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Research Article

Cancer Immunology Research

Serine Proteases Enhance Immunogenic Antigen Presentation on Lung Cancer Cells

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Abstract

Immunotherapies targeting immune checkpoints have proven efficacious in reducing the burden of lung cancer in patients; however, the antigenic targets of these reinvigorated T cells remain poorly defined. Lung cancer tumors contain tumor-associated macrophages (TAM) and neutrophils, which release the serine proteases neutrophil elastase (NE) and proteinase 3 (P3) into the tumor microenvironment. NE and P3 shape the antitumor adaptive immune response in breast cancer and melanoma. In this report, we demonstrate that lung cancer cells cross-presented the tumor-associated antigen PR1, derived from NE and P3. Additionally, NE and P3 enhanced the expression of human leukocyte antigen (HLA) class I molecules on lung cancer cells and induced unique, endogenous peptides in the immunopeptidome, as detected with mass spectrometry sequencing. Lung cancer patient tissues with high intratumoral TAMs were enriched for MHC class I genes and T-cell markers, and patients with high TAM and cytotoxic T lymphocyte (CTL) infiltration had improved overall survival. We confirmed the immunogenicity of unique, endogenous peptides with cytotoxicity assays against lung cancer cell lines, using CTLs from healthy donors that had been expanded against select peptides. Finally, CTLs specific for serine proteases–induced endogenous peptides were detected in lung cancer patients using peptide/HLA-A2 tetramers and were elevated in tumor-infiltrating lymphocytes. Thus, serine proteases in the tumor microenvironment of lung cancers promote the presentation of HLA class I immunogenic peptides that are expressed by lung cancer cells, thereby increasing the antigen repertoire that can be targeted in lung cancer. *Cancer Immunol Res; 5(4); 319–29. ©2017 AACR.*

Introduction

Cytotoxic T lymphocytes (CTL) recognize and lyse cancer cells presenting neoantigens in the context of HLA class I molecules (1, 2). Reported mechanisms of lung cancer attenuation of the antitumor immune response include expression of T-cell inhibitory molecules such as PD-L1 by lung cancer cells to evade CTL activity (1) and recruitment of regulatory T cells (Treg) by tumor-associated neutrophils (TAN; ref. 3). This inhibition can be overcome by immune-checkpoint blockade therapies targeting PD-1 and CTLA-4 on the T cells, thus permitting recognition of tumor neoantigens presented on HLA class I molecules (1, 2).

The HLA-A2–restricted, leukemia-associated antigen PR1 (VLQELNVTV), derived from neutrophil elastase (NE) and proteinase 3 (P3) has proven to be an effective leukemia target in preclinical animal models as well as clinical trials (4–9). PR1 serves as an exogenous antigen on the surface of solid tumors, but has not been reported for lung cancer (6–8, 10). In breast cancer, NE cleaves the endogenous antigen–encoded protein cyclin E, generating an HLA-A2–restricted, immunogenic antigen, ILLDWLMEV (11). NE also increases expression of surface HLA-A, B,C on breast cancer cell lines, H2023 lung cancer cells, and MEL526 melanoma cells (12). In the solid tumor setting, NE and P3, along with other serine proteases, are delivered to cancer cells by TAN and tumor-associated macrophages (TAM; ref. 10). In early-stage lung cancer, TAN, which can comprise up to 25% of cells of the tumor, can activate T cells (13).

In view of (i) the demonstrated capacity of NE to elicit immunogenic peptide presentation from exogenous and endogenous proteins on solid tumors (10, 11), (ii) previous studies reporting cell-extrinsic perturbations to the immunopeptidome (14), and (iii) improved outcomes in lung cancer patients that correlated with neoantigen presentation on the tumor cell surface (1), we hypothesized that serine proteases alter the immunopeptidome

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by inducing immunogenic presentation, including but not limited to PR1, on the lung cancer cell surface. These peptides may elicit CTL recognition, and therefore could serve as targets for existing and novel immunotherapies.

In this study, we demonstrated that lung cancer cells cross-presented the leukemia-associated antigen PR1 and were lysed by PR1-targeted immunotherapy. Increased surface expression of HLA class I molecules on lung cancer cells was induced by NE, which led to enhanced presentation of novel self-antigens and mutant peptides. We validated the immunogenicity of novel self-antigens using *in vitro* assays and found CTLs specific for these novel, endogenous self-antigens in lung cancer patients. Additionally, we provided evidence that TAM could alter the antitumor adaptive immune response.

