eradication of developing tumors. The equilibrium phase is when transformed cells are held in check by the immune system. It is during this phase that the selection of less immunogenic variants of the tumor occurs. Experimental evidence of an equilibrium state was published by Koebel et al. using a chemical carcinogenesis mouse model in which wild-type C57BL/6 or 129/SvEv mice were injected with a low dose of MCA.¹⁰ Mice with small stable tumors that were identified at the injection site were selected; mice that had tumors that continued to progress were removed from the study. At day 200 of the experiment, one cohort of mice was treated with control immunoglobulin and none developed additional tumors. Mice in the second cohort received a mixture of antibodies depleting CD4⁺ and CD8⁺ T cells and neutralizing IFN- γ . Progressively growing tumors developed in 60% of these mice. Experiments were repeated in RAG-2^{-/-} mice and very few developed late-forming tumors arguing against de novo transformation of tumors and supporting the importance of adaptive immunity in the equilibrium phase. These investigators also noticed that a small percentage of MCA-challenged wild-type mice untreated or treated with control antibody developed late appearing sarcomas. They therefore questioned whether sarcoma cells that did or did not escape equilibrium could be distinguished on the basis of immunogenicity. Tumor cells that had been maintained in equilibrium formed progressively growing tumors in RAG-2^{-/-} mice but were rejected in wild-type mice suggesting that they were highly immunogenic. In contrast, cells from late-forming sarcomas which grew spontaneously from mice in equilibrium formed tumors when transplanted into either RAG-2^{-/-} or wild-type mice. From this they concluded that tumor cells held in equilibrium have an unedited phenotype whereas those that spontaneously escape equilibrium are edited. By showing that tumor cells in equilibrium are highly immunogenic (unedited) whereas those that exit equilibrium

spontaneously are less immunogenic (edited), they defined a process (equilibrium) between elimination and escape.¹⁰

The escape phase of immunoediting occurs when tumor variants that have acquired insensitivity to immunologic detection or elimination begin to expand in an uncontrolled fashion.⁹ Changes allowing for escape can occur at the level of the tumor cell or the tumor microenvironment. One change that occurs at the level of the tumor cell is the down-regulation or loss of major histocompatibility complex (MHC) class I protein which renders the cells invisible to the immune system (reviewed by Browning and Bodmer).¹¹ Changes in the tumor microenvironment are more complex. As will be discussed in detail below, factors produced by immune cells present in the tumor microenvironment can be immunosuppressive. In addition, other immune cells including regulatory T cells and myeloid-derived suppressor cells are recruited to the tumor microenvironment where they suppress effective anti-tumor immune responses (reviewed by Mittendorf and Sharma).¹²

Although there is much to be learned, it is clear that the immune system plays a critical role in the development and progression of tumors. This was acknowledged in 2011 when Hanahan and Weinberg included “evading immune destruction” as a new hallmark of cancer and identified inflammation as an enabling characteristic for the acquisition of this and other hallmarks.¹³

Evading Immune Destruction – A New Hallmark of Cancer

In 2000, Hanahan and Weinberg proposed six hallmarks of cancer to provide a foundation for understanding cancer biology.¹⁴ These hallmark capabilities include: sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death. In the subsequent years, significant progress has been made in understanding the mechanisms by which each hallmark contributes to the neoplastic process. In addition, new observations have been made leading to an update of the hallmarks in 2011.¹³ In this update, Hanahan and Weinberg describe two enabling characteristics that

underlie the hallmarks of cancer including genomic instability and inflammation. They also identify two emerging hallmarks – reprogramming of energy metabolism and evading immune destruction.

Tumor-Associated Inflammatory Response

In the 19th century, Rudolf Virchow was the first to postulate a link between inflammation and cancer when he described infiltration of leukocytes into tumors. Pathologists have since identified that many tumors are infiltrated by inflammatory cells. This inflammatory infiltrate resembles that found in non-neoplastic tissues leading to the suggestion that tumors are “wounds that never heal”.¹⁵ Improvements of markers for immune cells has allowed for more accurate identification of the inflammatory cells present in the tumor microenvironment, and it is now appreciated that virtually all solid tumors contain immune cells at various densities ranging from subtle infiltration requiring cell type-specific antibodies for identification, to gross inflammation apparent by standard staining techniques.¹⁶ In the last decade, studies have shown that this tumor-associated inflammatory response enhances tumor formation and progression by helping neoplasias acquire the hallmark capabilities. There are multiple mechanisms by which this occurs (reviewed by Hanahan and Coussens).¹⁷ As an example of this, tumor associated macrophages (TAM) have been shown to influence tumor angiogenesis. Using a transgenic mouse mammary tumor virus model, Lin et al. showed an increase in the number of TAM in premalignant lesions just prior to the angiogenic switch that occurs before malignant transformation. When they depleted TAM, there was a reduction in vascular density resulting in delayed tumor progression and metastasis. Reintroduction of the TAM resulted in an increase in vascular density and enhanced tumor progression.¹⁸ These findings have been corroborated in clinical studies showing a correlation between a high number of TAM and increased vascular density which suggests that TAM may promote angiogenesis.¹⁹⁻²¹ Other bone marrow-derived myeloid cells including neutrophils, dendritic cells (DCs), and mast cells have been shown to play a role in the formation and maintenance of blood vessels in tumors through the production of soluble mediators regulating angiogenesis including vascular endothelial growth factor (VEGF), transforming growth factor- β (TGF- β), tumor necrosis factor- α (TNF- α) and platelet-derived growth factor (PDGF); chemokines such as

CXCL12 and IL-8/CXCL8; and other factor including matrix metalloproteinases, serine proteases, histamine, reactive oxygen species and nitric oxide (reviewed by Murdoch et al.).^{21, 22} The role of the inflammatory component supporting angiogenesis is just one example; there are data supporting the role of inflammatory cells in the acquisition of other hallmark capabilities as well.¹⁷

Historically it was thought that inflammatory responses reflected the immune system's attempt to eradicate tumors. The fact that the inflammatory response may enhance tumorigenesis and progression by helping neoplasias acquire hallmark capabilities represents a paradox. Emerging data actually suggests that many inflammatory cells have a dual function during tumor development displaying both tumor-promoting and tumor-suppressive capabilities. This represents an active area of investigation as improved understanding of the pro- and anti-tumor activities of inflammatory cells will be critical in the development of effective immunotherapy.

NEUTROPHILS

Of all cell types present within the tumor microenvironment, neutrophils have received the least attention.²³ This may be due to the fact that neutrophils have been viewed simply as short-lived effectors of innate immunity with a primary role of clearing extracellular pathogens as part of an acute inflammatory response. More recent data has identified a broader range of functions for neutrophils which suggest that these cells are important in the activation, regulation, and effector functions of both innate and adaptive immune responses and that they have a role in the pathogenesis of several disease processes including cancer.²⁴

Neutrophils – More Than Just Phagocytes

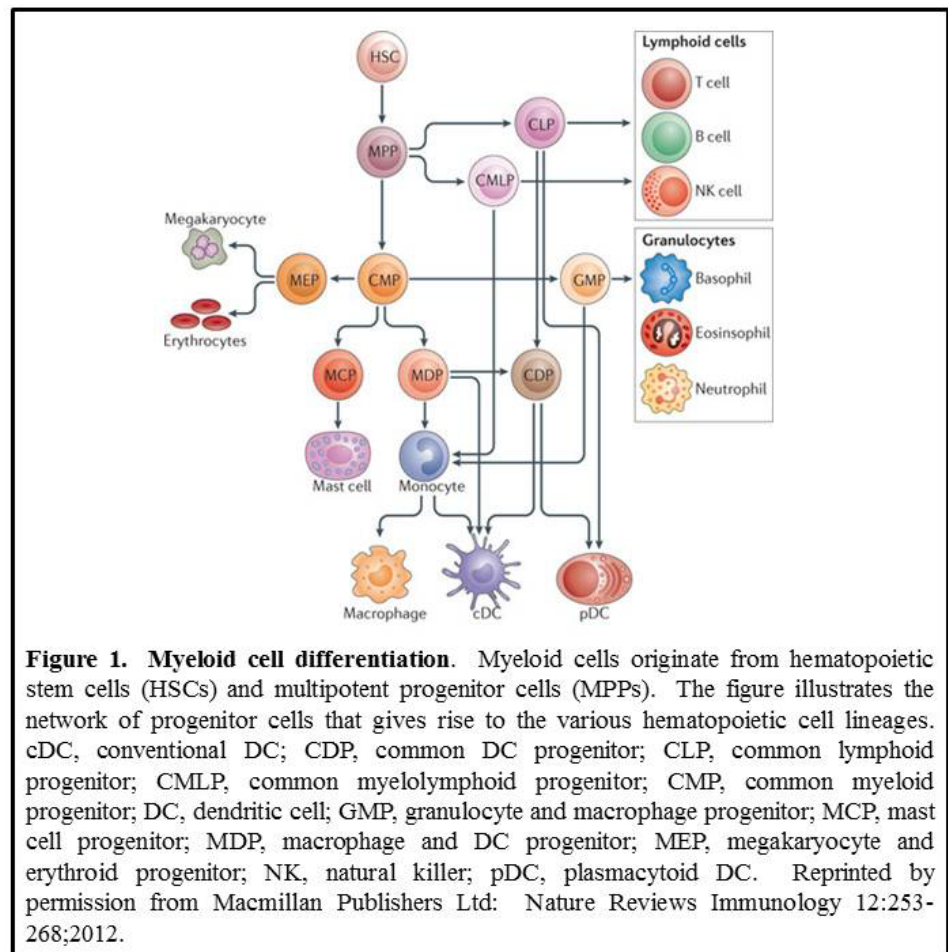
Myeloid cells, which arise from multipotent hematopoietic stem cells that mature through sequential differentiation steps, are the most abundant hematopoietic cells in the human body. There are three groups of terminally differentiated myeloid cells – macrophages, DCs and granulocytes (Fig. 1). Neutrophils represent a type of granulocyte; a cell type characterized by cytoplasmic granules and

specific nuclear morphology.

Neutrophils are the predominant population of circulating leukocytes accounting for 50-75%.²⁵

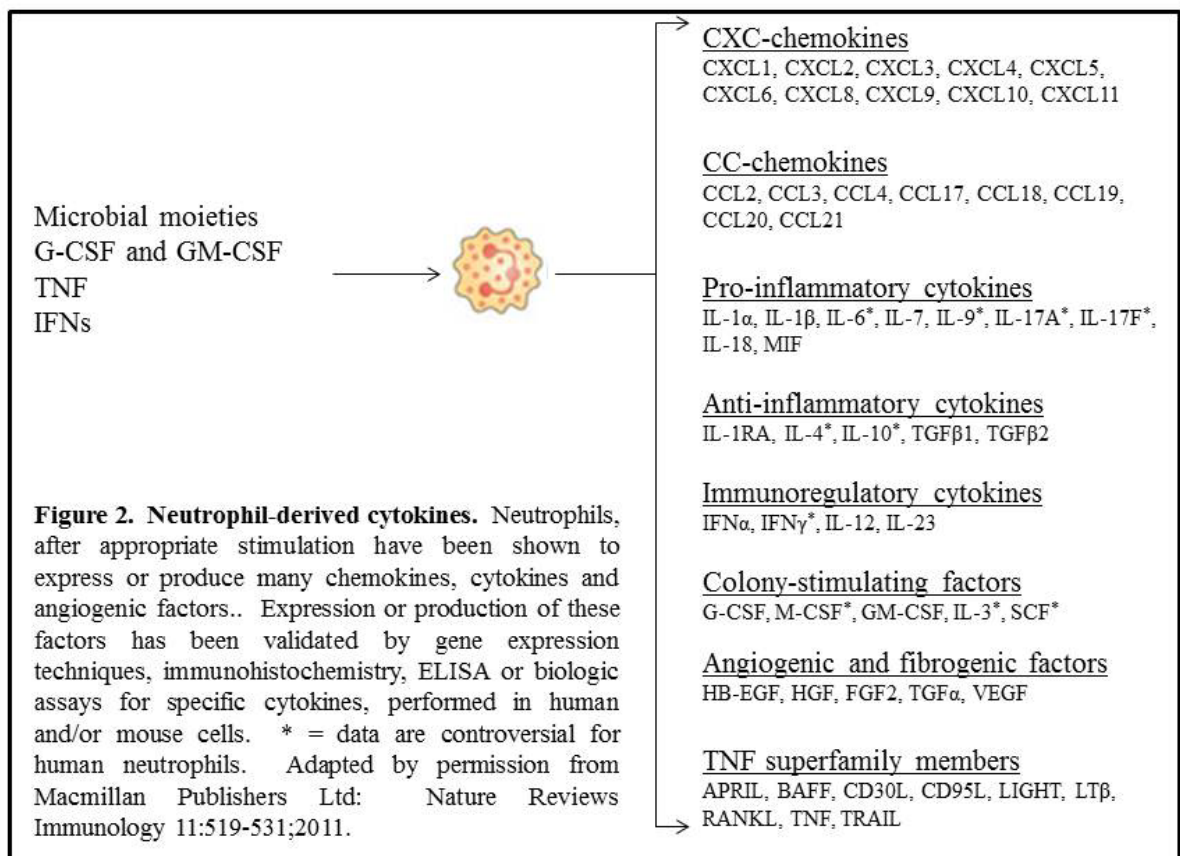
Neutrophils are the first cells to be recruited to areas of inflammation. They function to contain

and clear infection by efficiently engulfing and degrading microorganisms using both oxidative and non-oxidative mechanisms (reviewed by Pham).²⁶ After a microorganism is engulfed, it is sequestered in a phagolysosome. Phagocytosis activates the membrane-bound NADPH oxidase system which generates reactive oxygen species (ROS) that are released into the phagolysosome where they mediate direct killing. This represents the oxidative arm of the neutrophil's antimicrobial action. The non-oxidative arm is mediated by cytotoxic agents contained within neutrophil granules. Briefly, once a microorganism is sequestered, fusion of neutrophil granules with the phagolysosome occurs resulting in release of antimicrobial proteases and peptides into the phagolysosome. A more detailed description of neutrophil granules and their content is included below. More recently it has been shown that neutrophils can release neutrophil extracellular traps (NETs). Described by Brinkmann et al. in 2004, NETs are web-like structures composed of chromatin derived from the neutrophil nucleus and



antimicrobial proteins to include proteases.²⁷ They are able to trap and kill microbes extracellularly, and by localizing toxic proteases, they may prevent collateral damage to adjacent tissue.

While neutrophils have classically been characterized by their ability to phagocytose, produce ROS and release lytic enzymes from their granules, advances over the past two and a half decades have demonstrated that this is an overly simplistic view. Although an entire review of neutrophil biology is outside the scope of this dissertation, an example of an important advance in the study of neutrophils was the demonstration that these cells can be induced to express genes encoding inflammatory mediators including chemokines and cytokines, complement components and Fc receptors.²⁸ As shown in figure 2, either spontaneously or following stimulation, neutrophils can express or produce numerous cytokines, chemokines and angiogenic factors. The expression or production of these factors has been confirmed by gene expression techniques, immunohistochemistry (IHC), enzyme-linked immunosorbent assays (ELISA) or cytokine-specific assays.²⁴ The most studied chemokine produced by neutrophils is IL-8.²⁸ Bazzoni et al. showed that after phagocytosis of yeast



opsonized with IgG, or exposure to lipopolysaccharide (LPS), neutrophils were able to release IL-8.²⁹ Neutrophils not only produce IL-8 but are themselves a primary target of IL-8 as they respond to IL-8 by releasing granule enzymes, generating respiratory burst activity and upregulating adhesion molecules. IL-8 is chemotactic for neutrophils suggesting a feedback loop whereby neutrophils release IL-8 to recruit additional neutrophils to the site of inflammation.³⁰ IL-8 is also chemotactic for basophils and T lymphocytes. In fact, the release of many factors by neutrophils facilitates crosstalk with other cells of the immune system. As another example, it has been shown that neutrophils secrete CXCL9, CXCL10 and CXCL11, all of which act on the CXCR3 receptor which is highly expressed on activated T cells, particularly Th1 CD4⁺ T cells, suggesting that neutrophils contribute to homing of Th1 cells to sites of inflammation.³¹⁻³³ In addition, Cassatella et al. showed that neutrophils stimulated by LPS secrete IL-12 which influences T cell differentiation.³⁴

Recently, Pelletier et al. showed cross-talk between human neutrophils and Th17 cells.³⁵ They found that supernatants from purified neutrophils stimulated with IFN- γ and LPS release CCL2 and CCL20 which are chemoattractants for Th17 cells. They also found that the release of CCL2 and CXCL10 mediated recruitment of Th1 cells, consistent with other studies. Interestingly, they showed that human Th17 cells could chemoattract neutrophils through the release of IL-8 suggesting that the neutrophil/Th17 cell interaction may create a proinflammatory loop that amplifies local accumulation of these two cell types. They confirmed this by showing co-localization of neutrophils and Th17 cells in gut tissue from patients with Crohn's disease and synovial fluid from patients with rheumatoid arthritis. This was consistent with studies showing co-localization of neutrophils and Th17 cells in mouse models of *Helicobacter pylori* infection and inflammatory bowel disease.^{36, 37} An important aspect of the study by Pelletier et al. was that they were able to enrich neutrophils to >99% purity from Ficoll-Paque-isolated granulocytes by removing contaminating cells with mAbs against CD3, CD19, CD56, CD36, CD49d and Gly-A. Many previous studies evaluating human neutrophils used only a Ficoll-Paque gradient that results in preparations that may more accurately be labeled granulocytic preparations as variable levels of contaminating eosinophils, monocytes and lymphocytes are present, potentially

altering experimental findings. They propose therefore that this may address some of the conflicting data in the literature showing discrepancies between mouse and human neutrophils and differences in the ability to express cytokines.³⁵

Taken together, findings from multiple studies discussed above suggest that, by secreting chemokines and cytokines that augment T cell responses, neutrophils enhance adaptive immune responses. Paradoxically, neutrophils also secrete cytokines to include IL-10 and TGF- β that can suppress T cell activation and proliferation (reviewed by Mantovani et al).²⁴ Cytokines and chemokines released from neutrophils also impact the localization and function of macrophages, dendritic cells and B cells.²⁴ Thus, as an initial step in the process of inflammation, neutrophils, have a significant impact on the subsequent immune response through a diverse range of function.

Tumor-Associated Neutrophils

Neutrophil recruitment to tumor sites

Given the range of neutrophil functions, it is not surprising that neutrophils have been identified as important players in the pathogenesis of several disorders including cancer. Neutrophils present in the tumor microenvironment have been referred to as tumor-associated neutrophils (TAN). Many cell types within the tumor microenvironment are capable of secreting neutrophil chemotactic substances. This includes the tumor cells themselves. Using a xenograft model, Sparmann and Bar-Sagi demonstrated that mutant Ras resulted in transcriptional upregulation of IL-8 expression and that this correlated with a significant inflammatory infiltrate in the tumors.³⁸ Although these investigators did not delineate the specific cell types in their inflammatory infiltrate, because IL-8 is a strong chemotactic factor for neutrophils, they postulated that Ras-induced IL-8 expression resulted in the recruitment of this cell type. In another mouse model, Verbeke et al. showed the chemokine GCP-2 (CSCL6) to be important in recruiting neutrophils to melanoma tumors, as an antibody to GCP-2 was able to decrease neutrophil recruitment to tumor sites and subsequently reduce tumor growth.³⁹ It is known that neutrophils move from the blood into tissues under the influence of other chemokines to

include CXCL1 and CXCL2, cytokines such as TNF- α and IFN- γ , growth factors (i.e. G-CSF) and cell adhesion molecules on their own surface (CD11b) and on the surface of endothelial cells (i.e. selectins).⁴⁰ It is likely that many of these factors play a role in recruiting neutrophils to the tumor microenvironment. In a recent study published by Fridlender et al., investigators used a transcriptomic approach in mice to show that the expression of CXCL1, CXCL2, and CCL-3 were significantly upregulated in neutrophils infiltrating tumor when compared with bone marrow neutrophils.⁴¹ These are additional data suggesting that TAN initiate a positive feedback loop by secreting chemoattractants that will recruit additional neutrophils to the tumor site.

Clinical reports of TAN

Several clinical studies have reported on the presence of neutrophils in human tumors with the majority suggesting that TAN confer a poor prognosis. In a study evaluating 121 consecutive patients undergoing nephrectomy for localized renal cell carcinoma, Jensen et al. performed IHC using antibodies against CD66b to quantitate TAN. On multivariate analysis, the presence of TAN was an independent prognostic factor associated with short recurrence-free survival, cancer-specific survival and overall survival (OS).⁴² More recent studies have shown that intratumoral CD66b⁺ neutrophils correlate with an adverse prognosis in colorectal carcinoma⁴³, resectable nonsmall cell lung cancer (NSCLC)⁴⁴ and hepatocellular carcinoma.⁴⁵ The studies by Illie et al. and Li et al. studying NSCLC and hepatocellular carcinoma respectively, also evaluated the ratio of neutrophils to CD8⁺ T cells. In both studies, an increased ratio was an even better predictor than the presence of TAN for poorer outcomes.^{44, 45} Although these studies suggest that TAN are most likely pro-tumorigenic, these findings are not universal as a study published by Caruso et al reported that a high neutrophil count was associated with a favorable prognosis in gastric cancer.⁴⁶ An important caveat to this study however is that the investigators did not use specific antibodies to evaluate for the presence of TAN, rather the number of neutrophils present were determined from standard hematoxylin and eosin stained sections.

TAN: Protumor role

Consistent with the presence of TAN correlating with a poor prognosis, there have been multiple mechanisms described by which TAN are involved in tumor development and progression. Experimental data exists suggesting a protumoral role for neutrophils with respect to tumorigenesis, tumor growth, angiogenesis, invasion, metastasis, and immunosuppression. This subject has recently been reviewed by Piccard et al.⁴⁷; select examples are highlighted below.

Angiogenesis. Several studies have proposed multiple different mechanisms by which neutrophils support angiogenesis by releasing pro-angiogenic factors including VEGF, IL-8, and matrix metalloproteinases (MMPs).⁴⁸⁻⁵⁰ Using the RIP1-Tag2 pancreatic islet carcinogenesis mouse model, Nozawa et al. showed that neutrophils are important mediators of the angiogenic switch through their release of MMP-9 which activates VEGF.⁵¹ A study by Queen et al., showed that neutrophils purified from healthy donors and co-cultured with breast cancer cells were able to secrete high levels of oncostatin M, a cytokine belonging to the IL-6 family. In turn, breast cancer cells stimulated with oncostatin M had increased VEGF production.⁵² They also demonstrated increased invasiveness in Matrigel assays.

Invasion and metastasis. In addition to the study by Queen et al. which showed increased invasiveness of breast cancer cells co-cultured with neutrophils, an early study by Welch et. al used rat mammary adenocarcinoma cells to demonstrate that neutrophils increased invasion through a reconstituted basement membrane barrier in an *in vivo* invasion assay, a process mediated by collagenase-IV and heparanase.⁵³ Subsequent studies have shown that neutrophil elastase, which will be discussed in detail below as a focus of this dissertation, can promote invasion and metastasis by degrading the extracellular matrix (ECM).⁵⁴ Supportive data was published by Doi et al. who showed that a neutrophil elastase inhibitor could reduce hepatic metastases induced by ischemia and reperfusion in a rat model.⁵⁵

Immunosuppression. Several studies have suggested mechanisms by which neutrophils may be immunosuppressive. Rotondo et al. showed that NSCLC cell lines could induce exocytosis of arginase from neutrophils. Arginase inhibits T cell proliferation by degrading arginine, resulting in decreased T cell responsiveness to CD3/TCR stimulation.⁵⁶ In another study, Fridlender et al. demonstrated that, under the influence of TGF- β , neutrophils are skewed to assume a protumorigenic phenotype. When these neutrophils were depleted, the activation status of CD8⁺ T cells, as determined using 4-1BB as an activation marker, increased providing evidence of another mechanism by which neutrophils can promote immunosuppression.⁵⁷

TAN: Antitumor role

Although many studies such as those discussed above suggest a protumor role for neutrophils, review of the literature identifies numerous studies showing an antitumor role as well. Experimental data exists suggesting an antitumor role for neutrophils with respect to their ability to mediate cytotoxicity, reject tumors and stimulate adaptive immunity. This subject was also included in the recent review by Piccard et al.⁴⁷; select examples are highlighted below.

