Microenvironment and Immunology

Breast Cancer Cell Uptake of the Inflammatory Mediator Neutrophil Elastase Triggers an Anticancer Adaptive Immune Response

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Abstract
There is little understanding of the impact of tumor-associated neutrophils (TAN) on adaptive immunity to tumors. In this study, we report the results of an investigation of the pathobiologic basis for the prognostic significance of neutrophil elastase, a serine protease found in neutrophil granules, in a model of cyclin E (CCNE)–overexpressing breast cancer. We established that neutrophil elastase was expressed by TAN within breast cancer tissues but not by breast cancer cells. Neutrophil elastase modulated killing of breast cancer cells by CTLs specific for CCNE-derived HLA-A2–restricted peptide (ILLDWLMEV). Breast cancer cells exhibited striking antigen-specific uptake of neutrophil elastase from the microenvironment that was independent of neutrophil elastase enzymatic activity. Furthermore, neutrophil elastase uptake increased expression of low molecular weight forms of CCNE and enhanced susceptibility to peptide-specific CTL lysis, suggesting that CCNE peptides are naturally presented on breast cancer cells. Taken together, our findings reveal a previously unknown mechanism of antitumor adaptive immunity that links cancer cell uptake of an inflammatory mediator to an effective cytolytic response against an important breast cancer antigen. Cancer Res; 72(13); 3153–62. ©2012 AACR.

Introduction
Neutrophil elastase is a serine protease normally expressed in neutrophil primary granules. It plays a role in antimicrobial defenses and inflammation and is aberrantly expressed in myeloid leukemia (1–3). Although neutrophil elastase is primarily restricted to hematopoietic cells of the myeloid lineage, it has been shown in breast cancer tissue extracts in which it was prognostic (4–6). Foekens and colleagues (4) showed that high levels of neutrophil elastase detected by ELISA in primary breast tumors were associated with poor metastasis-free, disease-free (DFS), and overall survival (OS; ref. 5). These results were corroborated by Yamashita and colleagues who determined that neutrophil elastase concentration correlated with DFS (4, 6). The prognostic value of neutrophil elastase has been attributed to its ability to degrade extracellular matrix, thereby promoting invasion and metastasis (7, 8). The source of neutrophil elastase in breast tumors is unknown and has been attributed to endogenous production by breast cancer cells (9, 10).

Cyclin E (CCNE), an important cell-cycle regulator, has also been shown to be prognostic in breast cancer. Overexpression of CCNE causes tumorigenesis by promoting the G1 to S-phase transition, increasing CCNE-associated kinase activity and causing genomic instability (11–14). Keyomarsi and colleagues showed that CCNE levels were more powerful determinants of DFS and OS than commonly used clinicopathologic prognostic factors, including tumor size, nodal status, clinical stage, and estrogen receptor expression (15). In tumors, the principal mode of CCNE deregulation is at the protein level. Some breast cancer cell lines and human breast cancers express tumor-specific low molecular weight (LMW) isoforms that are more active than full-length CCNE and are resistant to cyclin-dependent kinase inhibitors (12, 16–19). Importantly, neutrophil elastase was shown to cleave CCNE into its LMW isoforms, suggesting that generation of LMW CCNE may be another mechanism linking neutrophil elastase expression and poor prognosis in breast cancer (18, 20).

The CCNE LMW isoforms have been described in other tumors including leukemia (21). We have investigated CCNE as a leukemia-associated antigen and identified the human leukocyte antigen (HLA)-A2–restricted CCNE-derived peptide...
CCNE144 (ILLDWLMEV) as a candidate target for immunotherapy. Importantly, the sequence for CCNE144 is contained in full-length CCNE and the LMW isoforms. CCNE144-specific CTLs (CCNE-CTL) were shown to specifically lyse leukemia cells overexpressing CCNE and its LMW isoforms (21). Because CCNE is aberrantly expressed in breast cancer, we hypothesized that it may represent a target for immunotherapy in breast cancer as well.