Materials and Methods

Cells, cell culture, and reagents

Healthy-donor peripheral blood mononuclear cells (PBMC) and polymorphonuclear leukocytes (PMN) were isolated from apheresis obtained from the MD Anderson Blood Bank by single or double Ficoll gradient, respectively, using Histopaque-1077 and Histopaque-1119 (Sigma-Aldrich). Four-digit, molecular HLA typing was performed for lung cancer cell lines: H2023 (A*02:01, A*02:01, B*07:02, B*08:01, C*07:01, C*07:02), H441 (A*02:01, A*03:01, B*38:01, B*44:03, C*12:03, C*16:02), DFCI024 (A*24:02, A*33:01, B*14:02, B*35:02, C*08:02, C*04:01), DFCI032 (A*02:01, A*02:01, B*44:02, B*51:01, C*05:01, C*05:01), H1299 (A*24:02, A*32:01, B*40:01, B*40:02, C*03:03, C*02:02), and HCC2935 (A*02:01, A*26:01, B*37:01, B*49:01, C*06:02, C*07:01) at the HLA Typing Laboratory at MD Anderson. The H441, U937 (histiocytic leukemia), and T2 cell lines were obtained from ATCC in 2012, 2014, and 2015, respectively. Cell lines were cultured continuously for 6 months or less. The remaining lung cancer lines were a generous gift from Dr. Adi Gazdar (University of Texas Southwestern) in 2009. Cell lines were maintained in RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptavidin (Invitrogen), and 10% fetal bovine serum that had been heat shocked at 56°C for 30 minutes. All cells were cultured at 5% CO_2 at 37°C. Cell lines were validated at the MD Anderson Sequencing and Microarray Facility using short-tandem repeat DNA fingerprinting and routinely checked for *mycoplasma* by PCR (PromoKine).

Purified neutrophil elastase (NE) and proteinase 3 (P3) were purchased from Athens Research. Recombinant human cytokines were purchased from R&D Systems. All primers were ordered from Sigma-Aldrich: *ELANE* (forward 5′-CACGGAGGGGCAGAGACC-3′; reverse 5′-TATTGTGCCAGATGCTGGAG-3′) and *PRTN3* (forward 5′-5′-GACCCCACCATGGCTCAC-3′; reverse 5′-ATGGGAAG-GACAGACAGGAG-3′). *GAPDH* (forward 5′-TAGACGGGAAGCT-CACTGGC-3′; reverse 5′-AGGTCCACCACCCTGTTGCT-3′) oligonucleotides served as loading controls. Real-time PCR amplifications were performed using an iCycler iQ thermal cycler (Bio-Rad Laboratories). Peptides of >95% purity were purchased from Bio-Synthesis Inc.

Flow cytometry, serotyping, and immunofluorescence

For flow cytometry, fluorochrome-conjugated antibodies to the following proteins were used: PR1/HLA-A2 complex clone 8F4-AlexaFluor-647 (in house), pan-HLA-A,B,C clone W6/32-FITC or phycoerythrin (PE), HLA-A2 clone BB7.2-FITC (BD Bioscience). Mouse anti-NE (Santa Cruz Biotechnology) and rabbit anti-P3 (NeoMarkers) were directly conjugated to fluorchromes (in house). Ghost Dye Violet 510 (Tonbo Biosciences) was used to select viable cells for all flow studies. Healthy donor PBMCs were serotyped with the anti-HLA-A2 (clone BB7.2). Peptide/HLA-A2 tetramers were generated by the MHC Tetramer Production Laboratory at Baylor College of Medicine and were used to determine CTL target specificity, as previously described (10, 11, 15). Briefly, patient samples were stained with CD8-PE/Cy7 (Tonbo), CD3-FITC (BioLegend), peptide/HLA-A2–tetramer–allophycocyanin (APC) or PE-conjugated, and the following pacific blue-conjugated lineage antibodies: CD4 (BD Biosciences), CD14 (BD Biosciences), CD16 (BD Biosciences), and CD19 (BioLegend). Healthy donor and patient samples were fixed with 4% paraformaldehyde. CTL frequency was determined as the percentage of live cells that were lineage$^-$CD3$^+$CD8$^+$, and peptide/HA-A2 tetramer$^+$. All staining was performed at 4°C for 30 to 45 minutes. Flow-cytometry events were collected on a BD LSRFortessa, analyzed using FlowJo software (Tree Star) and graphed using GraphPad Prism software. Antibody-binding capacity was determined using Quantum Simply Cellular anti-Mouse IgG (Bangs Laboratories, Inc.). Intracellular staining was performed with BD Perm/Wash (BD Biosciences). Confocal images were captured on a Leica Microsystems SP2 SE confocal microscope with a 63×/1.4 oil objective at the Optical Microscopy Core Laboratory (MD Anderson). Leica LCS software (version 2.61) was used for the image analysis.