Cytotoxicity and Tumor Rejection. As early as 1981, there were reports of direct killing of tumor cells by neutrophils *in vitro*.⁵⁸ A subsequent study reported in the early 1980s, suggested that neutrophils could mediate direct cytotoxicity *in vivo* as well. In this study, patients with ascites were administered intraperitoneal injections of OK-432 derived from group A streptococcus pyogenes, and in one patient they reported complete resolution of cancer cells in the ascites fluid which correlated with an increased number of intraperitoneal neutrophils. Neutrophils collected from the ascites were able to lyse tumor cells *in vitro*.⁵⁹

Animal studies have confirmed the ability of neutrophils to mediate tumor rejection. Using a model of B16 melanoma in C57BL/6 mice, Neville et al. showed that direct intratumoral injection of neutrophils led to a 50% reduction in tumor growth.⁶⁰ Using a murine colon adenocarcinoma cell line transduced to express G-CSF, Colombo et al showed that increased G-CSF at the tumor site led to

neutrophil recruitment and tumor rejection.⁶¹ Concordant with that, in experiments where neutrophils were deleted with antibodies, other investigators showed inhibition of tumor growth and metastases.^{62, 63}

Stimulate Adaptive Immunity. As was discussed above, neutrophils are able to secrete chemokines that recruit T cells, and pro-inflammatory cytokines that promote T-cell differentiation, proliferation and cytokine production.^{31, 32, 34} There are other studies confirming antitumor effects mediated by neutrophils and CD8⁺ T cells interacting. Photodynamic therapy (PDT) destroys tumor tissue by multiple mechanisms include direct killing, microvascular disruption and inflammation.⁶⁴ Using a murine tumor model, Kousis et al. have shown that PDT regimens that generate significant neutrophil infiltrates generate tumor-specific primary and memory CD8⁺ T cell responses.⁶⁵ When CXCR2^{-/-} mice that are defective in neutrophil tracking to peripheral tissues or mice depleted of neutrophils were used, PDT did not elicit a strong antitumor CD8⁺ T cell response. As yet another mechanism by which neutrophils may contribute to an antitumor immune response, other studies have demonstrated a role for neutrophils as antigen presenting cells. Beauvillain et al. showed that neutrophils can cross-present ovalbumin to naïve CD8⁺ T cells from OT1 transgenic mice. Following injection of neutrophils in their model, OT-1 CD8⁺ T cells were able to proliferate and secrete IFN- γ .⁶⁶

Tumor-Associated Neutrophil Phenotype: N1 vs N2 TAN

Polarization of tumor associated macrophages has been well characterized (reviewed by Allavena et al.).⁶⁷ Briefly, classically activated macrophages (M1) induced by IFN- γ are antitumoral. They are characterized by high antigen presenting capacity, IL-12 and IL-23 production, and activation of type I T-cell responses. They also have cytotoxic activity by releasing nitric oxide, ROS and TNF. Alternatively activated macrophages (M2) are induced by IL-4, IL-10, IL-13, immune complexes and glucocorticoids. They are poor antigen presenting cells, have an IL-12^{low}IL-10^{hi} phenotype, suppress T-helper type I adaptive immunity, and promote angiogenesis and tissue remodeling. M2 macrophages are therefore protumor. The literature clearly suggests a dual role for neutrophils in tumor biology and

recently, Fridlender et al. provided evidence for protumoral (N2) and antitumoral (N1) TAN; analogous to M1 and M2 macrophage polarization.⁵⁷

Using both flank and orthotopic mouse models of NSCLC and mesothelioma, Fridlender et al. showed that TGF- β blockade led to an influx of CD11b⁺/Ly6G⁺ TAN.⁵⁷ The 1A8 anti-Ly6G antibody was used as it is expressed only on neutrophils allowing investigators to differentiate neutrophils from macrophages (CD11b⁺Ly6G⁻).⁶⁸ There was no significant change in the Ly6G⁻ macrophages following TGF- β blockade. There was also no change in the percentage of CD11b⁺/Ly6G⁺ cells in the blood or spleen, showing that only the intratumoral population of neutrophils increased. The mechanism by which CD11b⁺/Ly6G⁺ TAN increased was multifactorial including increased expression of neutrophil attracting chemokines and cytokines as well as upregulation of ICAM-1 expression on endothelial cells. The CD11b⁺/Ly6G⁺ TAN which were labeled as N1 TAN had increased antitumoral activities including enhanced expression of T cell-attracting chemokines, proinflammatory cytokines, lower levels of arginase, and more capability for tumor cell killing both *in vivo* and *in vitro*. Depletion of N1 TAN impaired intratumoral CD8⁺ T cells and led to increased tumor growth, providing further evidence of the antitumor function. TAN in control mice not treated with the TGF- β blockade were labeled as N2 TAN and they had a protumor phenotype. When N2 TAN were depleted, tumor growth slowed.⁵⁷

In a recent review on TAN, Gregory and Houghton suggested that the changes seen in N1 TAN did not represent a unique transcriptional program rather an increased state of activation i.e. the response to TGF- β blockade was generation of the same products at higher levels.⁶⁹ In support of this hypothesis, these authors point to a second study demonstrating the alteration of neutrophil function *in vivo*. In a study by Jablonska et al. tumors in IFN- β knockout mice grew faster and were more highly vascularized than tumors in wild-type controls. Neutrophils from the IFN- β ^{-/-} mice had increased expression of proangiogenic factors including VEGF, CSCR4 and MMP-9, and expression of these genes was reversible ex-vivo with IFN- β .⁷⁰ Therefore, the “polarization” of TAN or their level of activation may be dictated by the tumor microenvironment.

In a recently published study, Fridlender et al. used microarrays to compare gene expression profiles in TAN versus naïve neutrophils derived from the bone marrow and to the granulocytic fraction of myeloid derived suppressor cells (MDSC).⁴¹ MDSC represent a heterogeneous population of suppressive immune cells that are defined in mice by their expression of the CD11b and Gr-1 surface markers.⁷¹ There are at least two subsets – granulocytic (Ly6G⁺) and monocytic (Ly6C⁺). MDSC are produced at high levels in cancer and have been shown to inhibit T cell activation. For the Fridlender et al. study, TAN and CD11b⁺Ly6G⁺ MDSC (G-MDSC) were obtained from the tumor and spleen respectively of mice growing AB12 mesothelioma. Naïve neutrophils were isolated from the bone marrow of non-tumor-bearing mice. RNA expression profiles were significantly different between the three groups with naïve neutrophils and G-MDSC being more closely related to each other than TAN.⁴¹ The most significant difference identified between TAN and other neutrophil populations was the significant upregulation of cytokines and chemokines suggesting an important role for TAN in recruiting other immune cells to the tumor. In addition, they found an upregulation in genes related to antigen presentation consistent with accumulating data showing that neutrophils may function as antigen presenting cells.⁷² In a recent review on TAN, Fridlender and Albeda indicate that when they compared RNA expression of N1 versus N2 TAN, they found that the majority of changes were upregulation of the same genes and pathways in the two subtypes. There were however some differences such as the upregulation of CCL-17, which attracts regulatory T cells, in N2 TAN versus N1.²⁵ Taken together, these data suggest that TAN are a distinct population of neutrophils however it remains unclear as to whether N1 and N2 TAN differ based on their transcriptional profile or represent differing states of activation dictated by factors present in the tumor microenvironment.

NEUTROPHIL ELASTASE

As was discussed above, neutrophils are efficient at engulfing and degrading microorganisms.⁷³ The non-oxidative arm of their antimicrobial action is mediated by the release of peptides and proteases from granules that occur after the granules fuse with phagolysosomes. There are four types of granules in neutrophils: primary (also known as azurophil), secondary (also known as

specific), tertiary (also known as gelatinase) and secretory granules.⁷⁴ A list of the granules and the protein constituents is shown in the table. Neutrophil elastase (NE) is a member of a family of structurally related serine proteases that also includes proteinase 3 (PR3) and cathepsin G (CG). They are found in primary granules. Although the antimicrobial function of these proteases has been extensively studied, as will be highlighted below, they are capable of modulating many biologic processes.

Table. Neutrophil granule content.

Azurophil Granules	Specific Granules	Gelatinase Granules	Secretory Granules
Neutrophil Elastase	Lactoferrin	Gelatinase	CR1
Proteinase 3	Lysozyme	Lysozyme	CR3 (CD11b-CD18)
Cathepsin G	Cathelicidin	Leukolysin	CD14
Myeloperoxidase	Collagenase	NRAMP1	CD16
Azurocidin	Leukolysin		FPR
Defensins	Cytochrome b558		
Bacteril permeability-increasing protein	NGAL		

CR, complement receptor; FPR, formyl peptide receptor; NGAL, neutrophil gelatinase-associated lipocalin; NRAMP1, natural-resistance-associated macrophage protein 1.

Table adapted from Nature Reviews Immunology 6:541-550;2006.

Biology of Neutrophil Serine Proteases

Neutrophil elastase, PR3 and CG have a high degree of homology with other serine proteases to include cytotoxic T lymphocyte-granule-associated granzymes and mast-cell chymase and tryptase. They all contain a conserved catalytic triad containing histidine, aspartic acid and serine residues which are separated in the primary sequence of the protein but are brought together at the active site in their tertiary structure.⁷⁵ The genes for NE, PR3 and CG all consist of five exons and four introns.^{76, 77} The genes encoding NE (formerly *ELA2*, now *ELANE*) and PR3 (*PRTN3*) are found in a cluster on chromosome 10 in mice⁷⁸ and chromosome 19 in humans.⁷⁹ In humans, the cluster on

chromosome 19 also includes the gene for azurocidin (AZU), a catalytic serine that does not have proteolytic activity.⁸⁰ The gene encoding CG (*CTSG*) is located in a separate cluster on chromosome 14 (both mouse and human). In humans, this cluster also includes gene encoding granzyme B and granzyme H.^{76, 81}

The synthesis of NE, PR3 and CG is regulated at the transcriptional level during granulocyte development than at the post-translational level before being stored within neutrophil azurophilic granules in their proteolytically active mature form. High levels of transcription of the genes encoding NE, PR3 and CG are limited to the promyelocytic stage of neutrophil differentiation in the bone marrow and are downregulated as neutrophils mature.⁷⁹ It should be noted that low levels of mRNA encoding NE and CG are detected in monocytes and mast cells⁷⁸ and that PR3 mRNA has been shown to be expressed by endothelial cells in humans.⁸²

All three serine proteases are synthesized as inactive prepro-proteins containing a signal peptide, an amino-terminal pro-peptide and a C-terminal pro-peptide.⁸³⁻⁸⁶ Subsequent processing has recently been reviewed by Korkmaz et al.⁸⁷ Briefly, shortly after synthesis, the amino-terminal peptide is cleaved by a signal peptidase leaving an N-terminal pro-sequence of two amino acids. Removal of the pro-dipeptide is required for activation of enzymatic activity. This is accomplished by dipeptidyl peptidase I (DPPI). In the absence of DPPI, N-terminal processing is incomplete and the pro-forms of serine proteases can be constitutively secreted or more easily degraded.^{79, 84, 88} N-terminal processing is essential for optimal storage of serine proteases in azurophil granules and usually occurs before or during transport to the granules.²⁶ NE, PR3 and CG also undergo C-terminal processing by an as-yet-unknown protease.^{86, 89} C-terminal processing is not required for enzymatic activity; however, data from Horwitz et al suggests that its retention might prevent normal trafficking of NE to azurophil granules.⁹⁰ These investigators showed that C-terminal processing reveals a docking site that allows NE to interact with the adaptor protein AP3 which is responsible for proteins shuttling from the Golgi to the granule compartment. If NE does not attach to AP3, it is routed to the cell surface where it would likely be inactive secondary to distortion of the catalytic site.²⁶ The definitive mechanisms responsible for serine

proteases tracking to granules is unknown although serglycin, an intracellular proteoglycan, has been shown to play a role in NE packaging into azurophilic granules.⁹¹ In addition to being stored in the azurophilic granules, immunostaining and electron microscopy have shown that NE is also localized in the nuclear envelope.^{92, 93}

The serine proteases are stored in azurophil granules in their active form. Once neutrophils are activated at inflammatory sites, these granules translocate to phagosomes and the plasma membrane where they release their contents. Regulated exocytosis of secretory granules has been studied in many cell types including neurons, neuroendocrine cells, endocrine cells and hematopoietic cells, but remains only partially understood. As reviewed by Burgoyne and Morgan, the process likely utilizes the same basic protein components in different cell types.⁹⁴ The translocation process has two steps. The first step depends on cytoskeleton remodeling and microtubule assembly. The second step involves interaction between soluble-N-ethylmaleimide-sensitive-factor accessory-protein receptors (SNAREs) present on the plasma membrane and the granule which facilitates fusion.⁹⁵ Multiple different SNAREs have been identified in neutrophils and it is likely that different granule SNAREs interact differently with the plasma membrane SNAREs thereby dictating the rate of exocytosis.^{95, 96} After they are released, the neutrophil serine proteases are fully active. Although NE and PR3 preferentially cleave after valine residues and CG favors hydrolysis of peptide bonds after aromatic amino acid residues, they have a broad range of substrates.^{26, 97} Their protease activity can be inhibited by endogenous serine protease inhibitors (serpins) including elafin and α_1 -proteinase inhibitor (NE and PR3) and secretory leukocyte protease inhibitor (NE and CG).^{97, 98}

Neutrophil Elastase in Infection

Neutrophil elastase plays a role in the killing of gram-negative bacteria.⁹⁹ Using targeted mutagenesis to generate strains of NE deficient mice, Belaouaj et al. showed that NE^{-/-} mice were more susceptible to sepsis and death following intraperitoneal infection with gram negative, but not gram positive, bacteria than wild-type mice. Further work by that group identified at least one mechanism by

which NE mediates killing of gram negative bacteria.¹⁰⁰ They showed that NE can degrade purified outer membrane protein A (OmpA) found on the surface of *E. coli*. *In vitro*, NE killed wild-type *E. coli* but not OmpA-deficient *E. coli*. *In vivo*, NE^{-/-} mice had decreased survival in response to sepsis induced by *E. coli*, but the absence of NE had no impact on survival in response to sepsis induced by OmpA-deficient *E. coli*. A study by Weinrauch et al. demonstrated a role for NE in controlling enterobacteria such as *shigella*, *salmonella* and *Yersinia*.¹⁰¹ Briefly, they showed that NE can cleave virulence factors on these bacteria at a lower concentration than required to degrade other proteins and in neutrophils in which NE is inactivated, the bacteria are able to escape from the phagolysosome leading to increased survival within the infected neutrophil.

Reeves et al suggested that serine proteases, not ROS, were the primary agents responsible for destroying bacteria.¹⁰² They found that mice deficient in proteases but normal with respect to superoxide production and iodinating capacity were unable to resist staphylococcal and candida infections. They also suggested that myeloperoxidase (MPO) protected serine proteases from inactivation by breaking down H₂O₂ to hypochloric acid. In contrast, Hirche et al. showed that MPO mediates oxidative inactivation of NE.¹⁰³ Activated neutrophils from MPO-deficient mice had increased NE activity. In addition, they found that MPO-deficient mice were susceptible to infection with *Klebsiella pneumonia* suggesting that, in mouse models, both MPO and NE are necessary for optimal killing of bacteria.

Finally, as was discussed above, NE released from neutrophils is a component of NETs. Within NETs, serine proteases are present at high concentrations able to degrade virulence factors and kill bacteria.²⁷ Serine protease, including NE, therefore have both intra- and extracellular effects mediating host defense against infection.

Neutrophil Elastase in Inflammation

In addition to having a role in host defense, NE has been associated with non-infectious, inflammatory processes. In order for NE to regulate inflammation, it must avoid or escape the effects of

the endogenous inhibitors present in the extracellular environment. The mechanism by which this occurs has not been definitively elucidated however there have been several proposed mechanisms as reviewed by Owen and Campbell.¹⁰⁴ One proposed mechanism is that a compartment is created between the neutrophil and ECM where the proteases are released and protected from the larger, high molecular-weight protease inhibitors.¹⁰⁴ Similarly, a proportion of serine proteases released from neutrophils bind to the plasma membrane and that tight binding makes them inaccessible to the larger inhibitors.¹⁰⁵ Finally, it has been suggested that large quantities of proteases can overwhelm the protease inhibitors.¹⁰⁴

Consistent with NE having a role in inflammation, several studies in animal models have shown that inhibition of NE reduces inflammation. For example, in a hamster model where acute lung injury is induced by endotoxin inhalation, Kawabata et al. showed an increase in inflammatory cell count and protein concentration in bronchoalveolar lavage fluid that peaked at 24 hours and correlated with NE activity in the fluid.¹⁰⁶ When hamsters were treated with an NE-inhibitor, there was a dose dependent change in the inflammatory cell count and protein concentration and histopathologic analysis of the lung tissue showed a decrease in hemorrhage and inflammation to include neutrophil infiltration. A role for NE in inflammation has also been shown in ischemia-reperfusion models¹⁰⁷ and models of collagen-induced arthritis.¹⁰⁸

Role of Neutrophil Elastase in Cancer

Clinical Reports of Neutrophil Elastase in Cancer

Several studies have investigated the prognostic significance of NE in cancer. In a study using extracts from 40 NSCLC tumors, Yamashita et al. used an enzyme immunoassay to measure immunoreactive NE (ir-NE) and looked for an association of ir-NE with clinicopathologic characteristics.¹⁰⁹ The assay measures both free-form NE and α 1-proteinase inhibitor complexed NE. They identified ir-NE in 34 specimens and the presence of ir-NE was significantly higher in stage IIIB disease versus stage I, II or IIIA. The presence of ir-NE was also higher in larger (T3 and T4) versus

smaller (T1 or T2) tumors. Patients with higher NE concentrations had shorter OS than those with lower NE concentrations. In a subsequent study, these investigators measured ir-NE in 144 NSCLC extracts.¹¹⁰ The concentration of ir-NE was again significantly higher in patients with clinical T4 tumors (versus clinical T1, T2 and T3). Tumors with aortic invasion had higher levels of NE than those involving other sites. This is an interesting observation given that the aorta is a rich source of elastin.

The prognostic significance of NE has also been evaluated in breast cancer. In a study using an ELISA assay to measure NE (in complex with α 1-proteinase inhibitor) in the cytosolic extracts of 1143 primary breast tumors, Foekens et al. showed that high levels of NE were associated with a poor prognosis.¹¹¹ In a Cox multivariate regression analysis correcting for standard prognostic factors, NE was independently associated with poor metastasis-free survival, relapse-free survival and OS. These data corroborated a previous report from Yamashita et al. who measured ir-NE in tumor extracts from 313 primary breast cancer patients who underwent mastectomy and axillary lymph node dissection.¹¹² Using an ir-NE concentration of 9.0 μ g/100 mg protein as the cutoff, patients were categorized as having high (n=52) or low (n=261) levels of NE. After a median follow-up of nine years, patients with high NE levels had significantly shorter disease-free survival (DFS). On univariate analysis, ir-NE level, tumor size, lymph node involvement, histologic grade, lymphatic vessel involvement and administration of adjuvant therapy were all significant prognostic factors. Multivariate analysis found that ir-NE level and lymph node involvement were the only independent prognostic factors. This group has published updated results after a median follow-up of 18.5 years and NE levels continue to be a significant indicator of prognosis.¹¹³

Iwatsuki et al. used IHC to look for NE in tumors from twelve patients with astrocytomas with varying degrees of malignancy.¹¹⁴ No NE was found in any of four low-grade astrocytoma cases. In contrast, NE was found in areas of tumor infiltration in 4/4 glioblastoma cases and 3/4 anaplastic astrocytoma cases suggesting that tumors with a greater degree of malignancy had more NE. These investigators concluded that NE released from neutrophils recruited to malignant gliomas aid in the process of infiltration.

NE and PR3 have also been shown to be aberrantly expressed in myeloid leukemia.¹¹⁵ My advisor, Dr. Molldrem, has identified a conserved nonameric HLA-A2-restricted peptide in NE and PR3 called PR1 (VLQELNVTV).¹¹⁶⁻¹¹⁸ In a study of 38 patients with chronic myelogenous leukemia (CML) treated with IFN- α 2b therapy or allogeneic bone marrow transplant, he found a strong correlation between the presence of PR1-specific CTL identified by tetramer staining and clinical response to therapy. These data suggest a role for T-cell immunity against the aberrantly expressed proteases in clearing malignant cells in CML.¹¹⁸ Other investigators have confirmed that the presence of PR1-specific CTL correlates with positive clinical outcomes following treatment.^{119, 120} In addition, in a study evaluating vaccination with the PR1 peptide, inoculated patients with acute and chronic myelogenous leukemias showed immunological and clinical responses including durable molecular remissions.¹²¹

Mechanisms of Neutrophil Elastase Promoting Cancer

The role of NE in cancer has largely been attributed to its ability to degrade ECM proteins (collagen, fibronectin, proteoglycan and cadherins) thereby promoting invasion and metastasis.^{54, 122} In addition to its ability to degrade ECM directly, another proposed mechanism by which NE may promote invasion is by activation of MMP-2. MMP-2, which is secreted as an inactive zymogen (proMMP-2), is an MMP involved in angiogenesis and tumor invasion. In *in vitro* experiments using HT1080 sarcoma cells incubated with purified NE, Shamamian et al. demonstrated dose- and time-dependent activation of proMMP-2.¹²³ The addition of neutrophil-conditioned media to cells expressing membrane-type 1 MMP (MT1-MMP) resulted in increased activation of MMP2 and ECM invasion. This effect was not seen when neutrophil-conditioned media was added to cells that do not express MT1-MMP suggesting that NE activation of proMMP-2 requires MT1-MMP. Finally, proMMP-2 activation by neutrophil-conditioned media was blocked by α 1-antitrypsin (NE inhibitor) but not by Batimastat (MMP inhibitor) showing that NE activation of MMP-2 is not dictated by MMP. Taken together, these data support an indirect role by which NE promotes tumor invasion.

Neutrophil elastase may also be involved in carcinogenesis via the TNF signaling pathway. TNF signaling is complicated with multiple downstream effects including activation of caspase 8 which mediates apoptosis (reviewed by Gaur and Aggarwal).¹²⁴ An early study by Scuderi et al. showed that the addition of NE to endotoxin-stimulated human peripheral blood mononuclear cells (PBMC) resulted in decreased TNF in the culture supernatant.¹²⁵ Co-culture of NE with TNF showed that NE degraded TNF. Porteu et al. showed that NE is able to remove an active fragment from the TNF receptor from the cell surface,¹²⁶ and subsequent to that, Van Zee et al. demonstrated that the shed TNF receptors consume circulating TNF.¹²⁷ Taken together, these data suggest that NE could effectively decrease TNF signaling resulting in continuous cell growth due to failure of activation of caspase 8. An *in vivo* study investigating the impact of NE on the development of ultraviolet (UV) light induced skin cancers in hairless mice, provided additional, supportive data suggesting an interaction between NE and TNF signaling. Briefly, UV radiation has been shown to cause keratinocytes to produce TNF- α .¹²⁸ In a model using NE-deficient mice, Starcher et al. showed that these mice formed fewer tumors than normal mice after UV irradiation.¹²⁹ They postulated that this could be due to NE cleavage of the TNF receptor.

Recently, Houghton et al. showed that NE promotes lung tumor growth using the LSL-K-ras mouse model.¹³⁰ When compared to LSL-K-ras/NE^{+/+} mice, LSL-K-ras/NE^{-/-} mice had decreased lung tumor growth and increased survival. The investigators were unable to identify a component of the ECM that was altered in their model. Instead, they showed that NE accelerated tumor cell proliferation via phosphatidylinositol-3 kinase (PI3K) hyperactivity. They found that NE entered tumor cell endosomes via clathrin coated pits; the first report of a secreted protease gaining access to a cell beyond the plasma membrane. Once internalized, NE degraded insulin receptor substrate-1 (IRS-1), a binding partner of the p85 regulatory subunit of PI3K.¹³¹ In turn, this increased the availability of PI3K to interact with the platelet-derived growth factor receptor promoting tumor cell proliferation.¹³⁰

We have recently published a study showing that breast cancer cells can take up NE resulting in an increased adaptive immune response against the novel tumor antigen cyclin E.¹³² Details

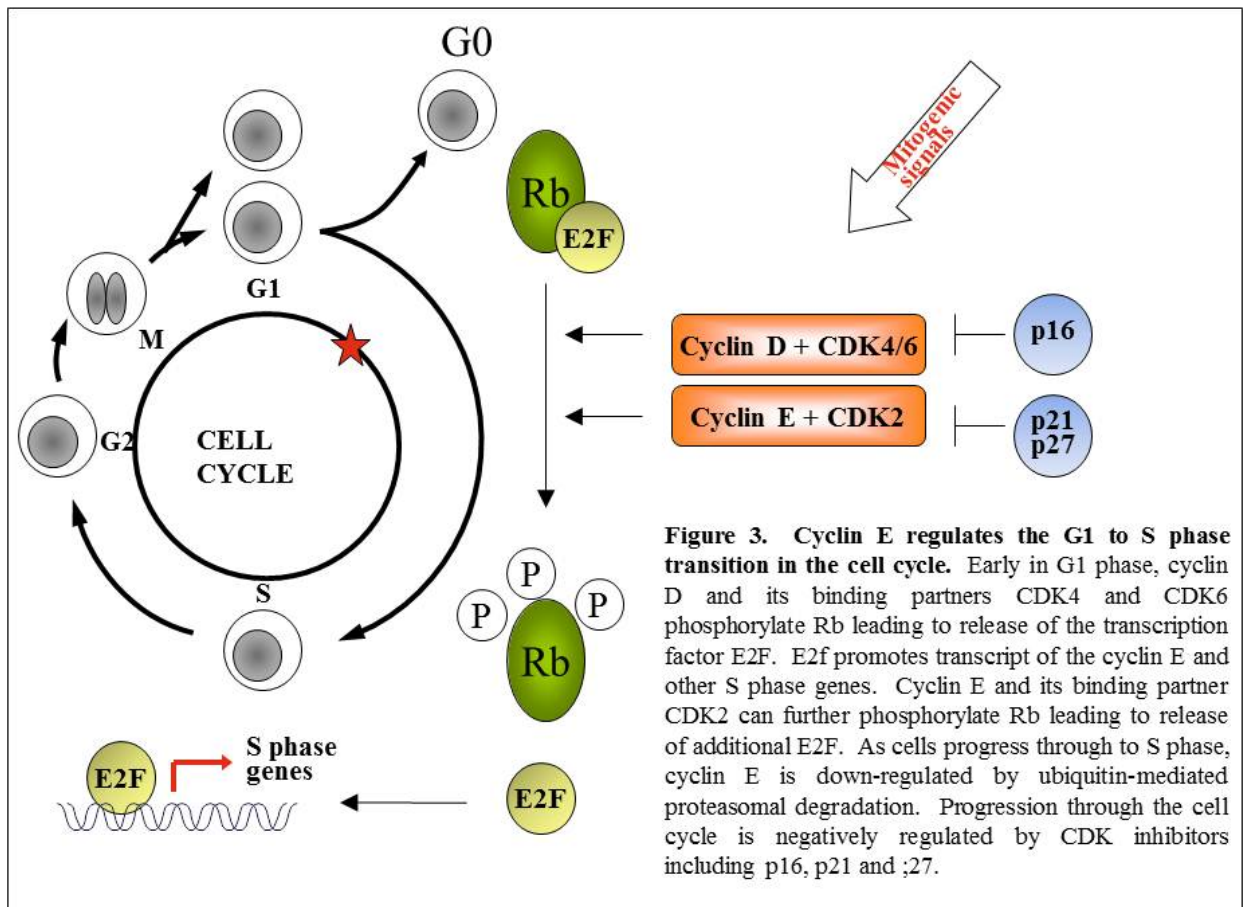
of that study comprise chapter 2 of this thesis. Taken together, these data suggest that there are multiple mechanisms by which NE may impact carcinogenesis.