Neutrophils and other myeloid cells are present in the tumor microenvironment, and because it has been shown that lung cancer cells can take up neutrophil elastase (22), we postulated that breast cancer cells may take up neutrophil elastase. Because neutrophil elastase has been shown to cleave full-length CCNE, we further hypothesized that neutrophil elastase uptake may lead to increased cleavage of CCNE to its LMW isoforms. The LMW isoforms lack the portion of the full-length protein’s amino terminus that contains the nuclear localization sequence, therefore, LMW CCNE isoforms have altered subcellular localization, accumulating in the cytoplasm in which they may be preferentially processed and presented as antigens complexed with HLA-I molecules (23, 24). This in turn could increase susceptibility to lysis by CCNE-CTL. In this article, we show that breast cancer cells lack endogenous neutrophil elastase expression but can take up neutrophil elastase at concentrations similar to that encountered in the tumor microenvironment because of the presence of activated tumor-associated neutrophils (TAN). Neutrophil elastase uptake resulted in increased LMW CCNE expression and susceptibility of breast cancer cells to specific lysis by CCNE-CTL. Taken together, these data provide strong evidence for a previously undescribed mechanism linking innate immunity and an adaptive immune response against a novel breast cancer antigen.

Materials and Methods

Patients, cells, and cell lines

Peripheral blood samples were obtained through an Institutional Review Board–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 Identifier Kit according to manufacturer instructions (Applied Biosystems). Breast cancer cells were cultured in Dulbecco’s Modified Eagle’s Medium with 10% FBS, 100 U/mL penicillin, and 100 µg/mg streptomycin. Media for HER-18 cells was supplemented with 0.5 mg/mL G418. U-937, JKT, T2, and HL-60 cell lines were cultured in RPMI-1640 (RPMI) with 10% FBS, 100 U/mL penicillin, and 100 µg/mg streptomycin. All cells were maintained in 5% CO2 at 37°C.

Western blot analysis

Whole cells lysates were generated in radioimmunoprecipitation assay 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 PCR

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 cRNA. cDNA was synthesized from 1 µg of cRNA 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’-CAGGAGGCGCAAGGCC-3’, reverse primer 5’-TATTTGCCGATCTGGGAG-3’), mammaglobin (forward primer 5’-AGCAGGCTGCAAGGCTCT-3’, reverse primer 5’-ATAAGAAAGAAGGTGTTG-3’), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an endogenous control (forward primer 5’-TAGAGGGGAAGCTACTGGA-3’, reverse primer 5’-AGGTCACACCACCTGTGCT-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 was 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 x 10⁶ cells were maintained in 6-well plates in media supplemented with various concentrations of neutrophil elastase at 37°C. At designated time points, cells were harvested, permeabilized, and stained with the following antibodies: Alexa-647- or Alexa-488-conjugated antineutrophil elastase (clone NP57; Santa Cruz), fluorescein isothiocyanate (FITC)-conjugated anti-EA-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 cocultured with MDA-MB-231 cells at a 1:1 ratio for 4 hours.

**Peptide-specific CTL lines and cell-mediated cytotoxicity assay**

Healthy donor HLA-A2⁺ peripheral blood mononuclear cells (PBMC) were stimulated with CCNE₁₉₄–₂₈₂ peptide, as previously described (25). 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, restimulation 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 (25). 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 x 10⁵ 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<sub>experimental</sub> – E<sub>medium</sub>)/(E<sub>control</sub> – E<sub>medium</sub>)) × 100, in which E is fluorescence emission and control group is targets alone.

**Staining for CCNE-CTL in breast cancer patients**

PBMCs 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 carry out statistical analyses and P values less than 0.05 were considered significant.

**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. 1A) or mRNA transcripts (Fig. 1B) in any of 5 cell lines 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. 1C). Immunohistochemistry carried out on breast tumor tissue showed neutrophil elastase in TAN within the tumor microenvironment, but not in breast cancer cells (Fig. 1D). Taken together, these data were consistent with our hypothesis that TAN present in the microenvironment are the primary source of neutrophil elastase in breast tumors.

**Soluble and cell-associated neutrophil elastase 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 non-meric peptide derived from neutrophil elastase that has been extensively investigated in myelogenous leukemias (25–28). In addition, Houghton and colleagues showed the ability of lung cancer cells to take up neutrophil elastase (22). 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. 2A). Cell viability was not affected by neutrophil elastase in the culture media (Supplementary Fig. S1). 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. S2). The extent of neutrophil elastase uptake varied among cell lines.

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ments showed concentration-dependent uptake of neutrophil elastase. Experiments were carried out. Concentration-dependent uptake of neutrophil elastase was required for uptake. Neutrophil elastase uptake whereas elastase was not detectable in any of the cell lines evaluated. C, RT-PCR evaluating for neutrophil elastase mRNA was carried out using RNA extracted from breast cancer cells isolated from tumors using LCM (LCM1, LCM2, and LCM3). Primers to mammoglobin (MGB-1) were used to confirm that LCM was specific for breast cancer cells. Positive controls included MDA-MB-453 breast cancer cells for mammoglobin and HL60 for neutrophil elastase. No LCM specimens showed neutrophil elastase mRNA. D, a breast tumor was evaluated by hematoxylin and eosin staining to confirm tumor cells and inflammatory infiltrate. TAN present in the microenvironment stained positive for neutrophil elastase by immunohistochemistry. NE, neutrophil elastase.

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 nonspecific proteins OVA or cathepsin G, a second serine protease (Fig. 2B), 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. 2C). 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. S3).

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 (65 kDa) or elafin (6 kDa), before its addition to culture media. Neutrophil elastase enzymatic activity inhibition by α-1 antitrypsin and elafin was confirmed (Supplementary Fig. S4). α-1 Antitrypsin inhibited neutrophil elastase uptake whereas elafin had no effect (Fig. 2D), 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 cocultured 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 μg/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. 3).

**Neutrophil elastase localization following uptake**

Confocal images had shown uptake of soluble neutrophil elastase into distinct cellular compartments (Supplementary Fig. S3). 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 μg/mL) media. At increasing time points, cells were harvested and costained 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

**Figure 1.** Breast cancer cells do not express endogenous neutrophil elastase. A, lysates harvested from breast cancer cell lines cultured in 0.5% serum media were used in Western blots showing no neutrophil elastase protein expression. The leukemia cell line U-937 served as a positive control. B, RNA extracted from cultured breast cancer cells was used to carry out RT-PCR using primers to neutrophil elastase. The leukemia cell line HL60 and T-cell line jurkat (JKT) served as positive and negative controls, respectively, for neutrophil elastase. No neutrophil elastase mRNA was detected in any of the breast cancer cell lines evaluated. C, RT-PCR evaluating for neutrophil elastase mRNA was carried out using RNA extracted from breast cancer cells isolated from tumors using LCM (LCM1, LCM2, and LCM3). Primers to mammoglobin (MGB-1) were used to confirm that LCM was specific for breast cancer cells. Positive controls included MDA-MB-453 breast cancer cells for mammoglobin and HL60 for neutrophil elastase. No LCM specimens showed neutrophil elastase mRNA. D, a breast tumor was evaluated by hematoxylin and eosin staining to confirm tumor cells and inflammatory infiltrate. TAN present in the microenvironment stained positive for neutrophil elastase by immunohistochemistry. NE, neutrophil elastase.
compartment (Fig. 4A). There was no evidence of neutrophil elastase uptake into lysosomes (Fig. 4B). Experiments were repeated following uptake of cell-associated neutrophil elastase that showed early uptake of neutrophil elastase with perinuclear localization (Fig. 4C and D).