CYCLIN E

Cyclin E and Cell Cycle Regulation

The E-type cyclins which includes cyclin E1 and cyclin E2 are central components of the cell cycle machinery. They have 48% sequence homology with some regions i.e. the cyclin box domain, being more conserved (75% homology).¹³³⁻¹³⁵ The two are thought to be functionally redundant as cyclin E1/E2 double knockout mice are embryonic lethal while single knockout mice have largely normal phenotypes.^{136, 137} More detailed studies (reviewed by Caldon and Musgrove) have identified additional roles for the E-cyclins outside of cell cycle regulation including endoreplication and meiosis which associate more closely with either cyclin E1 or cyclin E2.¹³⁸ However, cyclin E1 has been more studied than cyclin E2 to include in the majority of studies investigating cyclin E as a driver of oncogenesis therefore, for the remainder of this thesis, all references to cyclin E (CCNE) are for cyclin E1.

Normal cell division is regulated by checkpoints in the cell cycle including the G1 to S phase checkpoint, of which CCNE is an important regulator (reviewed by Johnson and Walker) (Fig. 3).¹³⁹ Briefly, as reviewed by Weinberg, the retinoblastoma (Rb) pathway is critical for passage of cells through G1 into S phase.¹⁴⁰ The pathway is regulated through sequential phosphorylation of Rb by cyclins and their associated cyclin-dependent kinases (CDKs). Early in the G1 phase, the cyclin D1/CDK4 and cyclin D1/CDK6 complexes phosphorylate Rb leading to a conformational change in the Rb protein and uncoupling from the E2F transcription Factor.¹⁴¹⁻¹⁴³ The CCNE promoter is transcriptionally regulated by E2F thus the release of E2F results in transcription of CCNE as well as other S phase genes.¹⁴⁴⁻¹⁴⁶ Complexed with CDK2, CCNE continues phosphorylating Rb with peak levels occurring at the restriction point late in G1.^{140, 147, 148} As cells progress through S phase, CCNE is down-regulated by ubiquitin-mediated proteasomal degradation.^{149, 150} Thus, in normal cells, cyclin E



levels oscillate during the cell cycle to ensure a limited window of activity. Progression through the cell cycle is negatively regulated by CDK inhibitors including members of the Cip/Kip family (p21 and p27) that inhibit CDK2 (reviewed by Sherr and Roberts).¹⁵¹

The critical role of CCNE in regulating the G1/S checkpoint was shown experimentally by Ohtsubo et al. Using mammalian fibroblasts engineered to constitutively overexpress CCNE they first demonstrated shortening of the duration of the G1 phase and decreased cell size suggesting that CCNE was rate-limiting in the G1 to S phase transition and that overexpression of CCNE promoted a faster transition.¹⁵² In a subsequent study, Ohtsubo et al showed that fibroblasts overexpressing CCNE had increased CCNE-dependent kinase activity and that microinjection of anti-CCNE antibodies during the G1 phase resulted in cell cycle arrest.¹⁵³

Cyclin E and Cancer

Role of Cyclin E Overexpression

The G1/S checkpoint is frequently deregulated in human tumors suggesting a role in carcinogenesis. A link between cyclins and oncogenesis was initially made when it was found that cyclin A and cyclin D were inappropriately expressed in tumors (reviewed by Hunter and Pines).¹⁵⁴ Subsequently, Keyomarsi and Pardee used breast cancer cell lines to show that various cyclins including A, B, D1 and E as well as CDKs 1 and 2 were overexpressed at both the mRNA (determined by Northern blot) and protein (determined by Western blot) levels compared to normal mammary epithelial cells.¹⁵⁵ CCNE specifically was overexpressed at the protein level in all 10 breast cancer cell lines evaluated compared to three mammary epithelial cell lines. Using the MDA-MB-157 breast cancer cell line, they found an 8-fold amplification of the CCNE gene which resulted in a 64-fold amplification of CCNE mRNA and overexpression of the CCNE protein. They also noted that the CCNE from these tumor cells had higher H1 kinase activity than CCNE from the benign mammary epithelial cells. In subsequent studies performed to better understand these alterations, Keyomarsi et al. showed that in contrast to what is seen in normal cells where CCNE protein and its kinase activity is cell cycle regulated, in tumor cells, CCNE is constitutively present and active across all phases of the cell cycle.¹⁵⁶

Additional support for a role of abnormal CCNE expression and the development of breast cancer was provided in a study by Bortner and Rosenberg.¹⁵⁷ Using the bovine β -lactoglobulin promoter to target expression of human CCNE to the mammary gland in a transgenic mouse model, they showed that lactating mammary glands overexpressing CCNE developed areas of hyperplasia which were not observed in control mice. In addition, more than 10% of the mice developed mammary carcinomas. Spruck et al. showed that CCNE overexpression in immortalized rat embryo fibroblasts and human breast epithelial cells caused chromosomal instability.¹⁵⁸ These authors hypothesized that

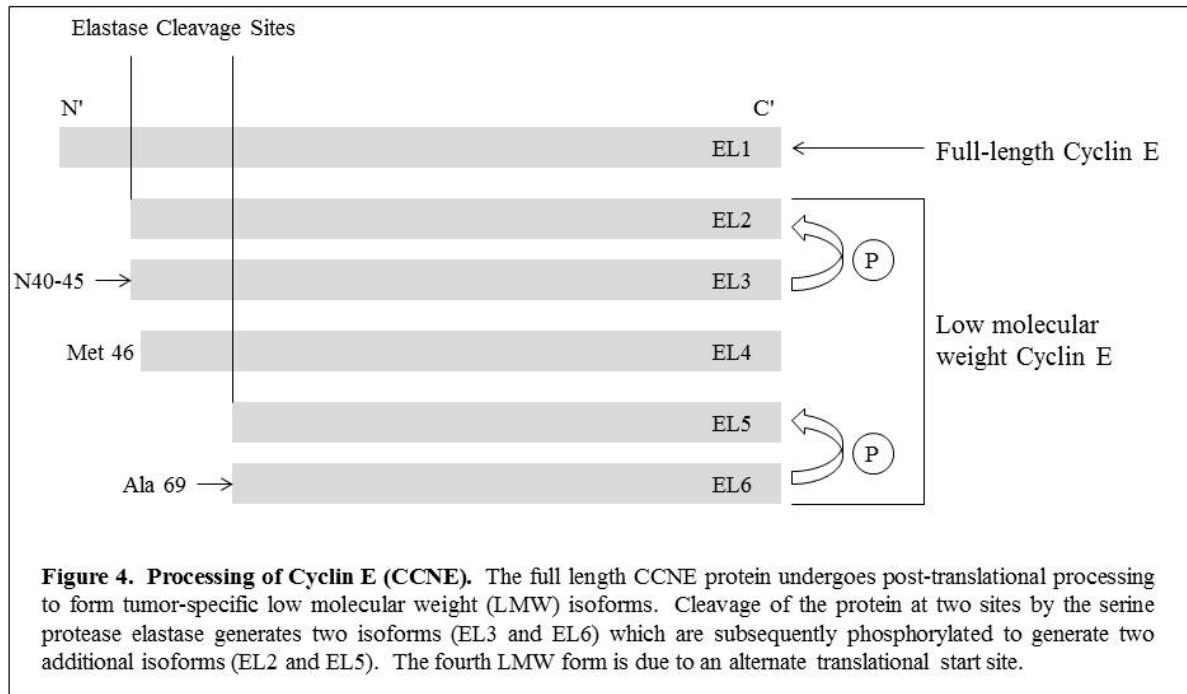
downregulation of CCNE/CDK2 kinase activity following the G1 to S phase transition, which is impaired in cancers with constitutive expression of CCNE, is required for chromosomal stability. Taken together, these data support a role for CCNE in cancer.

Low Molecular Weight Cyclin E

In the early study by Keyomarsi and Pardee, multiple bands ranging from 35-50 kDa in size were observed on the Western blot probing for CCNE on lysates from the MDA-MB-157 breast cancer cell line.¹⁵⁵ They referred to these as “cyclin E (-like) proteins” which were tumor specific, as western blots performed on lysates from mammary epithelial cells had only a single band at 50 kDa. Because there was a single mRNA transcript, they deduced that these “cyclin E (-like) proteins” were generated post-translationally. In a study performed to confirm these findings *in vivo*, Keyomarsi et al. generated lysates from frozen surgical specimens. In 16 paired cases of breast cancer and adjacent nontumorous tissue, they found that these “cyclin E (-like) proteins”, subsequently renamed low molecular weight (LMW) forms, were expressed in the tumors but not the adjacent normal tissue.¹⁵⁹ They made a similar observation in paired samples of lung, gastric, prostate, renal, pancreatic and colon cancer. Other investigators have shown LMW CCNE in ovarian cancer¹⁶⁰ and melanoma.¹⁶¹ My supervising professor, Dr. Molldrem, has identified LMW CCNE in leukemia.¹⁶²

Generation. Subsequent work performed in the Keyomarsi laboratory demonstrated that the tumor-specific LMW isoforms are generated by post-translational proteolytic processing of the full-length (FL) CCNE protein.¹⁶³ In addition to the FL protein (50 kDa), they showed 5 LMW forms (Fig. 4) ranging in size from 33 to 49 kDa. Using site-specific mutations and transient transfections of FLAG-tagged CCNE constructs, they were able to identify two protease-sensitive domains in the FL protein. Proteolysis by the serine protease elastase (in this study they utilized porcine pancreatic elastase) generated four of the five LMW forms which mimicked those observed *in vivo*. Cleavage between N40/N45 generated LMW-EL3 and between A69/D70 generated LMW-EL6.¹⁶³ LMW-EL2 and LMW-EL5 are phosphorylated forms of LMW-EL3 and LMW-EL6 respectively.¹⁶⁴ The FL form of

CCNE has been termed EL1. The EL2/EL3 doublet has been termed cyclin E-truncation 1 (Trunk 1; T1), while the EL5/EL6 doublet has been termed cyclin E-truncation 2 (Trunk 2; T2). LMW-EL4 is formed due to an alternate translational start site at M46.¹⁶³



Localization. The FL CCNE protein includes a canonical nuclear localization sequence, RSRKRK, at amino acids 27-32.¹⁶⁵ Recognizing that the LMW forms lack the nuclear localization sequence, Delk et al. hypothesized that the LMW forms may have altered subcellular localization.¹⁶⁶ Subcellular fractionation experiments performed using breast and ovarian cancer cell lines showed that LMW CCNE expression was predominantly cytoplasmic while FL CCNE was found in both the nucleus and the cytoplasm. Using a protein complementation assay, they showed that LMW CCNE could bind CDK2 in the cytoplasm and that this LMW-CCNE/CDK2 complex had kinase activity.

Function. Multiple studies have confirmed that the LMW forms of CCNE are biologically active. In addition to showing that the LMW forms of CCNE are generated by post-translational cleavage by elastase, Porter et al. also showed that these LMW forms are hyperactive.¹⁶³ Breast cancer and mammary epithelial cell lines engineered to overexpress the T1 and T2 forms of LMW CCNE were

able to activate CDK2 and phosphorylate substrates (histone H1 and GST-Rb in standard kinase assays) more efficiently than FL CCNE. This hyperactivity facilitated deregulation of the G1 to S transition providing a growth advantage for cells expressing LMW CCNE. This was an important observation given the previous description of LMW CCNE as being tumor-specific. In a study by Wingate et al, a baculovirus insect expression system was utilized to overexpress three forms of CCNE – EL, T1 and T2.¹⁶⁷ In these studies, they showed that the LMW CCNE/CDK2 complexes are resistant to inhibition by the CDK inhibitors p21 and p27 when compared to FL CCNE/CDK2 complexes despite the fact that the complexes bind the CDK inhibitors equally. They also showed that when FL and LMW CCNE were co-expressed, p27 preferentially binds the LMW forms but is unable to inhibit its activity. This suggests another possible mechanism by which LMW CCNE contributes to tumorigenesis; resistance to inhibitory activity of p21 and p27.

Evidence also suggests that LMW CCNE confer resistance to endocrine therapy in breast cancer. In the estrogen receptor (ER+) breast cancer cell line MCF-7, the addition of tamoxifen, a selective estrogen receptor modulator causes growth arrest. Dhillon and Mudryj showed that exogenous expression of CCNE could abrogate this effect.¹⁶⁸ In a study by Akli et al. MCF7 cells engineered to overexpress LMW CCNE showed a reduction in growth arrest in response to fulvestrant, another estrogen receptor antagonist.¹⁶⁹ These investigators subsequently studied the effects of LMW CCNE expression on susceptibility to aromatase inhibitors; another form of endocrine therapy. Using a model system in which they transfected MCF7 cells with aromatase (MCF7/Ac1), they found that MCF7/Ac1 cells infected with LMW CCNE exhibited resistance to the G1 arrest induced by the addition of an aromatase inhibitor. This effect was abrogated by the addition of Roscovitine, a CDK-inhibitor suggesting the effect was due to increased CCNE/CDK2 kinase activity.¹⁷⁰

Finally, there is also evidence linking LMW CCNE with metastases. Using a transgenic mouse model in which the T1 form of LMW CCNE (LMW-E-T1) is expressed under control of the MMTV promoter, Akli et al. showed that LMW CCNE contributed to lung metastases by interrupting the ARF-p53 pathway.¹⁷¹ In a study that corroborated these findings, Bales et al. used a melanoma

xenograft model and found that when SB-2 melanoma cells engineered to express LMW-E-T1 or LMW-E-T2 were injected into mice, 100% of the mice (10 in each group) developed lung metastases.¹⁶¹ No mice in the vector alone group and 40% of mice in the EL group developed lung metastases. Taken together, these data suggest that LMW CCNE has a role in driving tumorigenesis.

Clinical Reports of Cyclin E in Cancer

The LMW forms of CCNE occur in approximately 25% of breast tumors.¹⁵⁹ In a study performed to determine the prognostic significance of LMW CCNE in breast cancer, Keyomarsi et al. measured FL and LMW CCNE levels by Western blot in breast tumor tissue from 395 patients. Immunohistochemistry was used to determine CCNE levels in a subset of 256 patients. The levels of CCNE were compared to established factors predictive for disease-specific (DSS) and OS including age, tumor size, nodal status, disease stage, ER and progesterone receptor (PR) status.¹⁷² Using Western blot, low levels of LMW CCNE were detected in 289 patients and high levels were detected in 106. After a median follow-up of 6.4 years, the 5-year DSS and OS rates were 91% (95% CI: 87-94%) and 84% (95% CI:80-88%) for patients with low levels of LMW CCNE versus 17% (95% CI: 10-25%) for both DSS and OS for patients with high levels of LMW CCNE ($p<.001$). On multivariate analysis, independent factors predictive of death from breast cancer included high total CCNE (FL + LMW) levels, high LMW CCNE levels, positive lymph nodes, stage IIIB or IV disease and ER negative disease. Of these factors, high total CCNE levels were the strongest predictor with a hazard ratio of 13.3. An interesting finding in this study was the relationship between CCNE and nodal status. Breast cancer patients with negative lymph nodes have stage I disease which generally predicts for a favorable outcome. However, in this study which included 114 patients with stage I disease, none of 102 with low CCNE levels died within five years of diagnosis while all 12 with high levels of CCNE died of disease. Overall these data suggest a potential role for the routine determination of CCNE levels in breast cancer patients as part of their standard pathologic evaluation. Prior to this becoming standard practice, a prospective validation study is required. In addition, the data from this study point out an important caveat. Levels of CCNE determined by IHC were not significantly associated with death from breast

cancer on multivariate analysis. Routinely obtaining lysates from breast tumors to perform Western blot analysis is not feasible in clinical practice. In addition, the antibody used to detect CCNE targets the protein's carboxy terminus therefore, when used for IHC, cannot differentiate FL from LMW CCNE. As discussed above, LMW CCNE has altered subcellular localization suggesting that cytoplasmic CCNE staining on IHC may serve as a surrogate for LMW CCNE expression. Additional studies are needed to confirm this.

In a study that I performed while working in the laboratory with Drs. Keyomarsi and Hunt, I utilized the subset of 117 patients with HER2-overexpressing tumors from Dr. Keyomarsi's initial cohort of 395. Patients with high total CCNE (n=59) had a 5-year DSS rate of 14% versus 89% for those with low total CCNE (n=58). Stratifying by LMW CCNE levels, we found that patients with high LMW CCNE (n=50) had a 5-year DSS rate of 10% versus 82% for patients with low LMW CCNE (n=67) levels ($p<.0001$). In vitro studies demonstrated that HER2 downregulation or decreased HER2-mediated signaling resulted in decreased expression and function of the LMW forms of CCNE. In vivo studies confirmed this relationship and we found synergistic cell killing when using agents that targeted HER2 (trastuzumab) and CCNE (roscovitine).¹⁷³

The clinical significance of CCNE has been evaluated in other malignancies. In ovarian cancer, CCNE expression and CCNE-associated kinase activity were found to have roles in predicting response to platinum-based chemotherapy.¹⁷⁴ In bladder cancer, FL and LMW CCNE were found to be overexpressed in grade 2 and 3 transitional cell carcinomas with LMW CCNE levels being associated with invasiveness and OS.¹⁷⁵ In addition, a recent meta-analysis of 14 studies showed that CCNE overexpression is a strong predictor of poor prognosis in lung cancer.¹⁷⁶

Taken together, these data suggest that CCNE, particularly the tumor-specific, hyperactive LMW forms, may play a role in multiple different malignancies, suggesting that there may be utility in targeting LMW CCNE therapeutically. As will be detailed in chapter 2 of this thesis, CCNE may be a

target for immunotherapy since its aberrant expression makes it a potentially targetable tumor associated antigen.

CHAPTER 2: CELLULAR UPTAKE OF NEUTROPHIL ELASTASE LINKS INFLAMMATION TO A NOVEL ADAPTIVE IMMUNE RESPONSE

INTRODUCTION

Activated neutrophils are the primary source of NE. However, NE has been found in breast cancer tissue extracts where increased levels were associated with a poor prognosis.¹¹¹⁻¹¹³ The source of NE in breast tumors is unknown but has previously been attributed to endogenous production.^{177, 178} Because neutrophils, the primary source of NE, are present in the tumor microenvironment and because it was shown that lung cancer cells can take up NE,¹³⁰ we hypothesized that breast cancer cells may take up NE as well.¹³²

Although the prognostic value of NE in cancer has generally been attributed to its ability to degrade the ECM thereby promoting invasion and metastasis, the mechanism by which NE confers a poor prognosis in breast cancer is unknown.^{54, 122} One mechanism by which NE may confer a poor prognosis in breast cancer is by its ability to cleave CCNE into its LMW forms. CCNE is an important regulator of the G1 to S phase cell cycle transition. Overexpression of CCNE has been demonstrated in multiple tumor types including breast cancer in which it is associated with a poor prognosis.¹⁷² The principal mode of CCNE deregulation in breast cancer is post-translational processing of the FL protein to LMW forms.¹⁶³ These LMW forms are tumor-specific and as discussed in detail above, are functionally hyperactive thereby promoting progression through the G1/S phase transition point.

The LMW forms of CCNE have been shown in other tumor types. My supervising professor, Dr. Molldrem, has identified LMW CCNE in leukemia and has investigated CCNE as a leukemia-associated antigen.¹⁶² He identified a human leukocyte antigen (HLA-A2)-restricted CCNE-derived peptide, CCNE₁₄₄₋₁₅₂ (ILLDWLMEV) as a target for immunotherapy. HLA-A2 binding assays confirmed peptide binding and standard cytotoxicity assays showed that CCNE₁₄₄₋₁₅₂-specific CTL (CCNE-CTL) could specifically lyse leukemia cells overexpressing CCNE. Importantly, the CCNE₁₄₄₋₁₅₂ peptide is present in FL CCNE and the LMW forms. Because CCNE is aberrantly expressed in

breast cancer, we hypothesized that it may represent an immunotherapeutic target in breast cancer as well.¹³²

Because the CCNE LMW forms are generated by cleavage of the FL protein by NE, we further hypothesized that uptake of NE could increase LMW CCNE expression. Since these LMW forms lack a nuclear localization sequence, they remain in the cytoplasm where they may be preferentially processed and presented as antigens complexed with HLA-I molecules on the cell surface.^{132, 166}

In this chapter, we demonstrate that breast cancer cells lack endogenous NE but can take up NE at concentrations comparable to that present in the tumor microenvironment suggesting TAN as the primary source of NE in breast cancer. We also show that NE uptake results in increased LMW CCNE expression and enhanced susceptibility to lysis by CCNE-CTL. These data therefore show a link between innate immunity and an adaptive immune response against CCNE, a novel breast cancer antigen.

METHODS

The majority of the data in this chapter have recently been published in a manuscript that I was the first author on.¹³² Per The University of Texas Health Science Center at Houston and The University of Texas MD Anderson Cancer Center Graduate School of Biomedical Sciences guidelines, the methods and results sections, including figures and figure legends are taken verbatim from that publication. References and figures have been renumbered ensuring continuity of this thesis.

Patients, cells and cell lines

Peripheral blood samples were obtained through an institutional IRB-approved protocol. MCF-7, MDA-MB-231, T47D, and MDA-MB-453 breast cancer cells, U-937, Jurkat (JKT), HL-60 and T2 cell lines were obtained from American Type Culture Collection. HER-18 was a gift from Dr. Mien-Chie Hung (MD Anderson Cancer Center, Houston, TX). Cell lines were validated by short tandem repeat (STR) DNA fingerprinting using the AmpF/STR Identifiler kit according to manufacturer instructions (Applied Biosystems). Breast cancer cells were cultured in Dulbecco's modified Eagle's medium with 10% FBS, 100U/mL penicillin, and 100µg/mg streptomycin. Media for HER-18 cells was supplemented with 0.5mg/ml G418. U-

937, JKT, T2 and HL-60 cell lines were cultured in RPMI-1640 (RPMI) with 10% FBS, 100U/mL penicillin, and 100µg/mg streptomycin. All cells were maintained in 5% CO₂ at 37°C.

Western blot analysis

Whole cells lysates were generated in RIPA buffer containing protease inhibitors (Santa Cruz Biotechnology). Lysates were run on 10% SDS-page gels then transferred to polyvinylidene fluoride membranes. After blocking, blots were probed with antibodies targeting neutrophil elastase (Santa Cruz Biotechnology) or CCNE (Santa Cruz Biotechnology).

RNA extraction and amplification, cDNA synthesis and reverse transcription polymerase chain reaction

Breast cancer cells were isolated from fresh frozen tumor samples (Origene) by laser capture microdissection (LCM) using an Arcturus PixCell laser capture microscope with an IR diode laser (Life Technologies, Applied Biosystems). Total RNA was extracted and purified using the Arcturus PicoPure RNA Isolation Kit (Life Technologies, Applied Biosystems). RNA integrity and quantity were evaluated by spectrophotometry (Nano Drop ND-1000 Spectrophotometer, Thermo Scientific). Before PCR, RNA was amplified using the Arcturus RiboAmp RNA Amplification Kit (Life Technologies, Applied Biosystems) to generate aRNA. cDNA was synthesized from 1µg of aRNA using the Roche Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science). For cultured cell lines, total cellular RNA was extracted and isolated using RNA STAT-60 RNA extraction reagent (Amsbio). cDNA was synthesized as described above.

Reverse transcriptase PCR (RT-PCR) reactions were carried out on an iCycler iQ thermal cycler (Bio-Rad Laboratories). Primer sequences used included neutrophil elastase (forward primer 5'-CACGGAGGGGCAGAGACC-3', reverse primer 5'-TATTGTGCCAGATGCTGGAG-3'), mammaglobin (forward primer 5'-AGCACTGCTACGCAGGCTCT-3', reverse primer 5'-ATAAGAAAGAGAAGGTGTGG-3'), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an endogenous control, (forward primer 5'-TAGACGGGAAGCTCACTGGC-3', reverse primer 5'-AGGTCCACCACCCTGTTGCT-3'); oligonucleotides from Sigma Aldrich).