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 that subsequently undergo phosphorylation to generate 2 sets of doublets (Fig. 5A; refs. 18, 29). LMW isoforms of CCNE lack a nuclear localization sequence and therefore accumulate in the cytoplasm (23, 24), which may facilitate

Figure 2. Soluble neutrophil elastase is taken up by breast cancer cells. A, MDA-MB-231 cells were maintained in media supplemented with neutrophil elastase (10 μg/mL). After 24 hours, cells were analyzed for intracellular neutrophil elastase. Neutrophil elastase uptake was significant when compared with unpulsed cells (t test; \( P < 0.01 \)). Neutrophil elastase expression in MDA-MB-231 cells was also compared with T2 cells and HL60, a cell line known to express significant endogenous neutrophil elastase. B, MDA-MB-231 cells were maintained in media supplemented with neutrophil elastase at various concentrations for 18 hours, after which neutrophil elastase uptake was determined. Cells maintained in media supplemented with OVA and cathepsin G (CG) were used to evaluate for nonspecific uptake. C, timing of neutrophil elastase uptake was determined using MDA-MB-231 cells maintained in media supplemented with neutrophil elastase (10 μg/mL). Cells were harvested at various time points and neutrophil elastase uptake determined. D, to determine whether neutrophil elastase uptake was enzyme dependent, neutrophil elastase was coincubated with 2 different inhibitors, elafin (6 kDa) and α-1 antitrypsin (65 kDa), before addition to media. After 4 hours, cells were harvested and neutrophil elastase uptake determined. All experiments were conducted in triplicate and mean MFI ± SD is shown. Comparisons were made using ANOVA with Tukey multiple comparison test. MFI, median fluorescence intensity; NE, neutrophil elastase.

Figure 3. MDA-MB-231 breast cancer cells are able to take up cell-associated neutrophil elastase. MDA-MB-231 cells were cultured with soluble neutrophil elastase (10 μg/mL), irradiated neutrophils (PMN), or irradiated mononuclear cells (MNC) at a 1:3 ratio for 4 hours. Cells were permeabilized and stained with FITC-conjugated neutrophil elastase antibodies, then analyzed using flow cytometry. MDA-MB-231 cells cocultured with irradiated PMN showed significantly greater neutrophil elastase uptake compared with cells cultured in soluble neutrophil elastase–supplemented media (ANOVA with Tukey multiple comparison test). Experiments were conducted in triplicate and mean MFI ± SD is shown. MFI, median fluorescence intensity; NE, neutrophil elastase.
ubiquitination and proteasomal processing for presentation on HLA-A1 molecules (30–33). 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 PBMCs 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. 5B; ref. 34). These assays showed that CCNE-CTL more effectively lysed HER18, which express more LMW CCNE than MDA-MB-231 (Fig. 5C). CCNE-CTL–specific cytolyis was confirmed using unpulsed T2 cells and T2 cells pulsed with CCNE (Supplementary Fig. S5). 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. 5B). Processing of CCNE by neutrophil elastase to LMW CCNE was confirmed using recombinant CCNE incubated with neutrophil elastase over a range of concentrations (5–100 μg/mL; Supplementary Fig. S6). Importantly, CCNE-CTL–specific lysis of neutrophil elastase–pulsed MDA-MB-231 cells was greater than that versus unpulsed cells (Fig. 5D). The cytotocicity assays were carried out 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 cytosis.

**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. PBMCs 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 6A shows 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. 6B). The frequency of CCNE144-152–specific CTL in breast cancer patients was greater than in healthy donors (P = .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.
Elastase Augments Adaptive Immune Response in Breast Cancer

**Discussion**

In this article, we identified a novel function of neutrophil elastase, a serine protease in the tumor microenvironment. We showed that neutrophil elastase is present in TANs and that breast cancer cells do not produce neutrophil elastase, suggesting TANs as the primary source of neutrophil elastase in breast cancer. We also showed that neutrophil elastase is specifically taken up by breast cancer cells. Importantly, after neutrophil elastase uptake, LMW isoforms of CCNE increase and breast cancer cells become susceptible to cytolysis by CTL specific for an HLA-A2--restricted peptide (CCNE144-152) that is contained within each LMW isoform. This data confirms that CCNE-derived peptides are naturally processed and presented on breast cancer cells. Therefore, we have established a novel mechanism linking neutrophil elastase, a protease secreted by cells of the innate immune system, to an adaptive immune response against a novel tumor antigen in breast cancer.