Immunohistochemistry

Following LCM, remaining tumor tissue was fixed in formalin and paraffin-embedded for immunohistochemistry. Tissue sections were deparaffinized and rehydrated. Nonspecific binding was blocked, after which sections were incubated with primary antineutrophil elastase monoclonal antibody (1:200; Clone NP-57, Dako). Slides were incubated with secondary anti-mouse IgG-biotin antibody (1:200; Vectastain Elite ABC Kit; Vector laboratories) then with the avidin-biotin peroxidase complex (1:100; Vectastain Elite ABC Kit) after which visualization was conducted with chromagen 3, 3'-diaminobenzidine (Dako). Sections of normal tonsil tissue with neutrophils were used as positive controls. Omission of the primary antibodies were used as negative staining control.

Confocal microscopy and flow cytometry analysis

To evaluate uptake of soluble neutrophil elastase, cells were maintained in low serum (0.5%) media supplemented with neutrophil elastase prepared from whole blood and purified to more than 95% (Athens Research and Technology). Cathepsin G (Athens Research and Technology) was prepared in an identical fashion, therefore used as a control to show specificity of uptake. After culture in media supplemented with neutrophil elastase over a range of concentrations, viability was assessed at 1, 4, or 24 hours by trypan blue exclusion assay or

by staining with SYTOX blue dead cell stain (Invitrogen). Neutrophil elastase activity was determined using a fluorescent substrate assay (Enzcheck Protease Assay; Invitrogen) according to the manufacturer's instructions. Dose and time course experiments were carried out. Briefly, 2×10^5 cells were maintained in 6-well plates in media supplemented with various concentrations of neutrophil elastase at 37°. At designated timepoints, cells were harvested, permeabilized and stained with the following antibodies: Alexa-647- or -488-conjugated antineutrophil elastase (clone NP57; Santa Cruz), fluorescein isothiocyanate (FITC)-conjugated anti-EEA-1 (BD Biosciences), or FITC -conjugated anti-LAMP-2 (eBioscience). Direct conjugation of antineutrophil elastase antibody was carried out using Alexa-647 and 488 conjugation kits (Invitrogen). Aqua live/dead stain (Invitrogen) was used to assess cell viability. Flow cytometry was done using the Cytomation CyAn flow cytometer (Beckman Coulter). Data were analyzed using FlowJo software (Tree Star Inc.). Confocal imaging was carried out using a Leica Microsystems SP2 SE confocal microscope. To evaluate uptake of cell-associated neutrophil elastase, neutrophils were isolated from healthy donors by double Ficoll, after which they were irradiated and co-cultured with MDA-MB-231 cells at a 3:1 ratio for four hours.

Peptide-specific CTL lines and cell-mediated cytotoxicity assay

Healthy donor HLA-A2⁺ peripheral blood mononuclear cells (PBMC) were stimulated with CCNE₁₄₄₋₁₅₂-peptide, as previously described.¹⁷⁹ Briefly, T2 cells were incubated with 20 µg/mL of CCNE for 90 minutes at 37°C and then irradiated and cultured with freshly isolated PBMCs at a 1:1 ratio. On days 7, 14, and 21, re-stimulation with CCNE-pulsed T2 cells was carried out, and the following day 20 IU/mL of recombinant human interleukin-2 (IL-2; Biosource International) was added. On day 25, CTLs were harvested and used in cytotoxicity assays as previously described.¹⁷⁹ Target cells, including T2 cells ± CCNE peptide and HLA-A2⁺ breast cancer cells, were stained with 10 µg/mL of Calcein-AM (Sigma Aldrich), washed and plated in a 60-well Terasaki tray (2×10^3 cells/10 µl/well). Effector cells (CCNE-CTL) were resuspended in 10 µl at increasing effector to target dilutions and added to target cells. After 4 hours, trypan blue was added as a quenching agent. Fluorescence was measured (FLx800 Microplate fluorescence reader, Bio-Tek Instruments) and the percentage of cell lysis was calculated as follows: % cytotoxicity = $(1 - (E_{\text{experimental}} - E_{\text{Media}}) / (E_{\text{Control}} - E_{\text{media}})) \times 100$, where E is fluorescence emission and control group is targets alone.

Staining for CCNE-CTL in breast cancer patients

PBMC from HLA-A2⁺ breast cancer patients and healthy donors were stained with aqua live/dead stain (Invitrogen) and the following antibodies; CD8 APC-H7 (BD Biosciences), CD3 PE Cy7 (BD Biosciences), CD4 pacific orange (Invitrogen), CCNE-APC-conjugated tetramer and the following pacific blue conjugated lineage antibodies: CD14 (BD Biosciences), CD16 (BD Biosciences) and CD19 (Biolegend). Data were acquired on a Canto flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc.). The frequency of CCNE-CTLs was determined as the percentage of cells that were alive, lineage⁺, CD4⁺, CD3⁺, CD8⁺ and CCNE-tetramer⁺.

Statistical Analysis

GraphPad Prism 5.0 software was used to perform statistical analyses and *P*-values less than 0.05 were considered significant.¹³²

Additional methods

In this chapter, we will show additional data not included in our recently published manuscript confirming that NE uptake occurs via clathrin-coated pits. Briefly, cells were pretreated with chlorpromazine (10 µg/ml) to inhibit clathrin coated pits after which soluble NE was added to the media. After 30 hours, cells were harvested, permeabilized and stained for intracellular NE. Data were acquired on the Canto flow cytometer and analyzed using FlowJo software.

RESULTS

Breast cancer cells do not produce endogenous elastase

Because breast cancer cells are not derived from myeloid hematopoietic progenitors, the source of neutrophil elastase in breast tumors is not fully understood. To investigate this, we evaluated cultured breast cancer cell lines for the presence of neutrophil elastase at the protein and mRNA level. We did not detect neutrophil elastase protein (Fig. 5A) or mRNA transcripts (

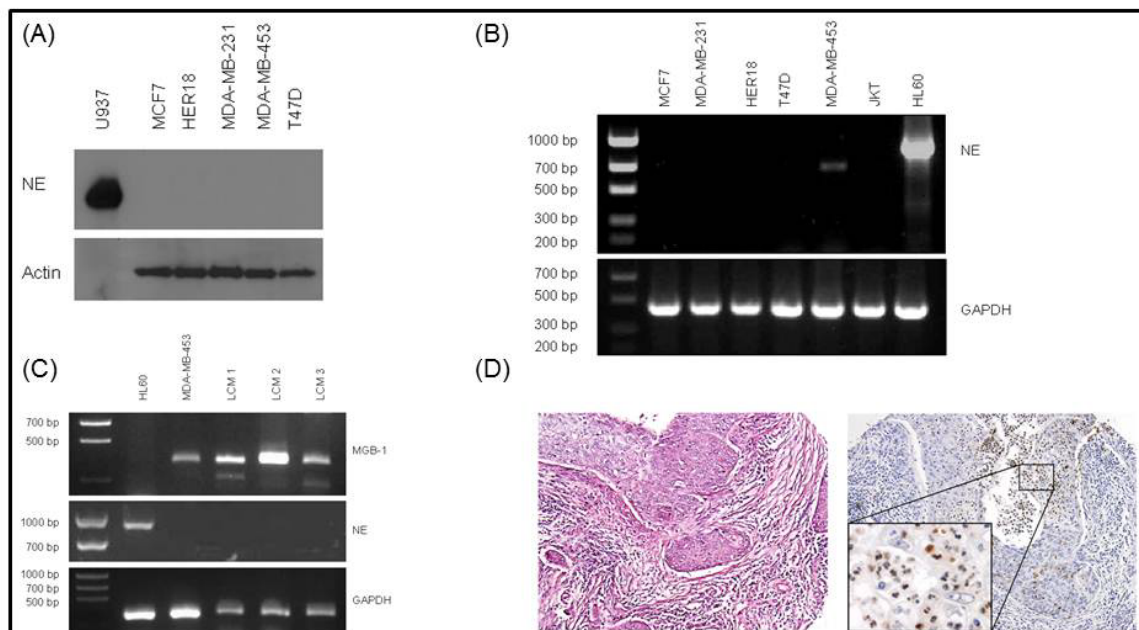
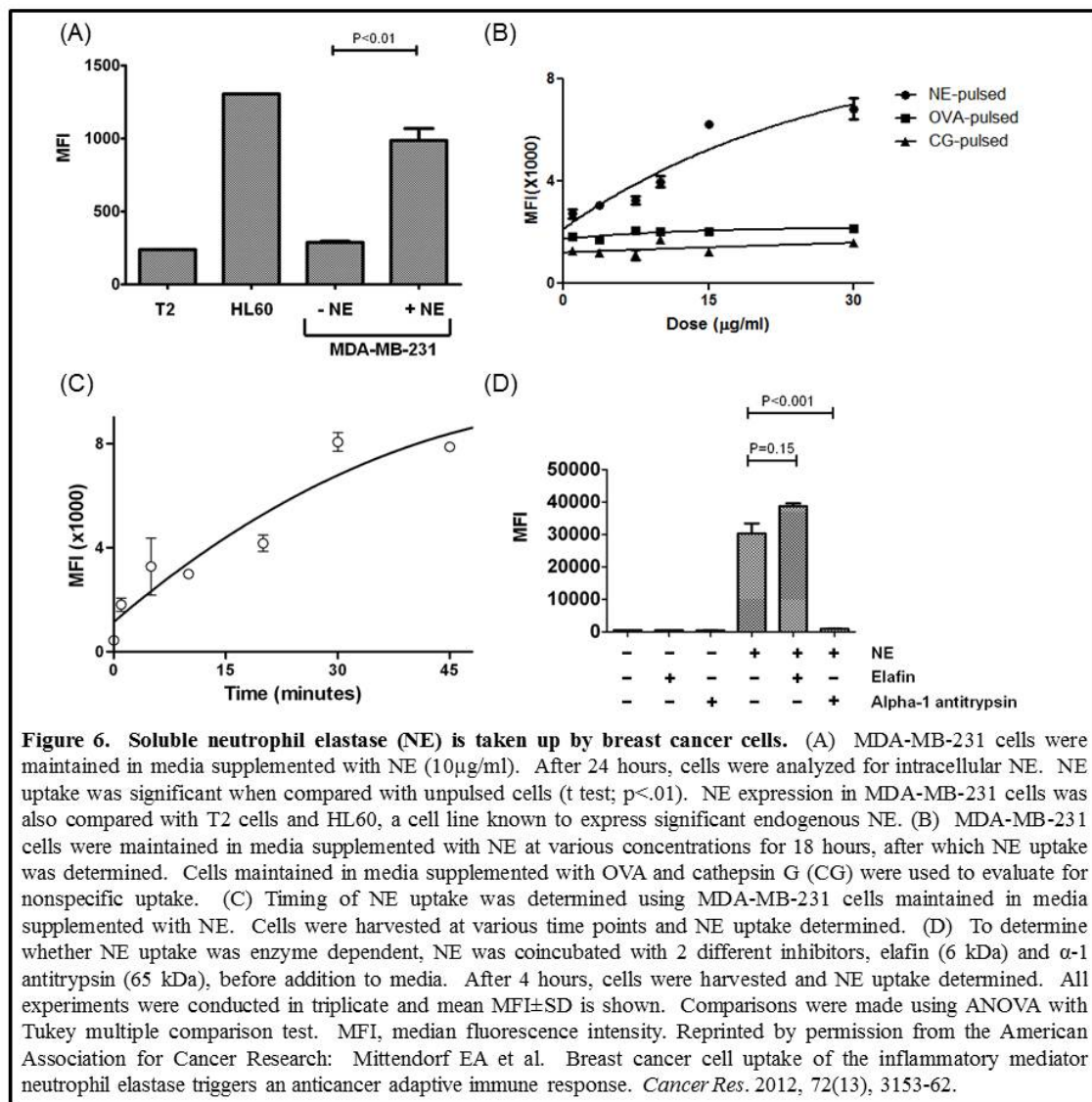


Figure 5. Breast cancer cells do not express endogenous neutrophil elastase (NE). (A) Lysates harvested from breast cancer cell lines cultured in 0.5% serum media were used in Western blots showing no NE protein expression. The human leukemia cell line U937 served as a positive control. (B) RNA extracted from cultured breast cancer cells was used to carry out RT-PCR using primers to NE. The leukemia cell line HL60 and T cell line jurkat (JKT) served as positive and negative controls, respectively, for NE. No NE mRNA was detected in any of the cell lines evaluated. (C) RT-PCR evaluating for NE mRNA was carried out using RNA extracted from breast cancer cells isolated from three primary tumors using LCM (LCM1, LCM2 and LCM3). Primers to mammoglobin (MGB-1) were used to confirm the LCM was specific for breast cancer cells. Positive controls included MDA-MB-453 breast cancer cells for mammoglobin and HL60 for NE. No LCM specimens showed NE mRNA (D) A breast tumor was evaluated by hematoxylin and eosin staining to confirm tumor cells and inflammatory infiltrate. This same tumor was evaluated by immunohistochemistry for NE. TAN present in the microenvironment stained positive for NE by immunohistochemistry. Reprinted by permission from the American Association for Cancer Research: Mittendorf EA et al. Breast cancer cell uptake of the inflammatory mediator neutrophil elastase triggers an anticancer adaptive immune response. Cancer Res. 2012, 72(13), 3153-62.

investigated. To evaluate whether the lack of expression was limited to cell lines, LCM was used to isolate breast cancer cells from primary tumors. Following RNA extraction, RT-PCR confirmed the lack of neutrophil elastase mRNA in all breast cancer specimens evaluated (Fig. 5C). Immunohistochemistry carried out on breast tumor tissue showed neutrophil elastase in TAN within the tumor microenvironment, but not in breast cancer cells (Fig. 5D). Taken together, these data are consistent with our hypothesis that TAN present in the microenvironment are the primary source of neutrophil elastase in breast tumors.

Soluble and cell associated NE are taken up by breast cancer cells

We have previously shown that antigen-presenting cells are capable of taking up soluble neutrophil elastase and that this uptake leads to cross-presentation of PR1, a nonameric peptide derived from neutrophil elastase that has been extensively investigated in myelogenous leukemias.^{118, 121, 179, 180} In addition, Houghton and colleagues showed the ability of lung cancer cells to take up neutrophil elastase.¹³⁰ We therefore investigated whether breast cancer cells can take up neutrophil elastase using MDA-MB-231 breast cancer cells cultured in neutrophil elastase-supplemented media. At 24 hours, flow cytometry was used to show neutrophil elastase uptake (3.4-fold increase in MFI vs. unpulsed). At that point, the extent of neutrophil elastase following uptake was 76% of the level of neutrophil elastase in HL-60, a promyelocytic



leukemia cell line known to express endogenous neutrophil elastase (Fig. 6A). Cell viability was not affected by neutrophil elastase in the culture media (Supplementary fig. 1). Experiments were repeated using additional breast cancer cell lines (HER18 and MDA-MB-453), which showed neutrophil elastase uptake at 1, 4 and 24-hour time points (Supplementary fig. 2). The extent of neutrophil elastase uptake varied among cell lines.

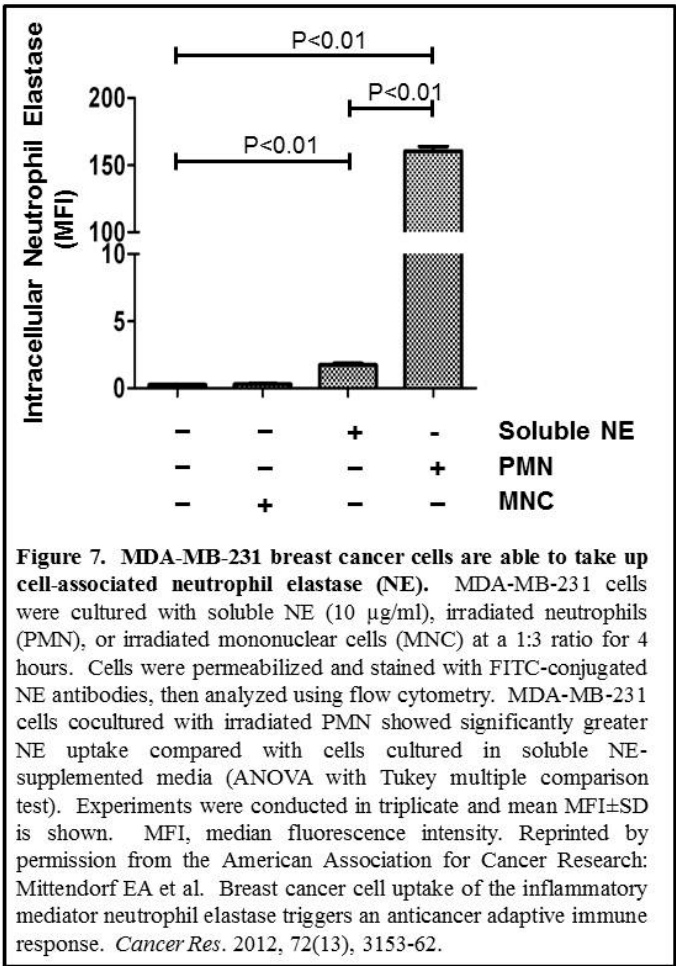
To further study soluble neutrophil elastase uptake by MDA-MB-231 cells, concentration-response and time course experiments were carried out. Concentration-response experiments showed concentration-dependent uptake of neutrophil elastase. Importantly, there was no uptake of the non-specific proteins OVA or cathepsin G, a second serine protease (Fig. 6B), suggesting neutrophil elastase uptake is antigen specific. Time course experiments showed that neutrophil elastase uptake was time-dependent and occurred as early as one minute after addition of soluble neutrophil elastase to the culture media (Fig. 6C). Confocal imaging confirmed early neutrophil elastase uptake by breast cancer cells and localization within distinct compartments, as shown by focal neutrophil elastase staining (Supplementary fig. 3).

We next sought to investigate whether enzymatic activity of neutrophil elastase was required for uptake. Neutrophil elastase was incubated with one of 2 neutrophil elastase inhibitors, α -1 antitrypsin (65kD) or elafin (6kD), prior to its addition to culture media. Neutrophil elastase enzymatic activity inhibition by α -1 antitrypsin and elafin was confirmed (Supplementary fig. 4). α -1 antitrypsin inhibited neutrophil elastase uptake whereas elafin had no effect (Fig. 6D) suggesting a potential steric-dependent but enzyme-independent mechanism.

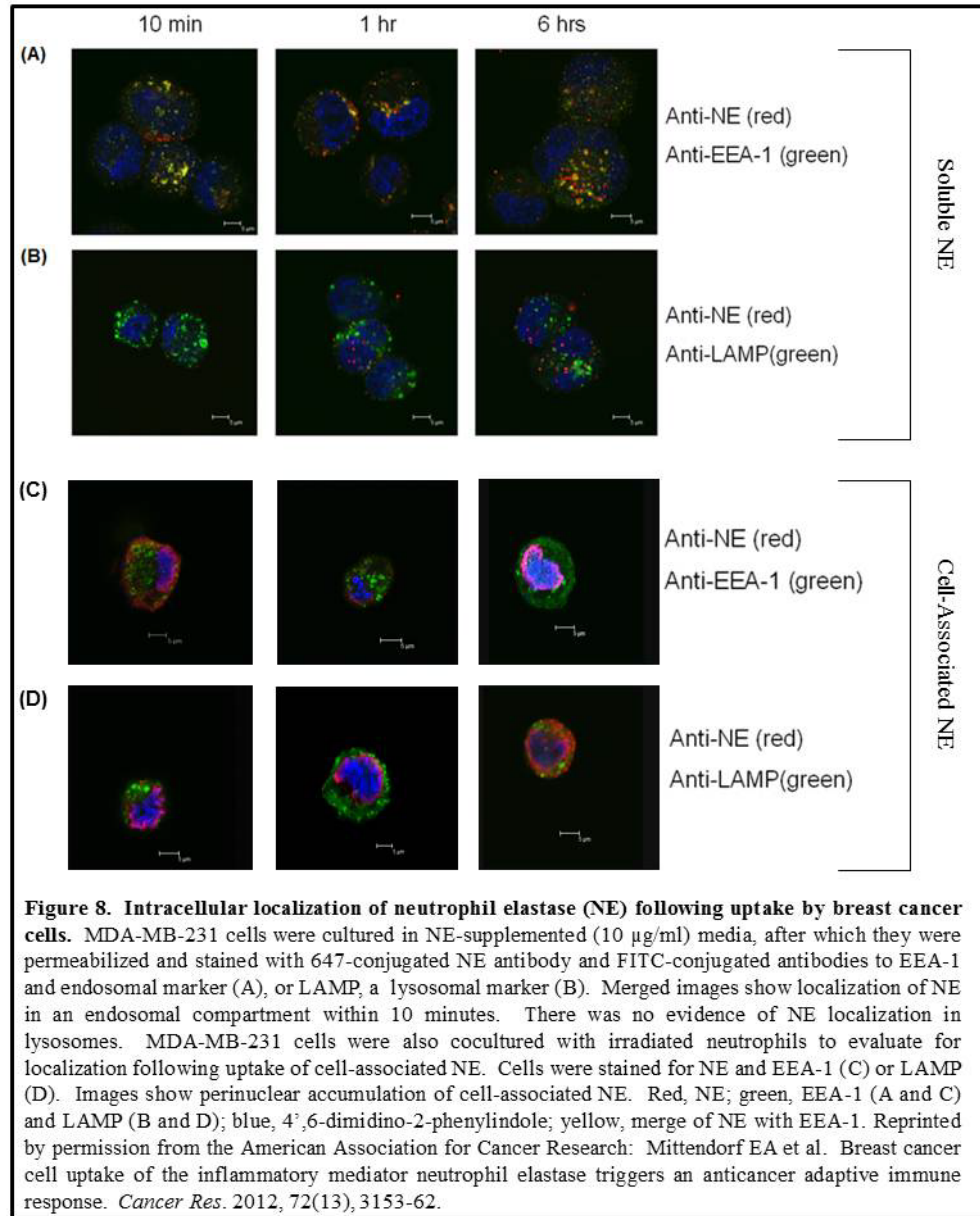
Having shown concentration and time-dependent uptake of soluble neutrophil elastase by MDA-MB-231 cells, we next investigated whether these cells could take up cell-associated neutrophil elastase. MDA-MB-231 cells were co-cultured with irradiated neutrophils as a source of neutrophil elastase or irradiated lymphocytes, which lack neutrophil elastase. Radiation induced cell death in approximately 50% of neutrophils and the concentration of neutrophil elastase in the culture at 4 hours was 16 μ g/ml. Uptake of neutrophil elastase was again determined using flow cytometry which showed greater uptake of the cell-associated neutrophil elastase than soluble neutrophil elastase ($P < .01$) (Fig. 7).

Neutrophil elastase localization following uptake

Confocal images had shown uptake of soluble



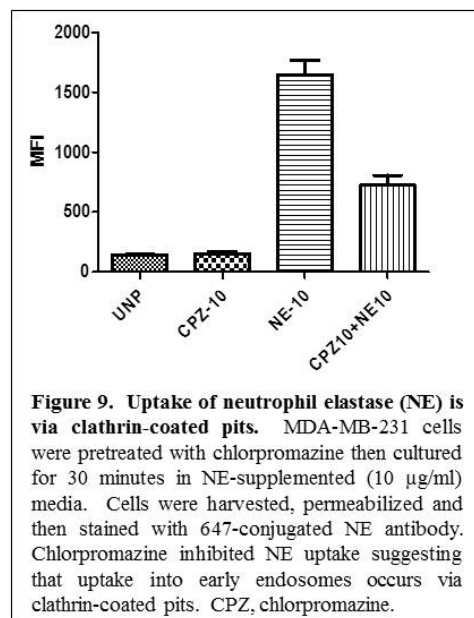
neutrophil elastase into distinct cellular compartments (Supplementary fig. 3). We therefore sought to determine the subcellular compartment to which neutrophil elastase localized after uptake. MDA-MB-231 cells were cultured in neutrophil elastase-supplemented (10 μ g/ml) media. At increasing time points, cells were harvested and co-stained for neutrophil elastase and either early endosomal antigen-1 (EEA-1) or lysosome-associated membrane protein (LAMP)-2 as markers for early endosomes or lysosomes, respectively. These experiments confirmed early uptake of neutrophil elastase as it was detected intracellularly within 10 minutes, and showed that soluble neutrophil elastase localizes to an early endosomal



compartment (Fig. 8A). There was no evidence of neutrophil elastase uptake into lysosomes (Fig. 8B). Experiments were repeated following uptake of cell-associated neutrophil elastase which showed early uptake of neutrophil elastase with perinuclear localization (Fig. 8C and 8D).

NE is taken up into clathrin coated pits

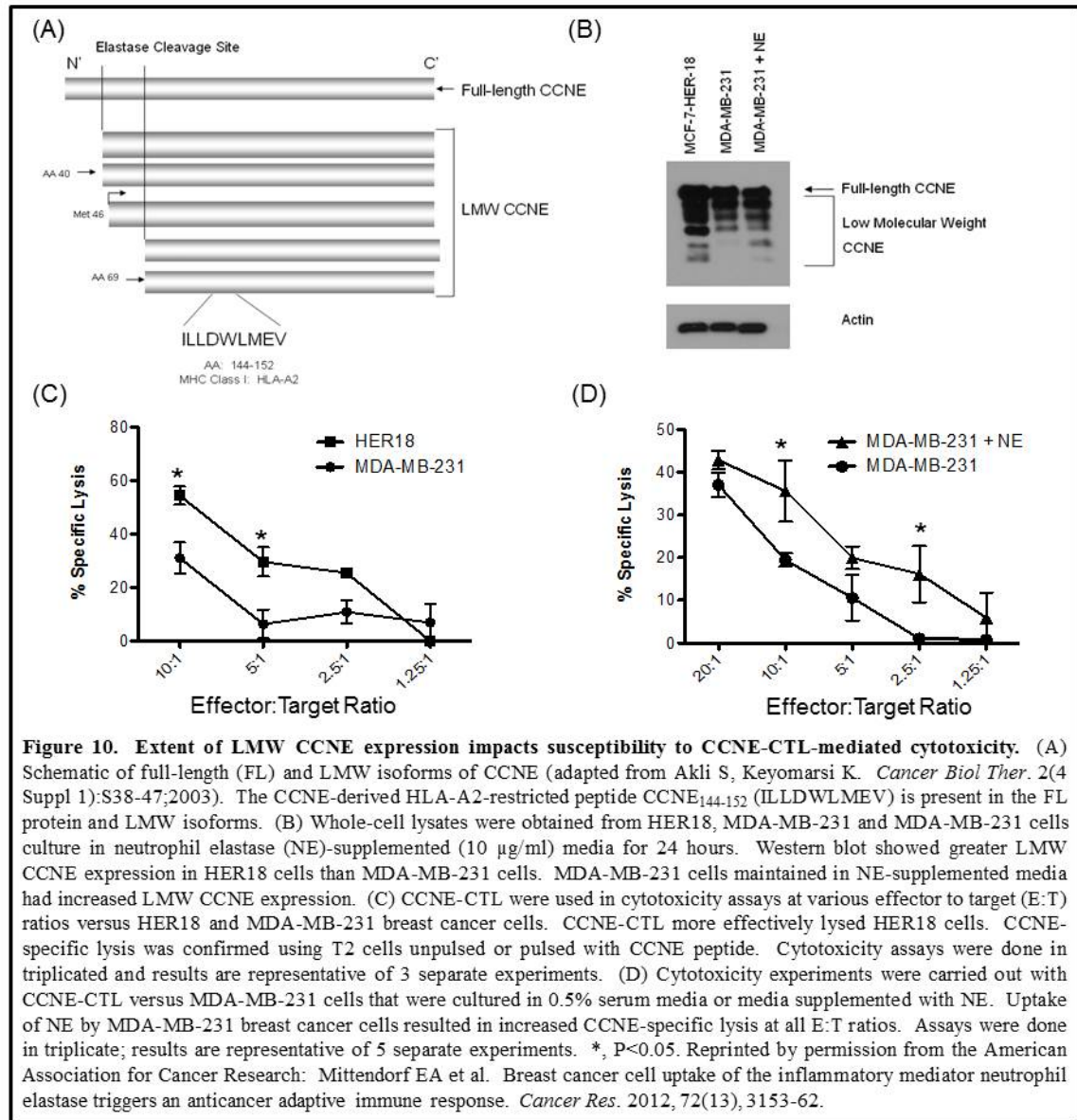
Having shown that NE localizes to early endosomes after uptake, we next sought to confirm that uptake was via clathrin-coated pits. MDA-MB-231 breast cancer cells were pretreated with chlorpromazine, an agent known to block clathrin-mediated endocytosis, after which soluble NE (10 μ g/ml) was added to the media. As shown in figure 9, pretreatment with chlorpromazine inhibited NE uptake consistent with the findings of Houghton et al. who showed uptake of NE into endosomes via clathrin coated pits in a lung cancer cells. These data were not included in our recent publication in *Cancer Research*.



Uptake of soluble elastase increases LMW CCNE expression and susceptibility to CCNE-CTL mediated cytotoxicity

Neutrophil elastase has been shown to cleave full-length CCNE at 2 sites giving rise to LMW isoforms which subsequently undergo phosphorylation to generate 2 sets of doublets (Fig. 10A)^{163, 164}. LMW isoforms of CCNE lack a nuclear localization sequence and therefore accumulate in the cytoplasm^{165, 166} which may facilitate ubiquitination and proteasomal processing for presentation on HLA-I molecules¹⁸¹⁻¹⁸⁴. We therefore hypothesized that cells with increased LMW CCNE would be more susceptible to lysis by CCNE-CTL, by virtue of increased HLA/CCNE₁₄₄₋₁₅₂ surface expression. To test this hypothesis, we expanded CCNE₁₄₄₋₁₅₂-CTL from PBMC from HLA-A2⁺ healthy donors. Lysis was tested using cytotoxicity assays. Initially, HER18 and MDA-MB-231 (both HLA-A2⁺), were used as targets because of differences in baseline LMW CCNE expression (Fig. 13B).¹⁷³ These assays showed that CCNE-CTL more effectively lysed HER18, which express more LMW CCNE than MDA-MB-231 (Fig. 10C). CCNE-CTL specific cytolysis was confirmed using unpulsed T2 cells and T2 cells pulsed with CCNE (Supplementary fig. 5). We next investigated the effect of neutrophil elastase uptake on LMW CCNE expression by MDA-MB-231 cells and whether this impacted susceptibility to lysis by CCNE-specific CTL. Western blot analysis confirmed that uptake of soluble neutrophil elastase resulted in increased expression of LMW CCNE (Fig. 10B). Processing of CCNE by neutrophil elastase to LMW CCNE was confirmed using recombinant CCNE incubated with neutrophil elastase over a range of concentrations (5 μ g/ml - 100 μ g/ml) (Supplementary fig. 6). Importantly, CCNE-CTL specific lysis of neutrophil elastase-pulsed MDA-MB-231 cells was greater than that versus unpulsed cells (Fig. 10D). The cytotoxicity assays were performed multiple times using CTL generated from different healthy donors with variable precursor frequencies of CCNE-CTLs thereby explaining differences in the absolute levels of CCNE-specific killing. Taken together, these data showed that exogenous neutrophil elastase such as may be present in the tumor microenvironment, can be taken up by breast

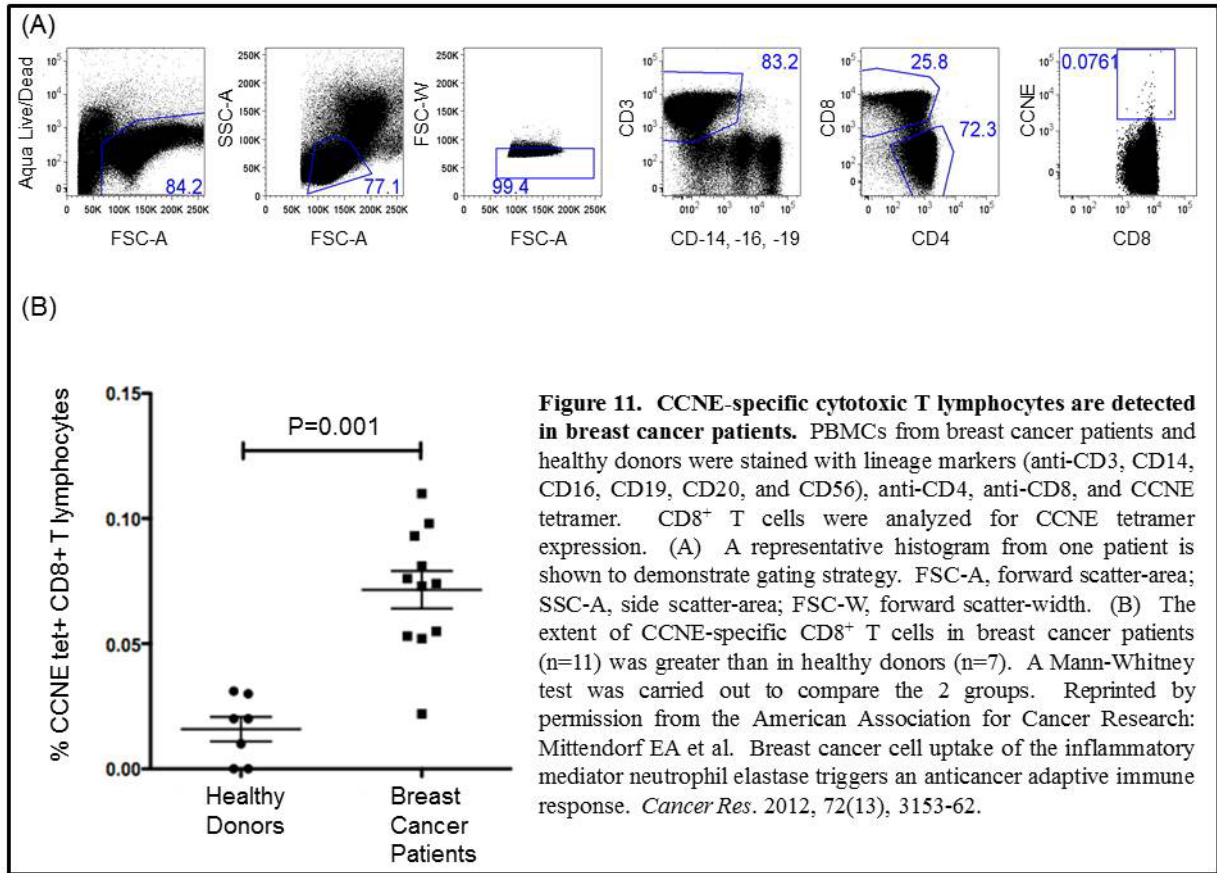
cancer cells exposing the CCNE-derived epitope and rendering the cells susceptible to CCNE-CTL-mediated cytotoxicity.



CCNE₁₄₄₋₁₅₂ tetramer positive CD8⁺ T cells are present in peripheral blood of breast cancer patients.

Having shown that breast cancer cells expressing CCNE are susceptible to lysis by CCNE-CTL, we next sought to confirm whether immunity to CCNE₁₄₄₋₁₅₂ is detected in breast cancer patients. PBMC were obtained from 11 HLA-A2⁺ breast cancer patients and 7 HLA-A2⁺ healthy donors and stained with CCNE₁₄₄₋₁₅₂ tetramer to assess the frequency of CCNE₁₄₄₋₁₅₂-specific CTL. Figure 11A demonstrates our gating strategy. All breast cancer patients had CCNE₁₄₄₋₁₅₂-specific CTL present at a low precursor frequency with the median number of CCNE₁₄₄₋₁₅₂-specific CTL=0.074±0.02 (Fig. 11B). The frequency of CCNE₁₄₄₋₁₅₂-specific CTL

in breast cancer patients was greater than in healthy donors ($P=0.001$). These data suggested that the CCNE₁₄₄₋₁₅₂ peptide is naturally processed in breast cancer patients resulting in immunity to CCNE and that vaccination with a CCNE₁₄₄₋₁₅₂ peptide could potentially augment the immunologic response against CCNE-expressing breast cancer targets.¹³²



DISCUSSION

In this chapter, we have identified a novel function for NE in breast cancer. We have shown that: 1) breast cancer cells do not produce NE but are capable of taking up NE in an antigen-specific manner; 2) NE is present in TANs suggesting TANs as the primary source of NE in breast cancer; 3) after NE uptake, LMW forms of CCNE increase leading to enhanced susceptibility of breast cancer cells to lysis by CCNE₁₄₄₋₁₅₂-CTL and 4) PBMC from HLA-A2⁺ breast cancer patients have a low precursor frequency of CCNE₁₄₄₋₁₅₂-CTL suggesting that the peptide is naturally processed and presented. We have therefore established a novel mechanism linking NE, a serine protease released by innate immune cells, to an adaptive immune response against CCNE, a novel breast cancer antigen.¹³²

Inflammatory cells, to include neutrophils, comprise a significant component of the tumor microenvironment. Until recently, neutrophils have received little attention due to their short half-life. Although their life span in the circulation is short (8-10 hours), they live longer at sites of inflammation due in part to positive effects of cytokines in the microenvironment on their survival.¹⁸⁵ In addition, tumor and inflammatory cells upregulate neutrophil-chemotactic substances including IL-8 which results in continuous recruitment of neutrophils to a tumor site.⁶⁹ Neutrophils themselves secrete IL-8 suggesting that there could be a positive feedback loop ensuring TAN recruitment.^{23, 30}

There is increasing interest in studying TANs and as has been discussed above, reports showing that for multiple tumor types to include renal cell carcinoma, colorectal cancer, NSCLC and hepatocellular cancer, TAN are associated with a poor prognosis.⁴²⁻⁴⁵ No studies have evaluated the role of TAN in breast cancer, however, it has been reported that the presence of NE in whole tumor extracts from breast cancer patients correlates with worse outcomes.¹¹¹⁻¹¹³ The source of NE in breast cancer is unknown. A study by Nguyen et al. showed NE protein in MDA-MB-231 cells by indirect immunofluorescence.¹⁸⁶ However, we were unable to identify NE protein in MDA-MB-231 or other cultured breast cancer cells by Western blots of whole cell lysates or by confocal microscopy. We were also unable to find NE mRNA transcripts. Importantly, using RNA isolated from breast cancer with single cell laser capture microdissection, we were unable to amplify NE mRNA transcripts. We believe therefore that we are the first to show that TAN within the tumor microenvironment is the source of NE in breast cancer. To our knowledge, the presence of NE mRNA in epithelial cells has not been shown definitively, thereby supporting our conclusion.

As was discussed in detail in the introduction of this thesis, TAN have multiple effects within the tumor microenvironment. Protumor effects include promoting invasion and metastasis, are in part due to the effects of NE which can remodel the ECM.⁵⁴ Recently, Houghton et al. demonstrated a novel mechanism by which NE could promote tumor growth via an effect directly on tumor cells. Using a lung cancer model, they showed that NE could be taken up by tumor cells into clathrin-coated vesicles and localized to early endosomes.¹³⁰ NE cleaved IRS-1 leading to hyperactivity of the PI3K

pathway and uncontrolled proliferation. We have confirmed that tumor cells can take up exogenous NE; both soluble and cell-associated. Uptake is antigen specific and both dose and time dependent suggesting a receptor-mediated mechanism. Uptake was inhibited by α -1 antitrypsin but not elafin suggesting that the mechanism is potentially steric-dependent but enzyme-independent. We have also shown another mechanism by which NE uptake could promote proliferation – generation of CCNE LMW forms. Conversely, NE may also potentiate an anti-tumor immune response by increasing the susceptibility of tumor cells to lysis by CCNE-CTL. The net effect of NE uptake by tumor cells likely depends upon multiple factors.

Cyclin E has characteristics of an ideal tumor-associated antigen (TAA). It is aberrantly expressed as tumor-specific LMW forms and overexpression of these LMW forms drives cancer cell proliferation.^{155, 163} Our data showing a low precursor frequency of CCNE₁₄₄₋₁₅₂-tetramer positive CTL in the PBMC of breast cancer patients confirms that this peptide is naturally processed generating adaptive immunity in patients. An immunotherapeutic strategy administering the CCNE₁₄₄₋₁₅₂ peptide combined with an immunoadjuvant may effectively augment a CCNE-specific CTL response.

Combined with previous studies showing that NE cleaves CCNE into LMW forms,¹⁶³ our data suggest that after uptake, NE increases availability of LMW CCNE in breast cancer cells which could enhance antigen processing and presentation of CCNE peptides. This is supported by cytotoxicity assays performed using CCNE-CTL versus HER18 and MDA-MB-231 cells which have comparable amounts of FL CCNE but differing LMW CCNE expression. HER18 cells have greater LMW CCNE expression and were more susceptible to lysis by CCNE-CTL. Furthermore, after uptake of soluble NE from culture media by MDA-MB-231 cells there was an increase in LMW CCNE expression and a concomitant increase in susceptibility to lysis by CCNE-CTL. Additional work is required to confirm that the LMW forms (versus FL CCNE) are the predominant source of CCNE₁₄₄₋₁₅₂ peptide. Because the LMW forms of CCNE are tumor specific, this could have important implications for an immunotherapy strategy targeting CCNE.

Although NE uptake could promote an anti-tumor immune response by increasing susceptibility to CCNE-CTL, it is also possible that NE uptake could promote tolerance due to anergy induced by antigen presentation in the absence of adequate co-stimulation. Breast cancer cells express MHC class I molecules and can present CCNE₁₄₄₋₁₅₂ and other peptides however they lack costimulatory molecules thus may be unable to stimulate naïve T cells. If this were the case, other strategies including antibodies targeting the CCNE₁₄₄₋₁₅₂/HLA-A2 conformational epitope or adoptive T-cell therapy could be used to overcome this tolerance.^{187, 188}

In conclusion, we have shown that breast cancer cells take up NE resulting in increased LMW CCNE expression and enhanced susceptibility to lysis by CTL specific for a novel HLA-A2-restricted CCNE-derived peptide. Therefore, we propose a mechanism linking NE derived from TAN in the tumor microenvironment, to an adaptive immune response against CCNE, a novel antigen that is cleaved into tumor-specific LMW forms after NE uptake. Additional investigation into the mechanism of NE uptake and the effects on antigen processing and presentation are indicated to improve our understanding of the interaction between inflammation and adaptive immunity in breast cancer.

CHAPTER 3: CROSS-PRESENTATION OF NE AND PR3 BY BREAST

INTRODUCTION

Neutrophil elastase and PR3 are serine proteases stored in neutrophil primary granules. The biology of these proteases as well as their role in infection, inflammation and cancer has been discussed in detail above. My advisor, Dr. Molldrem has identified PR1, a conserved nonameric HLA-A2-restricted peptide in NE and PR3.¹¹⁶⁻¹¹⁸ PR1-specific CTL have been detected in the peripheral blood of patients with myeloid leukemia where they correlated with positive clinic outcomes following IFN- α 2b therapy or allogeneic bone marrow or stem cell transplant.¹¹⁸⁻¹²⁰ Antigen processing and presentation has been extensively studied (reviewed by Neefjes et al.).¹⁸⁹ HLA class-I molecules are expressed by all nucleated cells and are capable of presenting intracellular antigens to CD8⁺ T cells. HLA class II molecules are expressed primarily on the surface of antigen presenting cells (APCs) and express extracellular antigens to CD4⁺ T cells. Cross-presentation is a mechanism whereby exogenously derived antigens can be complexed with HLA class I molecules for presentation to CD8⁺ T cells. It was originally thought that cross-presentation was restricted to subpopulations of APCs.¹⁹⁰⁻¹⁹² However, a report by Francois et al. showed that mesenchymal stromal cells could cross-present soluble exogenous antigens in a mouse model.¹⁹³

Recently, in a study that I was involved in, Alatrash et al. showed that NE and P3 are cross-presented by normal donor APCs and leukemia, and that cross-presentation by leukemia cells rendered them susceptible to PR1 targeted therapy.¹⁹⁴ Because we showed that NE could be taken up by breast cancer cells,¹³² we hypothesized that uptake of NE and PR3 by breast cancer may lead to cross-presentation making them susceptible to PR1-targeted therapy. In this chapter, we demonstrate that PR3, like NE, is taken up breast cancer cells and that this leads to cross-presentation increasing susceptibility of breast cancer cells to killing by PR1-targeted therapies including PR1-CTL and 8F4, a novel T-cell receptor-like immunoglobulin G2a antibody with high binding affinity for the PR1/HLA-A2 complex.¹⁸⁸ Furthermore, we show PR1 in association with HLA-A2 on the surface of primary

breast tumors and the presence of PR1-CTL in peripheral blood from breast cancer patients. Taken together, these data demonstrate the ability of breast cancer to cross-present NE and PR3 and suggest that PR1 may be a broadly expressed antigenic epitope.

METHODS

Patients, cells and cell lines

Peripheral blood samples were collected as part of an Institutional Review Board-approved protocol. PBMC and neutrophils were enriched using standard Histopaque 1077 and 1119 (Sigma) gradient centrifugation, respectively. Breast cancer frozen tissue blocks were obtained from Origene. MCF-7, MDA-MB-231, T47D, and MDA-MB-453 breast cancer cells, U-937, Jurkat (JKT), HL-60 and T2 cell lines were obtained from American Type Culture Collection. HER18 cells were provided by Dr. Mien-Chie Hung (MD Anderson Cancer Center, Houston, TX). Cell lines were validated by STR DNA fingerprinting. Breast cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100U/mL penicillin, 100µg/mg streptomycin, and 10% FBS. G418 (0.5mg/ml) was added to HER18 media as a selective agent. U-937, JKT, T2 and HL-60 cell lines were cultured in RPMI-1640 (RPMI) with 100U/mL penicillin, 100µg/mg streptomycin, and 10% FBS. All cells were maintained in 5% CO₂ at 37°C.

Western blot analysis

Whole cells lysates were generated by suspending cell pellets in RIPA buffer containing protease inhibitors (Santa Cruz Biotechnology). Lysates were separated by electrophoresis on 10% SDS gels under reducing conditions then transferred to PVDF membranes. After blocking, blots were probed with antibodies targeting PR3 (NeoMarkers) or GAPDH (Sigma).

RNA extraction and amplification, cDNA synthesis and reverse transcription polymerase chain reaction

As previously described, breast cancer cells were isolated from fresh frozen tumor samples by LCM. Total RNA was extracted and purified, and RNA integrity and quantity were determined by

spectrophotometry. Prior to PCR, RNA was amplified using two rounds of T7-based amplification yielding 2.5µg of aRNA. cDNA was synthesized from 1µg of aRNA. For cultured cell lines, total cellular RNA was extracted and isolated and cDNA was synthesized as described above.¹³² For RT-PCR, the primer sequences for PR3 included: forward primer 5'-GACCCCACCATGGCTCAC-3', reverse primer 5'-ATGGGAAGGACAGACAGGAG-3'. The primer sequences for mammaglobin and GAPDH were included above (chapter 2).

Flow cytometry analysis and confocal microscopy

To evaluate protein uptake, cells were maintained in reduced serum media (0.5% FBS) containing 10 µg/ml NE or PR3 (Athens Research and Technology) or EndoGrade ovalbumin (Ova) (Hyglos). To evaluate cell-associated uptake of neutrophil proteases, breast cancer cells were co-cultured with irradiated neutrophils at a 1:1 ratio (breast cancer : irradiated-cell). Cells were permeabilized and stained with alexa- 647 directly conjugated anti-PR3 (Clone MCPR3-2; Thermo Scientific) antibody and analyzed by flow cytometry. Aqua live/dead stain (Invitrogen) was used to assess viability. Data were analyzed using FlowJo software (Tree Star Inc.,). Confocal imaging to show PR3 intracellular localization was performed using a Leica Microsystems SP2 SE confocal microscope. Antibodies used for confocal imaging included alexa-647 directly conjugated anti-PR3 and FITC-conjugated LAMP-2 antibody (eBioscience) to stain for lysosomes. For experiments investigating cross-presentation, cells were surface-stained with fluorescently-conjugated 8F4 antibody as previously described.¹⁸⁸

Immunohistochemistry

Cryopreserved breast tumor tissue was formalin fixed then paraffin-embedded for IHC. Prior to staining, tissue sections were de-paraffinized, re-hydrated and quenched for endogenous peroxidase activity. Non-specific binding was blocked with 10% normal horse serum then incubated with primary WGM2 anti-PR3 mAb clone (1:10) (Abcam). Slides were washed and incubated with secondary anti-mouse IgG-biotin antibody (1:200) (Vectastain Elite ABC Kit) followed by avidin-biotin

peroxidase (1:100) (Vectastain Elite ABC Kit). Chromagen 3, 3'-diaminobenzidine (Dako) was used for staining visualization. Staining for neutrophils in normal tonsil tissue was used as a positive control. Sections of normal tonsil tissue with neutrophils were used as positive controls. Negative controls were stained as described with omission of primary antibodies.

Peptide-specific CTL lines and cell-mediated cytotoxicity assay

PR1-specific CTLs were expanded by stimulating PBMC from HLA-A2⁺ healthy donors with PR1-peptide as previously described.¹⁷⁹ Briefly, T2 cells were washed in RPMI 1640 medium then incubated with 20µg/mL of PR1 for 90 minutes at 37°C. PR1-loaded T2 cells were then irradiated and cultured with freshly isolated PBMC at a 1:1 ratio in RPMI 1640 medium supplemented with 10% human AB serum. Cultures were re-stimulated with peptide-pulsed T2 cells on days 7, 14, and 21. The following day, 20 IU/mL of recombinant human IL-2 (Biosource International) was added. On day 25, CTLs were harvested and used in standard cytotoxicity assays as previously described.¹⁷⁹ Briefly, target cells were stained with 10µg/mL of calcein-AM (Sigma Aldrich) for 90 minutes at 37°C, washed then co-incubated with peptide-specific CTLs at varying E:T ratios at 37°C. After 4 hours, trypan blue was added and fluorescence was measured using an automated fluorescence reader (FLx800 Microplate fluorescence reader, Bio-Tek Instruments). The percent specific cytotoxicity was calculated as: $(1 - (E_{\text{experimental}} - E_{\text{media}}) / (E_{\text{control}} - E_{\text{media}})) * 100$, where E = fluorescence emission and the control group was targets alone.