Our results, combined with previous studies that show neutrophil elastase cleaves CCNE into LMW isoforms, strongly suggest that after uptake, neutrophil elastase increases substrate availability of CCNE fragments in breast cancer cells, which could augment antigen processing of CCNE peptides. In support of this hypothesis, we showed that HER18 cells are more susceptible to cytolysis by CCNE-CTL compared with MDA-MB-231, which express similar amounts of full-length CCNE but less of the LMW isoforms compared with HER18. Furthermore, after uptake of soluble neutrophil elastase from culture media, LMW isoform expression increased in MDA-MB-231 cells, and the cells were more susceptible to lysis by CCNE-CTL. Additional studies must be done to confirm whether full-length CCNE or the LMW isoforms are the predominant source of CCNE144-152 peptide. However, our data support the discovery of CCNE as a breast cancer antigen with potential implications for immunotherapy strategies targeting CCNE. Because the LMW isoforms are tumor specific and their overexpression drives breast cancer cell proliferation (12, 16–19), they have characteristics of an ideal tumor-associated antigen. Importantly, using tetramer staining of PBMCs from breast cancer patients, we provide evidence of a low precursor frequency of CCNE144-152 CTL, suggesting that...
adaptive immunity against the peptide is increased in patients. A peptide vaccine incorporating the CCNE<sub>144–153</sub> peptide with an immunoadjuvant may, therefore, be effective in augmenting a CCNE-specific CTL response. High-grade, triple-negative tumors have been found to have an intense immune cell infiltrate (35), suggesting that patients with such tumors might be candidates for a CCNE-targeting vaccine.

Although neutrophil elastase uptake could promote antitumor immunity by enhancing susceptibility to a CCNE-specific immune response, it is also possible that neutrophil elastase uptake in the setting of chronic inflammation in the tumor microenvironment could promote tolerance resulting from anergy if antigen presentation occurred in the absence of adequate costimulation. Although breast cancer cells express MHC class I molecules and can present CCNE<sub>144–152</sub> and other peptides, they lack costimulatory molecules and may therefore be unable to stimulate naive T cells. Nevertheless, strategies such as adoptive T-cell therapy or antibodies that target the CCNE<sub>144–152</sub>/HLA-A2 conformational epitope (36, 37) might overcome such tolerance.

Within tumors, the major effects of TAN are thought to promote tumor growth. For instance, neutrophil-derived cytokines enhance tumor growth by producing factors such as IL-8 and oncostatin M that promote angiogenesis, invasion, and metastasis (38–40). Neutrophil-derived proteases, including neutrophil elastase, degrade cytokines, chemokines, and their receptors and are important for remodeling the extracellular matrix (7, 8). By these mechanisms, neutrophil-derived products impact tumor proliferation, vessel density, and metastatic potential (41). A study by Houghton and colleagues showed neutrophil elastase uptake by lung adenocarcinoma cells, in which it localized to endosomes and induced tumor cell proliferation by cleaving insulin receptor substrate-1 inducing hyperactivity of the phosphatidylinositol-3-kinase pathway and uncontrolled proliferation (22). Our study confirms that cancer cells take up exogenous neutrophil elastase. The study by Houghton and colleagues showed neutrophil elastase is taken up into clathrin-coated vesicles, and we show that neutrophil elastase uptake is antigen specific and time and dose dependent, suggesting a receptor-mediated mechanism. Our study provides an additional potential mechanism of neutrophil elastase–induced tumor cell growth by increasing CCNE LMW isoforms. Conversely, neutrophil elastase can also potentiate antitumor immunity by increasing the susceptibility of breast cancer cells to lysis by CCNE-CTL. Thus, the net effect of neutrophil elastase uptake in tumor cells likely depends upon additional factors. Because the timing and subcellular localization of neutrophil elastase after uptake is similar in distinct cancer cells, it is possible that there is a common uptake mechanism. If so, such a mechanism could be critical for controlling cell growth and modulating responsiveness to adaptive immunity. Experiments are ongoing in our laboratory to explore this possibility.