Complement-mediated cytotoxicity assay

To determine if cross-presentation increases breast cancer susceptibility to 8F4, we performed a complement-mediated cytotoxicity assay as previously described.¹⁸⁸ MDA-MB-231 cells were cultured in NE or PR3 supplemented media for 24 hours then incubated with calcein AM (Sigma Aldrich), washed and resuspended in serum-free RPMI. One million breast cancer cells were mixed with increasing doses of 8F4 antibody or isotype antibody as a negative control and incubated for 10 minutes at 37°C. Standard rabbit complement (5µL) (Cedarlane Labs) was added and cells were

incubated for 60 minutes at 37°C. Supernatant from BB7.2 hybridoma (source for anti-HLA-A2) and digitonin (Promega) were used as positive controls. Fluorescence was measured and specific killing was calculated as described above.

Staining for PR1-CTL in breast cancer patients

PBMC from HLA-A2⁺ breast cancer patients were stained with the following antibodies; CD3 PE Cy7 (BD Biosciences), CD8 APC-H7 (BD Biosciences), CD4 pacific orange (Invitrogen), PE-conjugated PR1/HLA-A2 dextramer (Immudex) and the following pacific blue-conjugated lineage antibodies: CD14 (BD Biosciences), CD16 (BD Biosciences) and CD19 (Biolegend). Aqua live dead stain (Invitrogen) was used to exclude dead cells. Data were acquired on a Canto flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc). The frequency of PR1-CTLs was determined as the percentage of live cells that were lineage⁻, CD4⁻, CD3⁺, CD8⁺ and PR1-dextramer⁺.

Confocal imaging of patient tissues

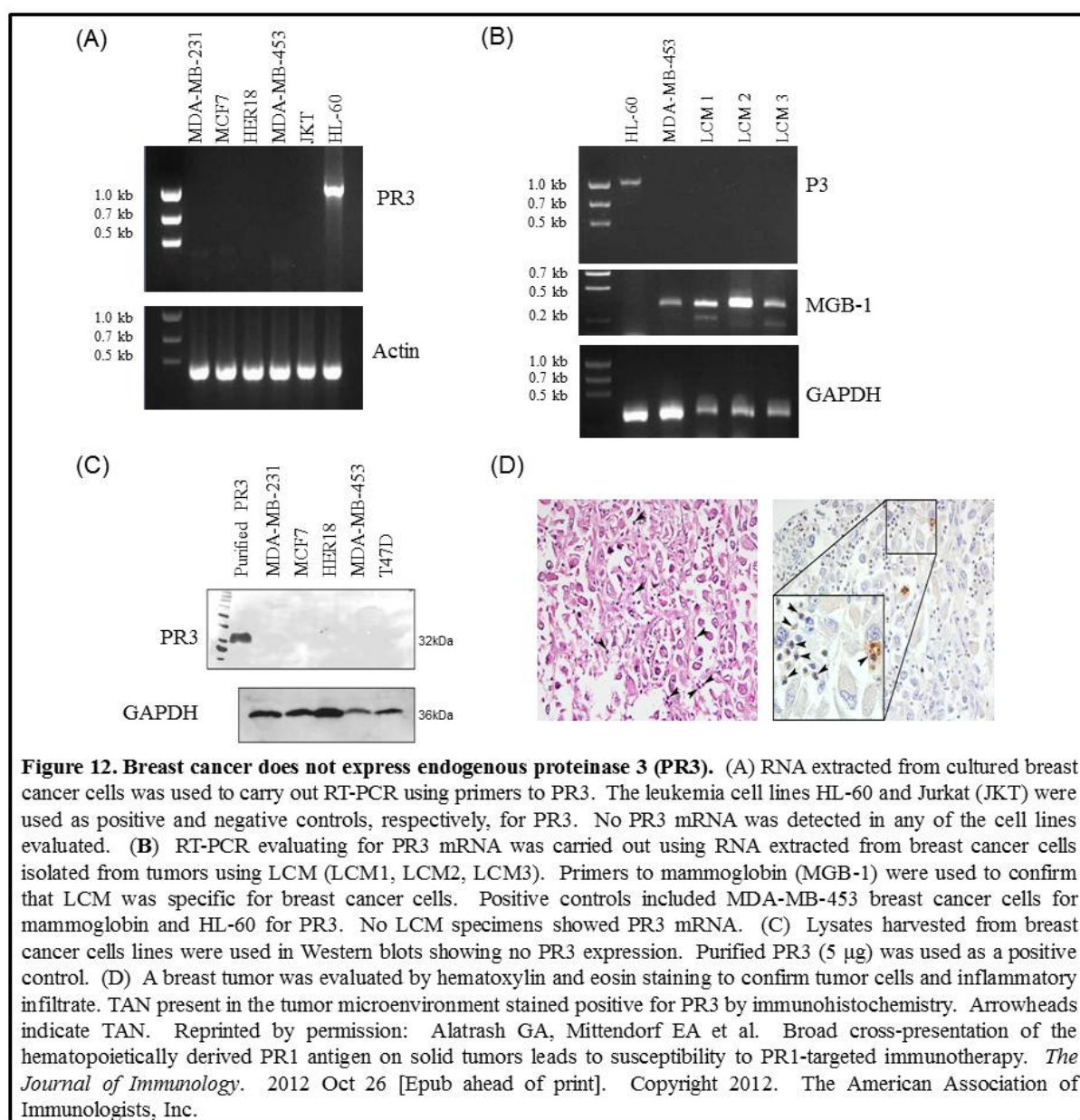
Cryopreserved breast cancer tissue specimens were fixed with cold acetone then stained with Alex-488 conjugated mouse anti-cytokeratin-7 (CD7) antibody (Abcam) as a breast marker and Alexa-647 conjugated 8F4 antibody.¹⁸⁸ ProLong Gold antifade reagent with dapi (Invitrogen) was added. Confocal imaging was performed using Leica Microsystems SP2 SE confocal microscope and Leica software (version 2.61) was used for image analysis.

RESULTS

Breast cancer cells lack endogenous PR3

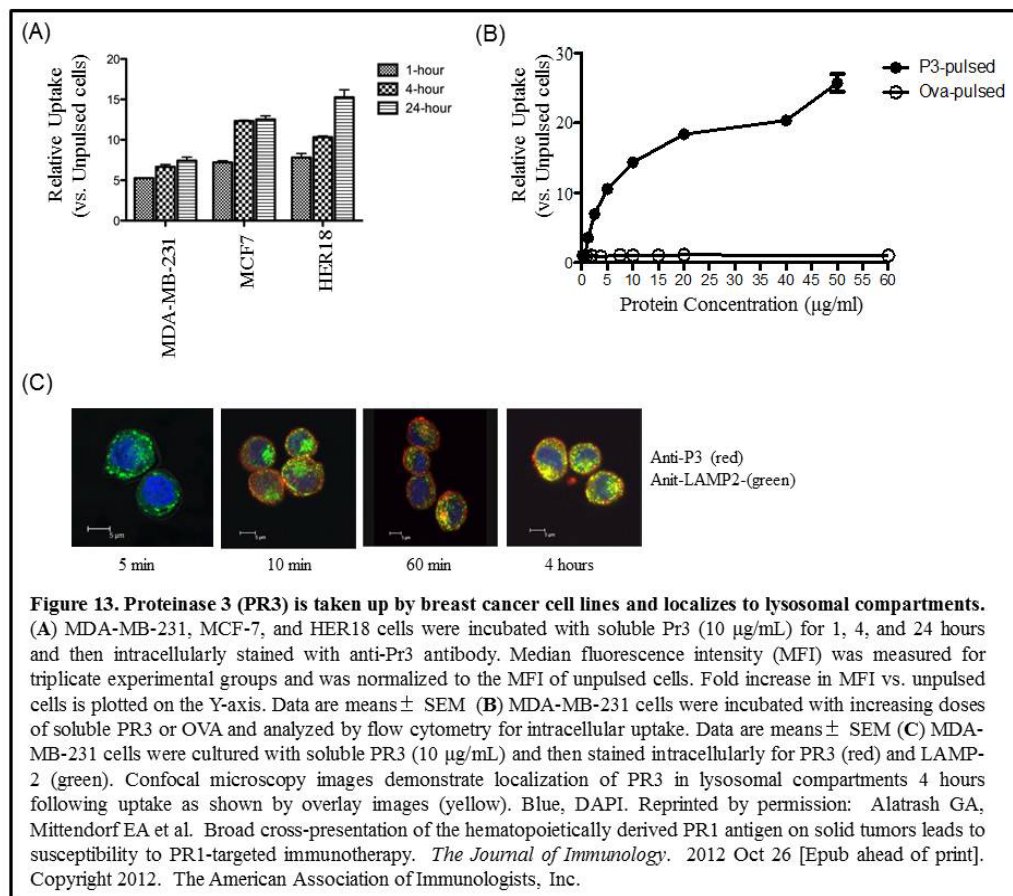
Having previously shown that breast cancer cells do not express endogenous NE but are able to take it up, we sought to determine if these cells express endogenous PR3. Breast cancer cell lines and primary tumor tissues were analyzed for PR3 expression at the mRNA and protein levels. PCR showed that MDA-MB-231, MCF-7, HER18 and MDA-MB-453 breast cancer cells lack PR3 mRNA (Fig.12A). In addition, breast cancer cells extracted from three primary breast tumors by LCM

also lacked PR3 mRNA (Fig. 12B). Western blots performed on whole cell lysates from the breast cancer cell lines confirmed the absence of PR3 protein expression (Fig. 12C). Finally, IHC staining of breast tumors detected PR3 in breast cancer tissue but the PR3 was limited to the inflammatory component of the tumor, not in the breast cancer cells (Fig. 12D). These data show that breast cancer cells do not express endogenous PR3, consistent with our previous findings that they do not express NE.¹³²

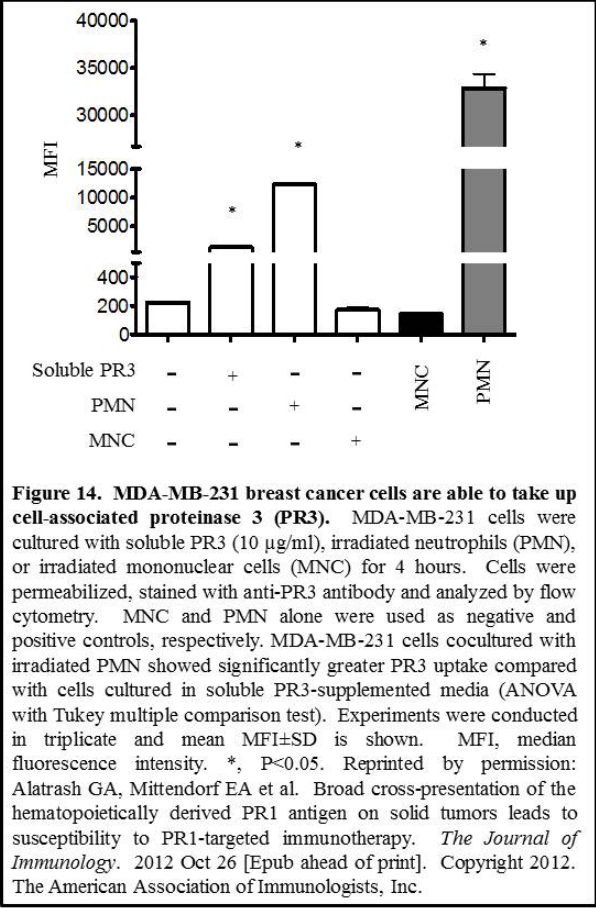


PR3 is taken up by breast cancer cells

Having shown that PR3 is not endogenously expressed by breast cancer cells, we next sought to determine if it could be taken up by breast cancer cells as we have shown for NE.¹³² The HLA-A2⁺ breast cancer cell lines MDA-MB-231, MCF-7 and HER18 were maintained in media supplemented with 10 μg of PR3. Cells were harvested at 1, 4 and 24 hours and then analyzed for intracellular PR3 expression using flow cytometry. A time-dependent increase in PR3 was seen in all three cell lines (Fig. 13A). Uptake of PR3 was dose dependent (Fig. 13B) however, unlike NE, the levels did not appear to plateau suggesting a non-receptor mediated process for P3 uptake. To determine PR3 localization after uptake, we performed confocal microscopy and showed co-staining with LAMP-2 demonstrating that PR3 localizes within lysosomes (Fig 13C). Antigen cross-presentation occurs in distinct cellular compartments.¹⁹⁵ Early uptake (1-4 hours) into lysosomal compartments may be an initial step in antigen degradation and processing for cross-presentation.¹⁹⁶

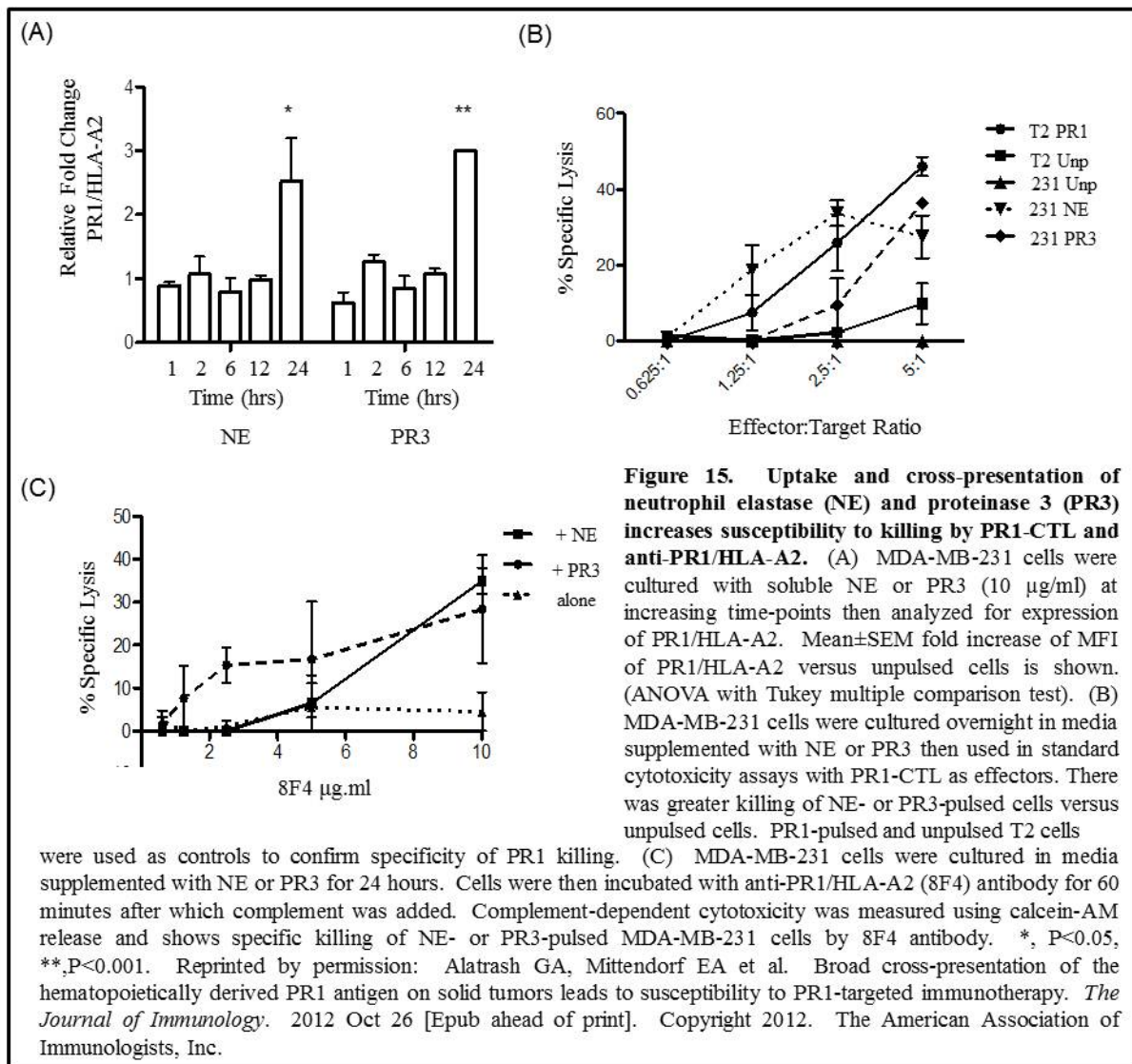


Since neutrophils are a significant component of the inflammatory response in the tumor micro-environment and because our data suggest TAN as the primary source of proteases in breast tumors, we next investigated the ability of breast cancer cells to take up cell-associated PR3. MDA-MB-231 cells were co-cultured with irradiated neutrophils or lymphocytes at a 1:1 ratio. Additional cells were maintained in PR3-supplemented media as a positive control. As shown (Fig. 14), breast cancer cells were able to take up cell associated PR3 and did so more efficiently than it took up soluble PR3 (average MFI = 12,292 versus 1,356; $p < 0.05$).



NE and PR3 are cross-presented by breast cancer cells

PR1 is a conserved nonameric HLA-A2-restricted peptide in NE and PR3.¹¹⁶⁻¹¹⁸ Having shown that breast cancer cells take up both NE and PR3, we next sought to determine if they can be cross-presented. MDA-MB-231 cells were cultured in media supplemented with NE or PR3 at increasing time points after which they were harvested and analyzed for PR1/HLA-A2 expression using 8F4, a mouse anti-PR1/HLA-A2 antibody. Significant cross-presentation from both NE and PR3 was seen at 24 hours with a 2.5- and 3-fold increase in PR1/HLA-A2 expression on the cell surface following culture in media supplemented with NE and PR3 respectively, compared with cells maintained in standard media (Fig 15A).



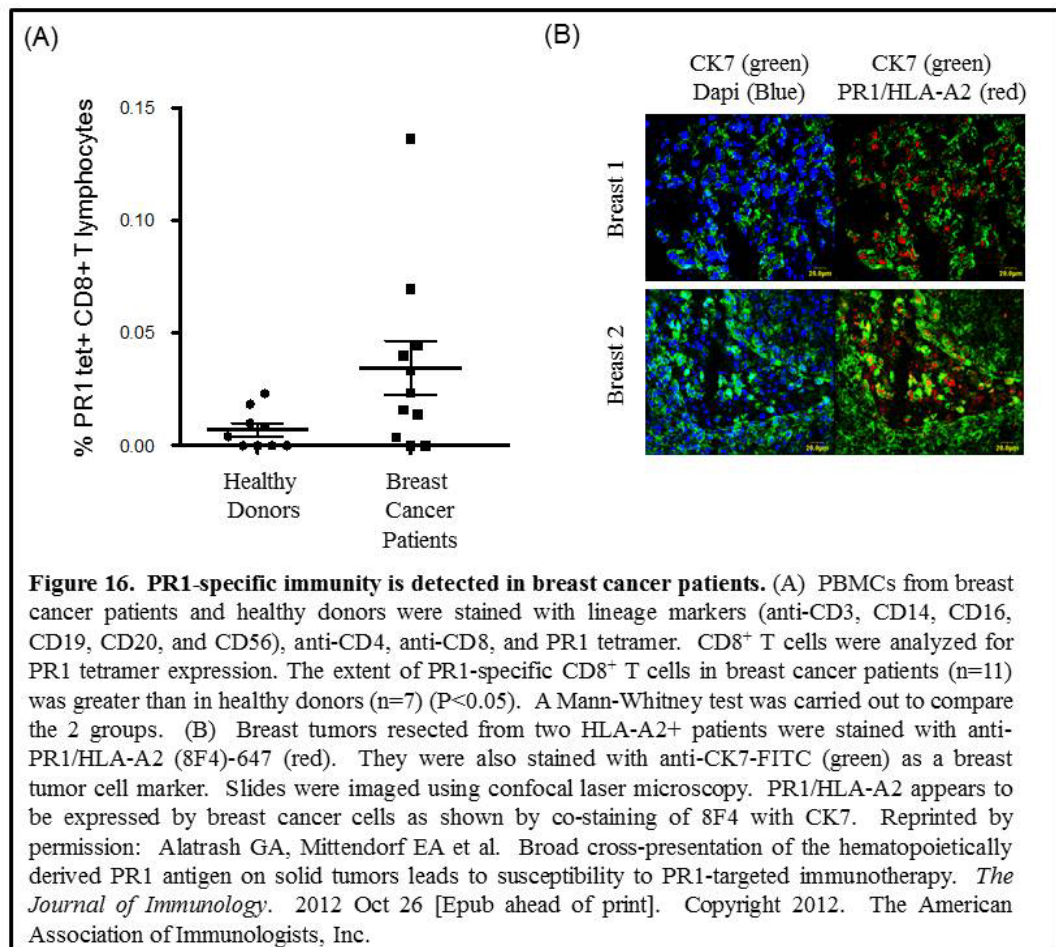
Cross-presentation of NE and PR3 renders cells susceptible to PR1-targeted therapy

PR1 has been effectively targeted in leukemia using a PR1 peptide vaccine,¹²¹ PR1-CTL,^{197,}
¹⁹⁸ and 8F4 (anti-PR1/HLA-A2 antibody).¹⁸⁸ We therefore investigated whether PR1/HLA-A2
 expression on breast cancer cells following NE or PR3 cross-presentation would render them
 susceptible to killing by PR1-CTL or 8F4 antibody. MDA-MB-231 cells were cultured in media
 supplemented with NE or PR3 for 24 hours then incubated with PR1-CTLs in a standard cytotoxicity
 assay. MDA-MB-231 maintained in standard media without NE or PR3 were not killed by PR1-CTL.
 Cross-presentation of NE and PR3 increased susceptibility of the cells to lysis by PR1-CTL (Fig. 15B).

Using 8F4 antibody in a complement dependent cytotoxicity assay, we also observed dose-dependent killing of MDA-MB-231 cells after NE or PR3 cross-presentation (Fig. 15C).

PR1-immunity is detected in patients with breast cancer

Having shown that breast cancer cells cross-present NE and PR3 leading to susceptibility to killing by PR1-targeted therapies, we next sought to confirm whether immunity to PR1 is detected in breast cancer patients. PBMC were obtained from 11 HLA-A2⁺ breast cancer patients and 9 HLA-A2⁺ healthy donors and stained with PR1 dextramer to assess the frequency of PR1-specific CTL. The median frequency of PR1-CTL in breast cancer patients was .05% of CD8⁺ T cells (range, .02-.2%), which was significantly greater than the frequency of PR1-CTL in healthy donors ($p < 0.05$) (Fig. 16A).



Next we evaluated HLA-A2⁺ primary breast tumors. Tissues were stained with 8F4 and anti-CK7 then evaluated using confocal microscopy. Both tumor samples showed co-staining of 8F4 with CK7 suggesting PR1/HLA-A2 expression on the breast cancer cells (Fig. 16B).

DISCUSSION

In this chapter, we have provided evidence of two known leukemia-associated antigens, NE and PR3, being taken up and cross-presented by breast cancer cells suggesting that NE and PR3 may be targetable antigens in solid tumors. Specifically, we have shown that similar to what we had previously found with NE, breast cancer cells are able to take up soluble and cell-associated PR3. After uptake, NE and PR3 are cross-presented as evidenced by increased PR1/HLA-A2 expression on the cell surface, and this cross-presentation leads to susceptibility to PR1-targeted therapies. Importantly, we show PR1/HLA-A2 expression on primary breast tumors and PR1-specific CTL in peripheral blood from breast cancer patients providing further evidence that studies evaluating PR1-targeted therapies in breast cancer are warranted.

Cross-presentation refers to the process whereby peptides derived from exogenous antigens are loaded onto MHC class I molecules for presentation to CD8⁺ T cells. It is critical for initiating immune responses against viruses and tumors. Cross-presentation is an important function of APCs and the majority of studies evaluating cross-presentation have focused on DCs, of which there are subtypes important for the process. Tissue DCs transport antigen from tissues to secondary lymphoid organs such as lymph nodes.¹⁹⁹ There, they transfer antigen to a subset of DCs capable of cross-presentation. In mice, these cells are marked by surface expression of CD8 α .²⁰⁰ We have previously shown that DCs take up and cross-present soluble NE and PR3 by 4 hours.¹⁹⁴ In the current study, although PR1/HLA-A2 expression was observed as early as one hour after addition of NE or PR3 to the culture media, maximal PR1/HLA-A2 expression was not observed until after 24 hours. Since breast cancer cells are not APCs, they may not be optimally equipped for rapid cross-presentation. Consistent with our findings, there are two previous studies showing cross-presentation by non-APCs, specifically

mesodermally-derived mesenchymal stromal¹⁹³ and endothelial cells.²⁰¹ In both of these studies, cross-presentation occurred at later time points (> 12 hours).

Licensed DCs upregulate expression of co-stimulatory molecules including CD80 and CD86 and downregulate inhibitory molecules including PDL1 (reviewed by Kurts et al.).²⁰² In contrast, tumor cells, to include breast cancer cells, lack co-stimulatory molecules therefore it is possible that cross-presentation of NE and PR3 by breast tumors would facilitate cross-tolerance in vivo. There is indirect evidence to support this as NE expression in breast cancer is a negative prognostic factor.¹¹¹⁻¹¹³ The possibility that cross-presentation of NE and PR3 by breast cancer cells may lead to cross-tolerance has implications for therapeutic strategies. Therefore, active immunization targeting PR1 may not be effective. However, passive immunotherapy strategies employing PR1-CTL or an anti-PR1/HLA-A2 monoclonal antibody may have antitumor activity in this setting.

Finally, the data in this chapter showing that breast cancer cells lack endogenous PR3 but are able to take it up is consistent with our previous data showing NE uptake.¹³² It is interesting to note that the kinetics of uptake differed. Whereas the dose-response curve for NE uptake plateaued suggesting a receptor-mediated mechanism of uptake, there was no plateau for PR3 uptake suggesting a different mechanism. In addition, whereas NE localized to early endosomes,¹³² PR3 was found in lysosomes. Both endosomal and lysosomal compartments are known to play a role in antigen cross presentation, providing further support for NE and PR3 cross-presentation by breast cancer cells.^{196, 203}

In conclusion, we have shown that breast cancer cells can take up and cross-present NE and PR3 leading to susceptibility to PR1-targeted therapy. Since inflammatory cells, to include neutrophils, are found in numerous solid tumors and can provide a source for NE and PR3, our findings identify cross-presentation as a novel mechanism that may render tumors susceptible to PR1 immunotherapies. Additional studies are required to determine how ubiquitous the process of NE and PR3 uptake is to include on other solid tumor types and non-malignant cells. If multiple tumor types can take up and cross-present NE and PR3, this would suggest broad applicability for PR1 immunotherapy.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

In this thesis, we have identified novel functions of NE, a serine protease in the tumor microenvironment. We show that NE is present in TANs and that breast cancer cells do not produce endogenous NE suggesting TANs as the primary NE source in breast cancer. We also demonstrate that NE is taken up by breast cancer cells in an antigen-specific manner. Importantly, after uptake, NE modulates adaptive immune responses by enhancing antigen presentation. Specifically, NE uptake leads to increased generation of LMW CCNE isoforms and enhanced susceptibility of breast cancer cells to lysis by CCNE-CTL. Furthermore, we have shown that NE, as well as PR3, a second neutrophil primary granule protease with significant homology to NE, is cross-presented by breast cancer cells leading to increased PR1/HLA-A2 on the cell surface and subsequent killing by PR1-targeted therapies including PR1-CTL and 8F4, an antibody targeting PR1/HLA-A2. Therefore, we have provided evidence for a novel mechanism linking NE, a protease secreted by innate immune cells, to adaptive immune responses against novel antigens in breast cancer. These findings have significant implications for cancer biology and tumor immunology.

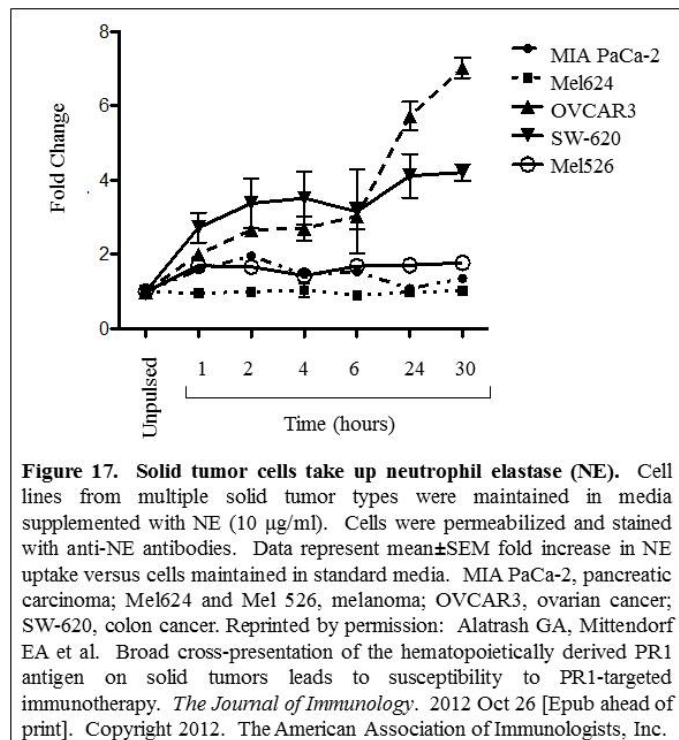
Uptake of neutrophil elastase

The majority of studies investigating the effects of NE in cancer have focused on its ability to promote invasion and metastasis through degradation of the ECM.^{54, 122, 123} Two other studies have demonstrated that NE can impact tumor cell proliferation by its effects on cell signaling. Studies have shown that NE can cleave the TNF receptor from the cell surface and that cleaved receptors can bind circulating TNF- α thereby decreasing TNF signaling and subsequently decreasing caspase 8-mediated apoptosis.^{126, 127, 129} A study by Houghton et al. showed that NE uptake by lung cancer cells can cleave IRS-1 leading to hyperactivity of the PI3K pathway resulting in increased proliferation.¹³⁰ Our studies confirmed the findings of Houghton et al. that cancer cells can take up exogenous NE. NE uptake in breast cancer cells led to increased expression of LMW forms of CCNE which have previously been shown to be hyperactive compared to the FL CCNE protein.¹⁶³ These hyperactive LMW forms promote

proliferation by shortening the G1/S phase transition of the cell cycle thus suggesting an additional potential mechanism for NE-induced tumor cell growth following uptake.

Importantly, we have provided further information regarding the mechanism of NE uptake by demonstrating uptake to be dose- and time-dependent suggesting a receptor-mediated mechanism. Because the timing and subcellular localization of NE after uptake is similar in different cancer cell types, it is possible that there is a common uptake mechanism. If so, such a mechanism could be important for controlling cell growth.

We have begun to investigate this in the laboratory where we have evaluated multiple different cell lines from various tumor types to include melanoma, ovarian cancer, pancreatic cancer and colon cancer, for their ability to take up NE (Fig. 17). We found that not all tumor types take up NE and for those that do, the extent of uptake varies. We are working to use this differential uptake to determine the receptor



involved using a computational approach. Briefly, in addition to the five cell lines shown in figure 17, we have evaluated the fold change in uptake for 15 additional cell lines of diverse tissue origins with gene expression data (from Affymetrix GeneChip Human Genome U133 Plus 2.0 array) available from a public database. Working with our biostatistical collaborator, Dr. Shoudan Liang, we have correlated NE uptake with the level of mRNA expression of membrane proteins thus identifying potential receptors that may mediate NE uptake. This line of investigation is currently being pursued by Dr. Celine Kerros, a post-doctoral fellow working in the laboratory of my advisor Dr. Molldrem.

Along with the report by Houghton et al., our demonstration of NE uptake by breast cancer cells represents the first studies showing the ability of a secreted protease to enter target cells. We are continuing to evaluate other cell types for NE uptake and our data suggests that this is a ubiquitous phenomenon shared among multiple solid tumors. Because NE has broad substrate specificity, it is likely that uptake provides NE access to a wide array of potential substrates affecting multiple biologic processes impacting tumor development and growth. As was demonstrated in chapters 2 and 3 of this thesis and will be discussed further below, uptake of NE also results in increased antigen presentation thereby making the tumor cells more attractive targets for adaptive immune responses and targeted immunotherapeutic approaches.

Cyclin E as a novel breast cancer antigen

We have provided data linking NE uptake to an adaptive immune response against CCNE in breast cancer. Specifically, we showed that increased expression of LMW CCNE after NE uptake leads to enhanced susceptibility to lysis by CCNE CTL.¹³² Importantly, using tetramer staining, we identified a low precursor frequency of CCNE₁₄₄₋₁₅₂ CTL in breast cancer patients confirming that the antigen is naturally processed. Our data therefore support the discovery of CCNE as a novel breast cancer antigen and suggest that CCNE-targeting immunotherapy to include a peptide vaccine combining CCNE₁₄₄₋₁₅₂ with an immunoadjuvant may augment the CCNE-specific-CTL response.

Previously, overexpression of CCNE and its LMW forms has been shown to be a poor prognostic factor in breast cancer.¹⁷² Because CCNE is aberrantly expressed in breast cancer and this aberrant expression drives proliferation, CCNE has characteristics of an ideal TAA. CCNE₁₄₄₋₁₅₂, the immunogenic epitope that we have identified, is expressed in both the FL and LMW forms of the CCNE protein (Fig. 9A). Our data strongly suggest that after NE uptake, there is increased substrate availability of the CCNE LMW fragments, which could facilitate antigen processing and presentation of CCNE peptides. The LMW forms of CCNE lack the nuclear localization sequence that is in the amino terminus of the FL protein. Consistent with this, Delk et al. have shown altered subcellular localization

of the LMW forms of CCNE with the majority being present in the cytoplasm.¹⁶⁶ This cytoplasmic localization may facilitate proteasomal-mediated degradation and CCNE peptide translocation by the transporter associated with antigen presentation (TAP) protein to the endoplasmic reticulum where the peptides would access MHC class I molecules.¹⁸⁹ In support of this hypothesis, we found that HER18 cells are more susceptible to killing by CCNE-CTL than MDA-MB-231 cells that have less LMW CCNE expression (Fig. 10). In addition, after uptake of soluble NE, there was an increase in LMW CCNE expression in the MDA-MB-231 cells and enhanced susceptibility of the cells to lysis by CCNE-CTL. Additional studies must be done to determine whether the LMW forms are the predominant source of CCNE₁₄₄₋₁₅₂ peptide. To evaluate this, we have discussed a collaboration with Dr. Khandan Keyomarsi whereby we would stably transfect MCF-7 breast cancer cells (HLA-A2⁺; high transfection efficiency, low baseline LMW CCNE expression) with the EL, trunk 1, and trunk 2 constructs that she has previously used to investigate the effect of FL versus LMW CCNE on cell cycle regulation.¹⁶³ The EL construct overexpresses FL CCNE, the trunk 1 construct overexpresses the EL2 and EL3 LMW forms and the trunk 2 construct overexpresses the EL5 and EL6 forms. These cells would be used as targets in standard cytotoxicity assays with CCNE-CTL as effectors. We would hypothesize that the MCF-7 cells transfected to overexpress LMW CCNE would be lysed more effectively than cells transfected with the EL vector or the empty vector control. This model system could also be used to further investigate ubiquitination and proteasome-mediated degradation of FL versus LMW CCNE; two processes required for antigen processing and presentation

Antigen discovery

Because NE has broad substrate specificity, it is possible that uptake of NE may generate novel antigens other than CCNE. There are however many challenges in identifying target antigens. First, most antigen-specific T-cell receptors (TCRs) have low affinity for their target MHC-peptide complex. This makes using biochemical techniques relying on high affinities difficult.²⁰⁴ In addition, the majority of TCRs are polyspecific and can be activated by the parent peptide as well as similar “mimotopes” that have amino acid exchanges.²⁰⁵ Finally, TCRs recognize peptide-MHC complexes that

have undergone complex intracellular antigen processing (reviewed by Vyas et al).²⁰⁶ Recently, Siewert et al. identified a technology for unbiased identification of antigenic peptides complexed with MHC class I molecules that directly addresses some of these difficulties.²⁰⁴ The methodology involves plasmid-encoded combinatorial peptide libraries and a single-cell detection system. Briefly, they use COS-7 cells as APCs. They co-transfect MHC class I cDNA and a plasmid-coded combinatorial peptide library into the COS-7 cells thereby overcoming the intricacies of protein processing. In parallel, they cotransfect T hybridoma cells with TCR- α and TCR- β chains²⁰⁷ and super GFP controlled by NFAT (nuclear factor of activated T cells). TCR transfectants are seeded on top of COS-7 APCs and only those that contact with a COS-7 cell presenting the correct antigen will light up. Highlighted APCs are isolated and the plasmid coding for the antigenic peptide can be isolated by subcloning.²⁰⁴ They validated their approach using the well-characterized TCR JM22 which is specific for HLA-A2 and the Flu₅₈₋₆₆ peptide. Purported benefits of this approach are that it is extremely sensitive; able to distinguish single mimotope-expressing cells from millions of negative cells. Because the APCs and T cells are kept in contact by gravity, this methodology overcomes the requirement for high affinities of TCRs to the peptide-MHC complexes. In addition, the technique uses immortalized cell lines that can be transfected and grown in large quantities. In theory, this would allow for the investigation of antigens for TCRs from single T cells recovered using LCM from tumor specimens rather than requiring T cell lines from peripheral blood.^{204, 207} These investigators have previously reported a method to obtain single T cells from archived human tissues.²⁰⁷ It is possible therefore that T cells could be isolated from tissue, their paired TCR α and β chains could be cloned and expressed, and their antigens characterized using the approach described above.

Uptake of NE by tumors with a significant inflammatory component may cause these tumors to express a broad array of neo-antigens not expressed in tumors lacking inflammation. Identification of such antigens may allow for the identification and development of immunotherapeutic strategies. One clinical scenario where this may be relevant is high-grade triple-negative breast cancer. These tumors have an intense immune cell infiltrate.²⁰⁸ It is possible therefore that uptake of NE from

the tumor microenvironment in triple-negative breast cancer may generate neo-antigens that could be targeted therapeutically. This would be an important finding because currently patients with triple-negative breast cancers have limited therapeutic options beyond standard chemotherapy regimens. Despite the fact that these regimens do have clinical activity as evidenced by high pathologic complete response rates (35-40%) to neoadjuvant chemotherapy, these patients have a paradoxical shortening of progression free- and OS²⁰⁹ suggesting a need for novel therapeutic strategies.

Enhanced antigen processing and presentation

The first human tumor associated antigen gene to be identified was melanoma-associated antigen 1 (*MAGE1*) which encodes the MZ2E antigen.²¹⁰ Since then, hundreds of naturally processed and presented tumor antigens have been identified²¹¹ and a list of these antigens is available at the cancer immunity peptide database (<http://archive.cancerimmunity.org/peptidedatabase/Tcellepitopes/htm>). Having shown that NE uptake enhanced presentation of the CCNE antigen, we would postulate that it may have a broader impact on other known antigens. There are several mechanisms by which this may happen. First, NE could increase degradation of the antigenic proteins leading to increased generation of peptides. Second, NE cleavage of peptides could create novel protein products that may be cleaved differently than the parent proteins, thereby uncovering novel peptides. Third, NE uptake may impact genes associated with antigen processing and presentation machinery which would enhance susceptibility to a wide range of antigens.

We have recently performed experiments looking at the effect of NE uptake on the susceptibility of HER2-expressing tumor targets to lysis by HER2-specific CTL. HER2 is a well described tumor antigen in breast cancer. Several peptides from the HER2 protein have been demonstrated capable of inducing HER2-specific CTLs including E75 (HER2₃₆₉₋₃₇₇:KIFGSLAFL), a nonamer derived from the protein's extracellular domain.^{212, 213} E75 is the immunodominant epitope from the HER2 protein and has been studied extensively in both the laboratory and the clinic. The

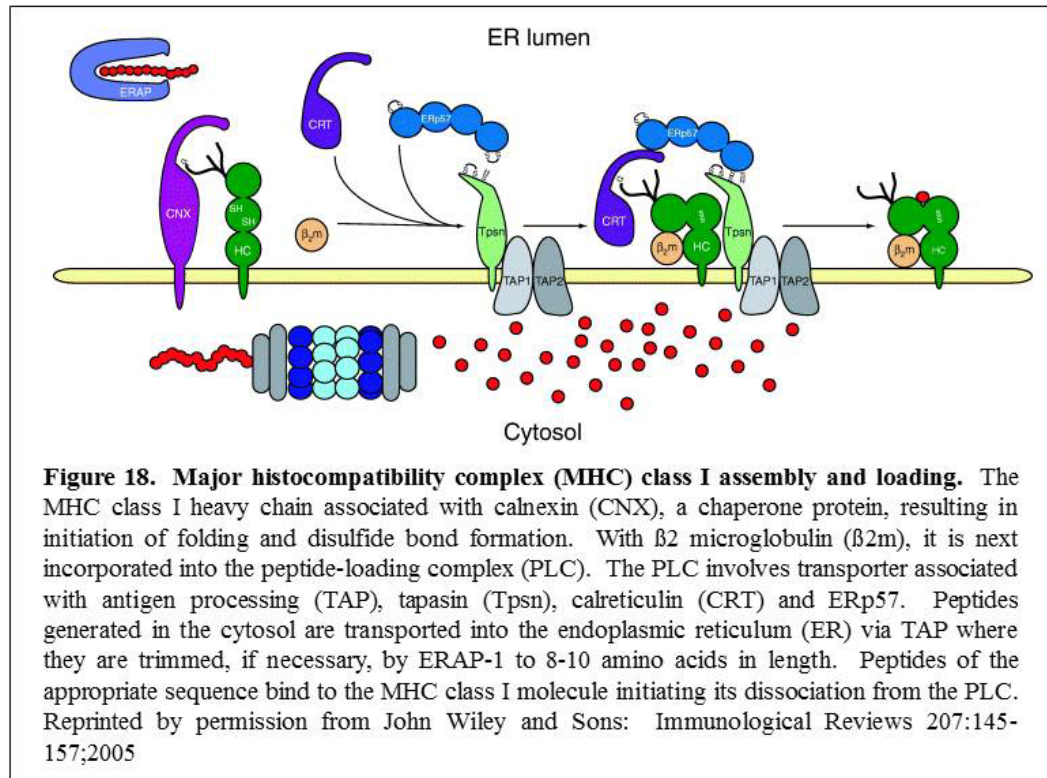
combination of E75 plus the immunoadjuvant GM-CSF has been evaluated in phase I and II clinical trials with reports demonstrating efficacy in appropriately selected patient populations.^{214, 215}

Interestingly, the vaccine appears to stimulate the most robust immune response in patients whose tumors have low to intermediate HER2 expression (versus HER2 overexpression which is the clinical standard required for patients to be treated with trastuzumab, the monoclonal antibody targeting HER2). We have generated preliminary data from cytotoxicity assays using E75-specific CTL as effectors and MDA-MB-231 (HLA-A2+; low HER2 expression) as targets. MDA-MB-231 cells maintained for 24 hours in NE-supplemented media were more susceptible to lysis by the E75-specific CTL. These experiments need to be repeated and additional studies performed looking at other TAA (i.e. MAGE, WT1). Further investigation into the mechanism must be undertaken however; these early results suggest that the effect of NE uptake on antigen processing and presentation may be more ubiquitous impacting the expression of multiple TAA derived-peptides complexed with HLA-A2 molecules on the tumor cell surface.

Furthermore, we have recently completed experiments using gene expression profiling that support the idea that NE uptake more broadly enhances antigen processing and presentation. Briefly, RNA was extracted from MDA-MB-231 cells maintained in standard media and NE-supplemented media for 1 and 13 hours. This was used in gene expression arrays on the Illumina platform, and data was analyzed using Ingenuity pathway analysis. This analysis demonstrated upregulation of components of the antigen presentation pathway. There were 10 genes in the pathway in particular that were upregulated after NE uptake: CANX (calnexin); HLA-DMA (MHC class II, DM alpha); HLA-DMB (MHC class II, DM beta); HLA-DOA (MHC class II, DO alpha); HLA-DPA1 (MHC class II, DP alpha 1); HLA-F (MHC class I, F); MR1 (MHC class I-related); PSMB9 (proteasome subunit, beta type, 9 also known as LMP2, large multifunctional protease 2); TAP1 (transporter 1); TAPBP (TAP binding protein, tapasin).

Several of the genes upregulated after NE uptake impact MHC class II presentation, while others have critical roles in MHC class I antigen presentation. Briefly, as reviewed by both Neefjes et

al.¹⁸⁹ and Cresswell et al.¹⁹⁵ and as illustrated in figure 18, the MHC class I heavy chain, a transmembrane glycoprotein, binds to the membrane-associated ER chaperone protein calnexin. At this stage, folding and the formation of disulfide bonds occurs. Once the MHC class I heavy chain



dissociates from calnexin, it binds $\beta 2$ -microglobulin and is incorporated into the peptide-loading complex (PLC). Other components of the PLC include the two transporter associated with antigen processing subunits (TAP1 and TAP2), the transmembrane glycoprotein tapasin, the soluble thiol oxidoreductase ERp57 and the ER chaperone calreticulin. Our gene expression data shows that the genes encoding three proteins described thus far, calnexin, TAP1 and tapasin are upregulated after NE uptake. Peptides are transported from the cytosol into the ER via TAP and if necessary, are trimmed by an ER-associated peptidase (ERAP) to 8-10 amino acids, the length required for association with MHC class I molecules. If the peptide has the correct sequence, it can bind the MHC class I- $\beta 2$ -microglobulin heterodimer which is then released from the PLC. The assembled MHC class I molecule leaves the ER and travels to the cell surface via the Golgi apparatus. Ongoing studies in the laboratory

are being performed to verify the gene expression data and further investigate the effects of NE uptake on these components of the antigen processing and presentation machinery.

If we prove that NE uptake has a more general effect on enhancing antigen processing and presentation, it would be relevant to determine if this effect extends beyond tumor cells to include virally infected cells. To evaluate this, a viral antigen model system could be used. Briefly, EBV-specific T cells have been generated and used clinically in the treatment of EBV-related infections and lymphoproliferative disease arising after hematopoietic stem cell transplant.²¹⁶ EBV-specific T cell clones are available and could be used as effectors in cytotoxicity assays versus lymphoblastoid cell lines pre- and post-NE uptake. If NE uptake enhanced presentation of viral antigens in addition to enhancing presentation of tumor antigens, this would be a significant observation as it would suggest an additional important role for neutrophils and NE in inflammation and infection.

Naïve T cell infiltration, activation and differentiation

Central tolerance occurs during lymphocyte development in either the thymus (T cells) or bone marrow (B cells). In the case of the former, T cells with high affinity receptors for MHC/self-peptide complexes are eliminated by the process of negative selection. T cells that survive negative selection leave the thymus and migrate to the periphery where they can encounter antigen, become activated, and differentiate into effector CTLs. In order for a T cell to become activated, it must bind the MHC/peptide complex via its TCR then receive a co-stimulatory signal from molecules such as CD80 or CD86 which are recognized by CD28 on the T cell surface. Co-stimulatory molecules including CD80 and CD86 are limited to APCs.²¹⁷ Although a full review of co-stimulation is outside the scope of this chapter, it should be noted that there are other co-stimulatory molecules on the T cell surface including OX40 (CD134) and 4-1BB (CD137), members of the TNFR family, which provide co-stimulation when bound by their ligands, OX40L and 4-1BBL respectively, as well as GITR, CD27 and HVEM.²¹⁸⁻²²⁰ T cells also have inhibitory molecules on their cell surface including CTLA-4, which is a homologue of CD28 that also binds CD80 and CD86.^{221, 222} Programmed cell death-1 (PD-1)

receptor is another inhibitory molecule on the T cell surface that can bind PD-L1 or PD-L2.^{223, 224}

CTLA-4 and PD-1 blocking antibodies are being used clinically.^{225, 226} Other inhibitory receptors include LAG-3, TIM-3, BTLA and VISTA.^{218, 219} Binding of the TCR without adequate co-stimulation induces anergy and the T cell subsequently undergoes apoptosis.

Although co-stimulatory molecules are critical in immune priming, the immune microenvironment where naïve T cells interact with APC is also important. The process of CD8⁺ T cell activation is largely thought to occur in secondary lymphoid organs including lymph nodes. Naïve T cells enter lymph nodes through high endothelial venules.²²⁷ Once in the lymph node, T cells encounter APCs presenting peptide-MHC ligands and can become activated. Several reports describe CD8⁺ T cell activation in tumor-draining lymph nodes.²²⁸⁻²³⁰ This is due either to direct priming by tumor cells that have migrated to the lymph node from the tumor site or cross-priming by DCs in the lymph node. Cross-priming is the initiation of a CD8⁺ T cell response to a cross-presented antigen; it is critical for the initiation of immune responses not only to tumors but to viruses that do not infect APCs.

Although relatively few studies have investigated tumors as sites for naïve T cell activation, tumors have features that may make them an attractive site for T cell priming. A tumor has a large supply of antigen and contains multiple cell types that function as APCs to include macrophages and DCs.²³¹ There are several reasons that the tumor has not been extensively investigated as a site for naïve T cell activation. One reason may be that, due to the presence of MDSCs, regulatory T cells, TGF- β , and indoleamine 2,3-dioxygenase, tumors are considered to be immunosuppressive. A second reason may be that cancer cells lack co-stimulatory molecules which are required for T cell activation. Another reason may be the fact that naïve T cells have high CD62L and CCR 7 expression which guides their migration to lymph nodes and low expression of adhesion molecules and chemokines that may guide them to tumors.²³² There are however studies demonstrating naïve T cell infiltration of peripheral tissues.^{233, 234} There are also studies where tumors engineered to express LIGHT, a member of the TNF superfamily, or lymphotoxin α can attract T cells.²³⁵ A more recent study by Thompson et al. used a model of C57BL/6 mice bearing B16-cOVA tumors to show that tumors can support activation of naïve

CD8⁺ T Cells.²³¹ Briefly, naïve OT-1 T cells were adoptively transferred into mice and within 24 hours, significant numbers of activated T cells were found within the tumors. Experiments performed in mice treated with FTY720, a phingosine 1-phosphate analogue that prevents T cell egress from lymph nodes or in mice lacking lymph nodes, confirmed that the tumor masses, without contribution from draining lymph nodes, supported activation of CD8⁺ T cells. The investigators further showed that these CD8⁺ T cells, once activated, could proliferate and had effector function. They confirmed their findings in a second tumor model using Lewis lung carcinoma (LLC) transfected with OVA. The authors suggest that tumor infiltration by naïve T cells may result from normal migration of these naïve T cells through nonlymphoid sites or due to their attraction to an inflamed nonlymphoid site. They further postulate that the chronic inflammation in a nonlymphoid site such as a tumor could drive the development of a tertiary lymphoid organ (TLO).²³¹ TLOs have a highly organized structure where T cells can interact with APCs.²³⁶ Additional work is needed to better understand the requirements for naïve T cell infiltration into tumors. However, given our findings that uptake of NE by tumor cells enhances antigen presentation, it is interesting to consider that the microenvironment required to activate CD8⁺ T cells is one with significant inflammation, to include neutrophils, which can secrete NE that enhances antigen presentation and in the appropriate microenvironment, may contribute to immune priming.