Inflammatory cells comprise a significant component of the tumor microenvironment (42–46). Included in these inflammatory cells are neutrophils that are derived from myeloid hematopoietic progenitor cells and produce proteases, including neutrophil elastase. Although neutrophils have a short life span in circulation, they survive longer within the inflammatory environment, possibly as a result of the effects of...
cytokines on their survival (47). In addition, upregulation of neutrophil–chemotactic substances, such as IL-8, results in continuous recruitment of neutrophils to the tumor site (41). Therefore, there is a growing interest in studying TANs. Jensen and colleagues showed that the presence of CD66b+ intratumoral neutrophils was an independent prognostic factor for shorter DFS and OS in clear cell renal cell carcinoma (48). Increased neutrophil infiltration is also associated with poor outcomes in bronchoalveolar carcinoma (49). To date, there have been no studies investigating intratumoral neutrophils in breast cancer. Two groups have shown that the presence of immunoreactive neutrophil elastase in whole tumor extracts in breast cancer patients correlates with poor clinical outcomes (4–6). However, neutrophil elastase is synthesized by bone marrow precursor cells that give rise to neutrophils, with neutrophil elastase mRNA being shown in these early myeloid progenitors (1, 50). To our knowledge, no study has definitely shown neutrophil elastase mRNA in epithelial cells, providing further support for our conclusion that neutrophil elastase in breast tumors is derived from TANs.

Nevertheless, a report by Nguyen and colleagues showed neutrophil elastase protein in MDA-MB-231 cells by indirect immunofluorescence (20), suggesting neutrophil elastase in tumors could be partially derived from breast cancer cells. However, in MDA-MB-231 cells, we were unable to identify neutrophil elastase protein by confocal microscopy or in immunohistochemistry (51). Neutrophils could not be identified in neutrophil elastase mRNA transcripts. We also found no neutrophil elastase in the supernatants of cultured breast cancer cells by an antineutrophil elastase ELISA (data not shown). Nevertheless, in MDA-MB-231 cells, we were unable to identify neutrophil elastase protein by confocal microscopy or in whole cell lysates, and we were unable to find neutrophil elastase mRNA transcripts. We also found no neutrophil elastase in the supernatants of cultured breast cancer cells by an antineutrophil elastase ELISA (data not shown). Most importantly, we were unable to amplify neutrophil elastase mRNA transcripts from RNA isolated from primary breast cancer cells with single cell LCM. Moreover, breast cancer biopsies stained with an antineutrophil elastase antibody and examined by immunohistochemistry confirmed that neutrophil elastase was present in neutrophils but not in breast cancer cells. We therefore believe our study is the first to show the cellular origin of neutrophil elastase in breast tumors is from TAN within the tumor microenvironment.

In conclusion, we show that breast cancer cells rapidly take up neutrophil elastase derived from neutrophils. After neutrophil elastase uptake, LMW CCNE isoforms are increased and the susceptibility of breast cancer to lysis by CTL specific for the novel HLA-A2–restricted CCNE144-152 peptide is increased. Thus, we propose a previously undescribed indirect mechanism linking neutrophil elastase derived from neutrophils, a component of the innate immune system, to an adaptive immune response against a novel breast cancer antigen that is cleaved after specific uptake of neutrophil elastase. Further investigation into the mechanisms regulating neutrophil elastase uptake and the subsequent effects on antigen processing are warranted to improve our understanding of the link between inflammation and breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: E.A. Mittendorf, G. Alatrash, J.J. Molldrem
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