Immunotherapy targeting CCNE and PR1

In this thesis, we have shown data suggesting increased expression of HLA-A2-restricted peptides (CCNE₁₄₄₋₁₅₂ and PR1) on the tumor cell surface following NE uptake. This suggests that NE uptake makes these cells more attractive targets for antigen specific T cells. With respect to specific immunotherapy strategies, there are active and passive strategies that could be utilized. For CCNE, our group is interested in an active immunization strategy using the CCNE₁₄₄₋₁₅₂ peptide mixed with an immunoadjuvant administered intradermally to elicit a CCNE-specific CTL response. Working with a statistical collaborator, Dr. Peter Thall, we have designed a phase I/II trial that will enroll breast cancer patients receiving neoadjuvant chemotherapy. The standard neoadjuvant chemotherapy regimen administered at MD Anderson includes weekly paclitaxel for 12 cycles followed by 4 cycles of 5-

fluorouracil, adriamycin and cyclophosphamide (FAC) administered every 3 weeks. The vaccine will be given during the FAC portion of their treatment with inoculations occurring 2 weeks after FAC dosing to correspond with early recovery of the patients' white blood cell counts, and therefore optimal immune priming. The trial's primary endpoints include assessing safety and toxicity as well as an immune response – the doubling of CCNE-CTL from baseline as measured by tetramer staining. Conduct of the trial will require completion of an Investigational New Drug application.

A PR1 vaccine has already been investigated in leukemia. A PR1 vaccine has already been investigated in leukemia. My advisor, Dr. Molldrem, who developed the PR vaccine showed its efficacy in patients with relapsed/refractory acute myeloid leukemia, CML or myelodysplastic syndrome.^{237, 238} In this group of patients (n = 66) with highly refractory disease who were treated with the PR1-peptide vaccine, 13 achieved complete remission (CR). Additionally, immunologic response (IR) (defined as a ≥ 2 -fold increase in PR1-CTLs) was observed in 25 of 53 patients (47%), and event-free survival (8.7 months) was longer in these patients than in unvaccinated patients (2.4 months) (P = 0.03); 9 of the 25 patients with IR showed CR. In a study enrolling 8 patients with myeloid malignancies, Rezvani et al. administered a vaccine combining 2 leukemia-associated antigenic peptides, WT1 and PR1 in Montanide adjuvant.¹²¹ The vaccine was well tolerated with only grade 1 to 2 toxicity. It was also effective in eliciting PR1-specific CTL as demonstrated using tetramer staining of PBMC after vaccination. It is possible that a PR1-peptide vaccine would be efficacious in breast cancer. Furthermore, our group is also interested in exploring a passive immunotherapy strategy, specifically treatment with 8F4, the monoclonal antibody complex identified in the laboratory of Dr. Molldrem that recognizes the PR1/HLA-A2 complex.¹⁸⁸ Preliminary data from animal models of leukemia suggest therapeutic efficacy of 8F4 in leukemia, therefore we are interested in further evaluating the use of 8F4 in solid tumors that cross-present NE and PR3.

Animal models of cross-presentation have shown varying results. Some models have demonstrated cross-presentation that remains localized to the tumor-draining lymph node, some show cross-tolerance, and others have shown induction of a weak, largely ineffective CTL response.^{202, 239-241}

Because many tumors in *in vivo* animal models lack intense inflammation and do not have the pathogen-associated molecular patterns that drive a strong CTL response, the weak responses may not be unexpected.²⁴² To further evaluate NE and PR3 cross-presentation and the efficacy of the 8F4 antibody targeting the PR1/HLA-A2 complex in vivo, my advisor, Dr. Molldrem, in collaboration with Dr. Gheath Alatrash, is using an in vivo xenograft mouse model with MDA-MB-231 breast cancer cells in NOD-scidIL2Rgamma^{null} mice. Preliminary studies have shown an inflammatory infiltrate in these tumors including granulocytes that secrete NE and P3 within the tumor microenvironment. In addition, further in vivo inflammation will be induced by intra-tumoral injection of lipopolysaccharide and recombinant TNF 1 week after tumor establishment. These reagents have been shown to induce acute inflammation with a marked neutrophil infiltrate peaking by 24 hours.^{243, 244} Completion of such in vivo studies will strengthen 8F4 phase I trials in patients with breast cancer.

The data presented in this thesis demonstrating CCNE to be a novel TAA and showing cross-presentation of NE and PR3 support the further investigation of such immunotherapeutic strategies. Ongoing work by our group is aimed at translating our findings to the clinic with the design and conduct of clinical trials evaluating CCNE- and PR1-targeted immunotherapy.

Modification of the tumor microenvironment to enhance response to immunotherapy

Immunotherapy, such as the strategies discussed above, have the potential to improve patient outcomes. It is possible that immunotherapeutic strategies could be augmented by incorporation with other treatments that modulate the tumor microenvironment. Examples of this include sequencing immunotherapy with standard chemotherapy regimens, as suggested above in the proposed CCNE vaccine trial, or using known immune stimulating agents such as IFN- α .

It was long thought that chemotherapy affected only the tumor cells without impacting other components of the tumor microenvironment to include immune cells. In fact, National Cancer Institute guidelines for drug screening, developed in the mid-1970s, have advocated using xenografts of human cell lines in immunodeficient mice.^{245, 246} However, there is now evidence that the immune system

contributes to the anti-tumor effects of chemotherapy (reviewed by Zitvogel et al.).²⁴⁵

Chemotherapeutic agents can elicit anti-tumor immunity in several ways including the induction of immunogenic tumor cell death and the stimulation of pro-inflammatory cytokine production. As an example of the former, anthracyclines and platinum-based drugs promote immunogenic cancer cell death by calreticulin exposure and high-mobility group box 1 (HMGB1) protein release.²⁴⁷⁻²⁴⁹

Calreticulin translocates from the ER of the tumor cell to the surface where it acts as an “eat-me” signal for DCs. HMGB1 is a nuclear protein released from dying tumor cells that is a ligand for TLR4, therefore it has a role in DC-mediated cross-presentation of tumors to T cells. Experiments have shown that tumor cells treated with anthracyclines then injected into mice can elicit a specific DC and antitumor T-cell response.²⁵⁰ DNA-damaging agents stimulate a complex response that includes activation of the p53 transcription factor. This in turn can promote the release of pro-inflammatory cytokines and chemokines that recruit neutrophils, macrophages and natural killer cells to the tumor site.²⁵¹ Put into context with our findings, chemotherapy could contribute to an enhanced response to vaccination with a CCNE-derived peptide vaccine in several ways. First, it could promote tumor cell death resulting in release of antigenic proteins including CCNE that are presented by DCs in the tumor microenvironment. Second, it could result in the recruitment of immune cells including neutrophils to the microenvironment. Once there, neutrophils would release NE which could be taken up by tumor cells enhancing CCNE antigen processing and presentation.

Another strategy to modulate the microenvironment thereby enhancing response to immunotherapy would be to administer an immune-stimulating agent such as recombinant IFN- α 2b which is currently approved for use in patients with high risk melanoma. In this patient population, IFN- α 2b has led to improvements in both DFS and OS.²⁵² IFN- α 2b has multiple mechanisms of action that include upregulation of MHC I molecules.²⁵³ It is possible therefore that if administered in patients with a tumor that has a significant inflammatory infiltrate, it may enhance response to CCNE- or PR1-targeted therapy by enhancing presentation of these antigens. Specifically, the neutrophils present in the microenvironment would release NE which could be taken up by the cancer cells thereby increasing

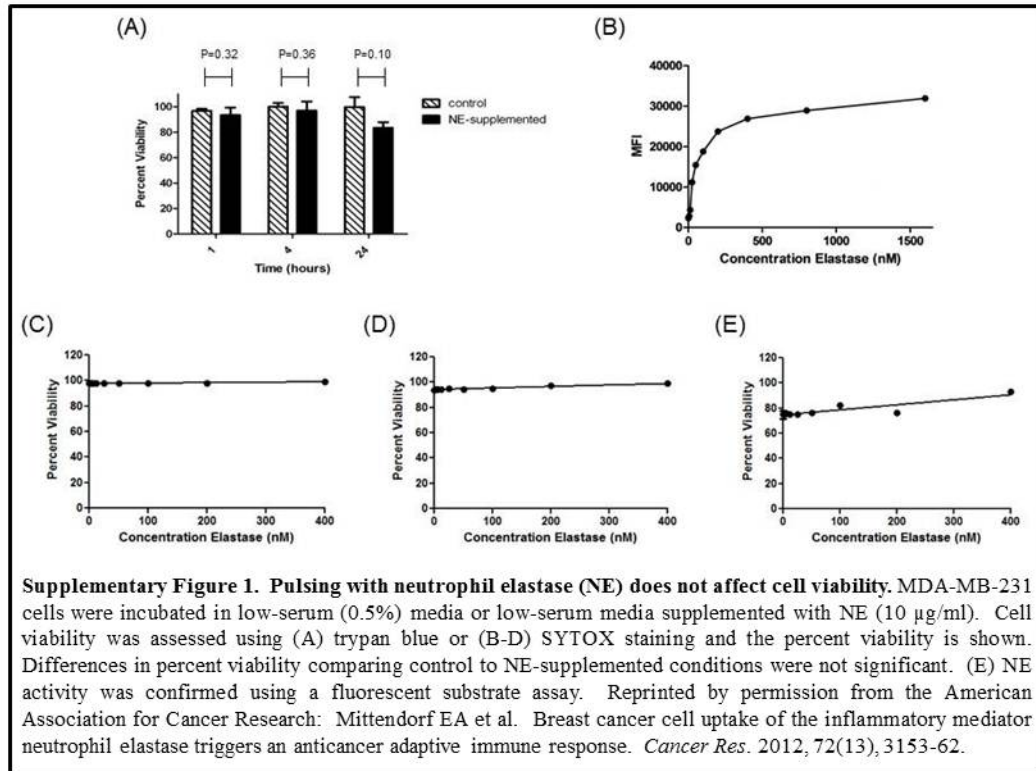
substrate availability for presentation on MHC class I molecules which may be upregulated by IFN- α 2b.

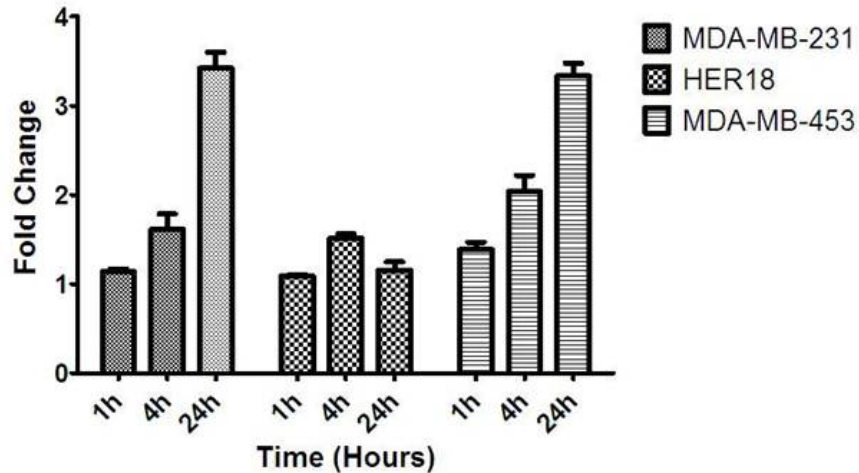
Conclusion

In conclusion, we have provided evidence for a novel mechanism linking NE, a protease secreted by innate immune cells, to adaptive immune responses against novel antigens in breast cancer. Specifically, we have shown that after uptake of NE, breast cancer cells become susceptible to killing by CCNE- and PR1-targeting therapies. Importantly, these initial studies evaluating the effects of NE uptake in breast cancer, have led to additional experiments which have provided evidence that NE uptake may have a more ubiquitous effect on antigen processing and presentation. Further studies, as described above, are required to further evaluate this mechanistically. This work has identified a line of investigation that will have broad applicability to immunotherapy against multiple tumor types.

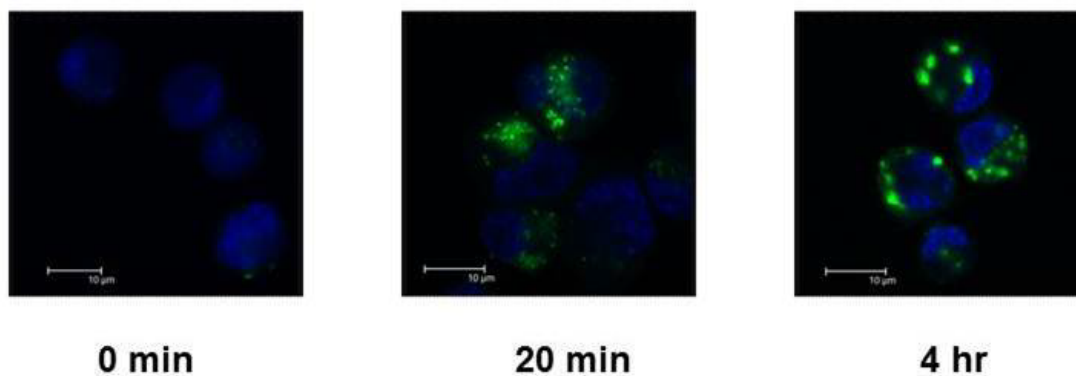
SUPPLEMENTARY FIGURES

All of the supplementary figures were included in our recently published manuscript (Mittendorf EA, et al. *Cancer Res* 72:3153-3162;2012) and are used with permission.

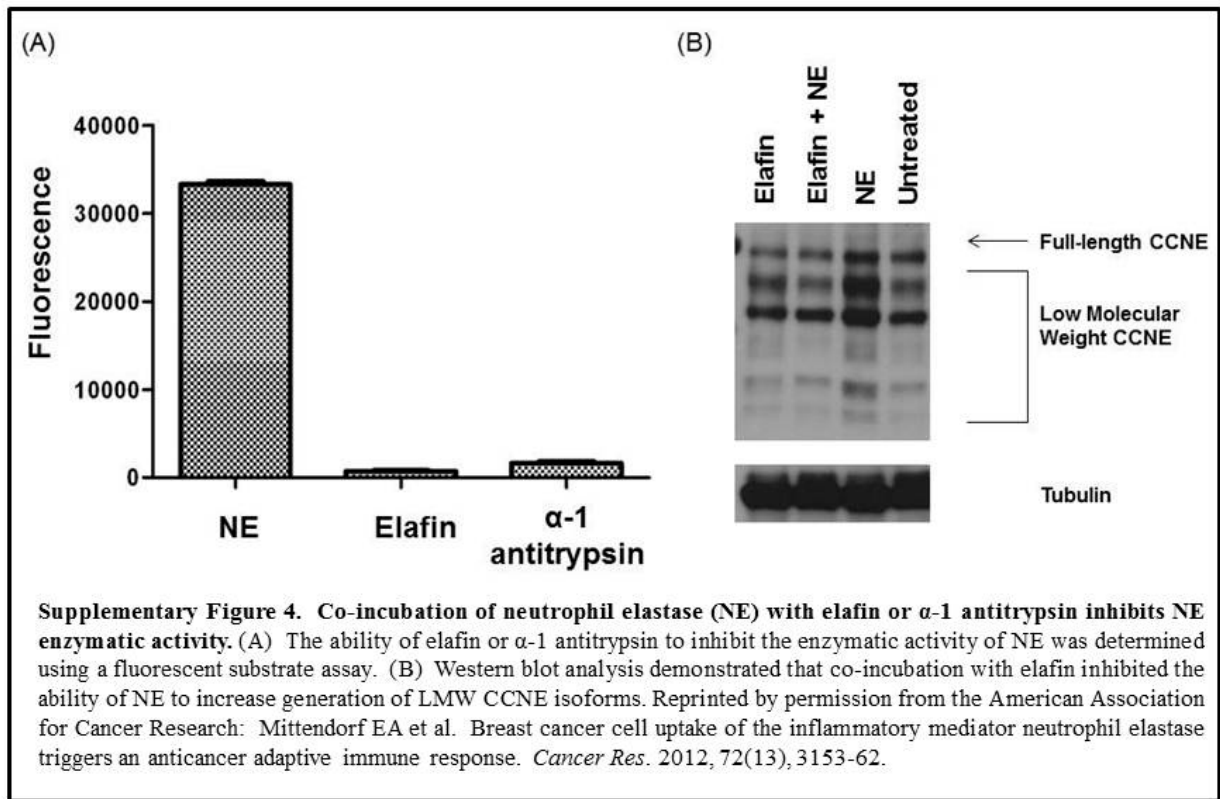


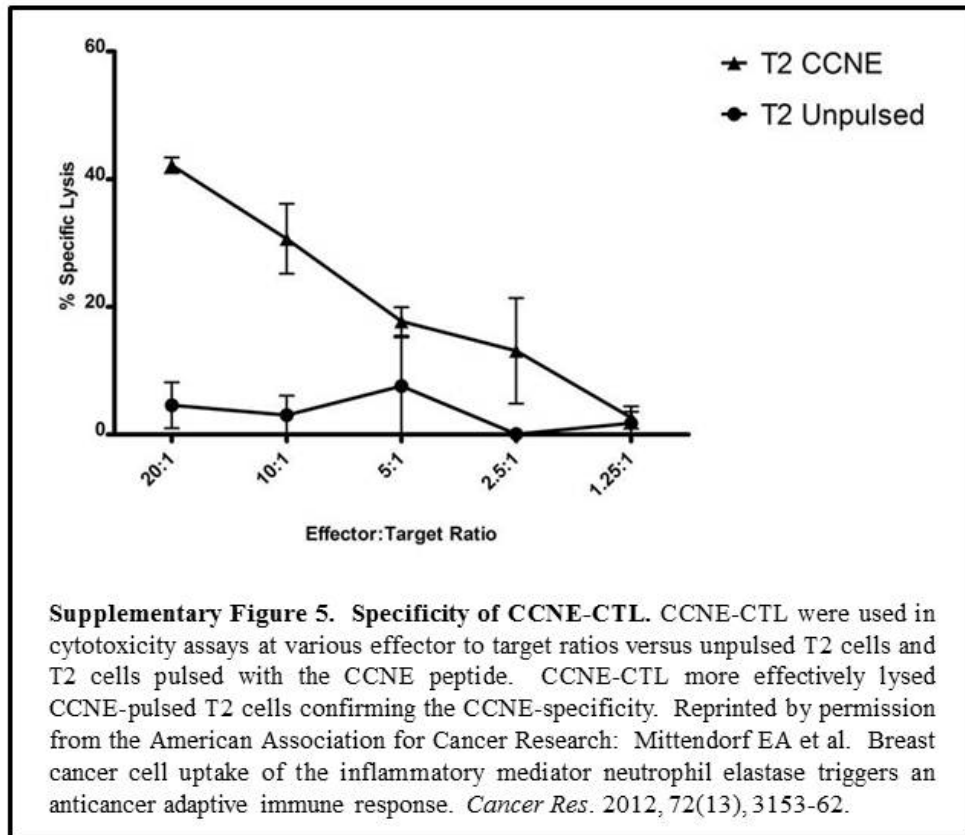


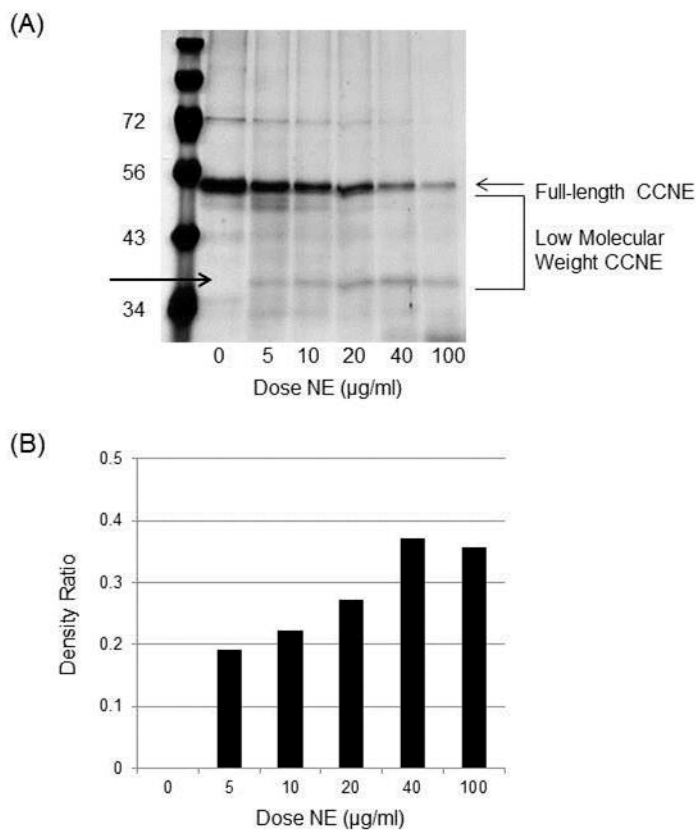
Supplementary Figure 2. Soluble neutrophil elastase (NE) is taken up by breast cancer cells. MDA-MB-231, HER18, and MDA-MB-453 breast cancer cells were pulsed with NE (10 $\mu\text{g}/\text{m}$) for 1 hour, 4 hours, and 24 hours. Cells were permeabilized and stained with a FITC-conjugated antibody to NE then analyzed using flow cytometry. Experiments were performed in triplicate. Data is expressed as mean fold-change \pm standard deviation in the MFI of anti-NE staining compared with unpulsed cells. MFI = median fluorescent intensity. Reprinted by permission from the American Association for Cancer Research: Mittendorf EA et al. Breast cancer cell uptake of the inflammatory mediator neutrophil elastase triggers an anticancer adaptive immune response. *Cancer Res.* 2012, 72(13), 3153-62.



Supplementary Figure 3. Soluble neutrophil elastase (NE) is taken up by breast cancer cells. MDA-MB-231 breast cancer cells were maintained for the indicated amount of time in NE-supplemented (10 μg/ml) media. Cells were permeabilized and stained with Alexa-488-conjugated anti-NE antibody (green). DAPI (blue) was used as a nuclear stain. Reprinted by permission from the American Association for Cancer Research: Mittendorf EA et al. Breast cancer cell uptake of the inflammatory mediator neutrophil elastase triggers an anticancer adaptive immune response. *Cancer Res.* 2012, 72(13), 3153-62.







Supplementary Figure 6. Neutrophil Elastase (NE) cleaves cyclin E (CCNE). Increasing concentrations of NE were added to 2 μg of recombinant human CCNE and incubated at 37° for 2 minutes after which the reaction was stopped by adding sample buffer (2% SUD / 10% glycerol / 5% β -mercaptoethanol). (A) Samples were run on a 10% SDS-PAGE gel and stained with silver stain, which demonstrated a decrease in the expression of full-length CCNE and concomitant increase in LMW CCNE with increasing concentration of NE. At the highest concentration of NE (100 $\mu\text{g/ml}$), there appears to be degradation of the LMW products as shown by the dimmer LMW CCNE bands. This degradation was not seen at the lower, more physiologic doses. (B) Densitometry analysis was performed to quantify the expression level of LMW CCNE in relation to total CCNE (full length + LMW) for each concentration of NE used. For purposes of this quantitative analysis, we used the density of the LMW isoform shown by the arrow in panel A. Reprinted by permission from the American Association for Cancer Research: Mittendorf EA et al. Breast cancer cell uptake of the inflammatory mediator neutrophil elastase triggers an anticancer adaptive immune response. *Cancer Res.* 2012, 72(13), 3153-62.

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VITA

Elizabeth Ann Mittendorf M.D. was born and raised in Fairfax County, Virginia. After graduating as the valedictorian of her high school class, she attended Duke University in Durham, North Carolina where she majored in Biomedical Engineering. She graduated magna cum laude in 1992. Following graduation from Duke, Elizabeth attended medical school at Case Western Reserve University in Cleveland, Ohio. She graduated in 1996 and remained in Cleveland to complete her general surgery training in the Case Western Reserve University Integrated Surgical Residency Program. Following completion of her surgical training in 2001, Elizabeth entered active duty in the United States Air Force. While in the Air Force, she spent the majority of her time at the Walter Reed Army Medical Center where she cared for active duty members and their families. While at Walter Reed she also worked in the laboratory of Dr. George Peoples who stimulated her interest in breast cancer immunotherapy. She continues to collaborate with Dr. Peoples in the design and conduct of HER2-derived peptide vaccine trials. Elizabeth separated from the Air Force in 2005 having attained the rank of Major. Following her active duty time, she came to the University of Texas MD Anderson Cancer Center where she completed the Surgical Oncology fellowship. During her time as a fellow, she worked in the laboratory of Drs. Kelly Hunt and Khandan Keyomarsi studying the relationship between HER2 and cyclin E in breast cancer. Following completion of her fellowship, Elizabeth remained on faculty in the Department of Surgical Oncology as a physician scientist in the surgical breast section. She also enrolled in the University of Texas Graduate School of Biomedical Sciences. Under the mentorship of Dr. Jeffrey Molldrem, she has studied neutrophil elastase as a link between innate immunity and adaptive immune responses in breast cancer. After graduate school, Elizabeth will continue in her position as a physician scientist at MD Anderson with the goal of continuing to build a breast cancer immunotherapy program.