Surface MHC class I expression following serine protease and cytokine incubation

Lung cancer cells were incubated for 24 hours with purified NE or P3 (10 μg/mL), or lysates from healthy-donor PMN at a 2:1 PMN:target cell ratio. Interferon-gamma (100 U/mL IFNγ) served as a positive control. The HLA-A2-binding peptide PR1 served as a negative control. The synergistic effects of NE and P3 with IFNγ was evaluated by coincubation. Surface MHC class I was evaluated by flow cytometry (described above).

Peptide extraction, mass spectrometry identification, and analysis

Peptides presented by HLA class I molecules were identified by mass spectrometry following isolation by immunoprecipitation. Briefly, HLA class I molecules were captured from 1×10^8 lysed cells per treatment, using anti-HLA-A,B,C (30 μg; clone W6/32 supernatant grown in house and purified by the Monoclonal Antibody Core Facility at MD Anderson) crosslinked to 100 μL of 50% w/v slurry of Protein A/G resin. Peptides were eluted in 0.1 N acetic acid and filtered using a 3-kDa Amicon Ultra column (Millipore). Peptides were fractioned using an off-line 1100 series HPLC system and were run on a nanoLC-MS/MS using LTQ-Orbitrap Elite. Sequest HT from Proteome discoverer was used for peptide identification. HLA class I–bound peptides were further inspected for mass accuracy and MS/MS spectra were validated manually. All identified HLA class I peptides were blasted against nonredundant NCBInr human entries to identify their corresponding source proteins (Gene ID). Predicted binding of discovered peptides was performed *in silico* using HLArestrictor v 1.2 (16) and NetHLApan version 3.0 (17, 18) under the following restraints: matched 4-digit HLA-A and HLA-B allele typing, length restricted to match discovered peptide, strong (50 nmol/L affinity or 0.5% rank) and weak (500 nmol/L or 2% rank) binding criteria.

For mutant peptide identification, a lookup table was generated from reported mutations with predicted binding to endogenous HLA molecules (19, 20). Briefly, reported missense mutations encoded within a peptide predicted to bind endogenous HLA class I alleles were accessed from the TRON cell line portal to create a lookup table for mutant peptides for mass spectrometry discovery. Predicted binding to endogenously encoded HLA class I alleles of the mutant neoantigens and wild-type counterparts for each cell line were examined. Peptides were assigned as mutant, wild-type, or potential, with the lattermost indicating the single amino-acid substitution was not resolved in that particular event. Peptides were quantified by peptide spectrum matches (PSM) and the fraction of peptides containing potential mutagenic peptides was compared with the potential mutagenic peptide fraction following coculture of lung cancer cells with NE or P3.

CTL expansions and cytotoxicity assays

CTLs specific for peptide/HLA-A2 complexes were generated as previously described (7, 8, 21). Briefly, T2 cells were loaded with peptide (30 μg) individually for 2 hours in 10% RPMI, pooled, irradiated, and washed four times to remove unbound peptide. Healthy donors were serotyped for HLA-A2 using fluorochorome-conjugated BB7.2 antibody. PBMCs were cultured in RPMI supplemented with 10% human AB serum (Gemini Bio-Products) and stimulated weekly with peptide-loaded T2 cells for 2.5 weeks; after 1 week in culture, IL (20 IU/mL) was added to the culture medium at the time of peptide stimulation (weekly).

Cytotoxicity was determined against T2 loaded with individual peptides. HIV GAG nonomer (BioSynthesis) served as an irrelevant peptide control (22). Target cells were fluorescently labeled with 5 μg of Calcein AM (BD Biosciences) for 15 minutes, excess Calcein AM was removed by repeat washes and 10,000 cells were seeded into a 96-well plate. CTLs and target cells were incubated for 4 hours at 37°C in a humidified incubator at the indicated effector-to-target ratios. Trypan blue (Sigma Life Sciences) was used as a fluorescence quencher, and fluorescence of live target cells was determined using a CytoFluor II plate reader (PerSeptive Biosystems). The percentage of specific cytotoxicity was calculated according to the following equation: $[1 - (\text{fluorescence}_{\text{target + effector}} - \text{fluorescence}_{\text{medium}}) / (\text{fluorescence}_{\text{target alone}} - \text{fluorescence}_{\text{medium}})] \times 100$ (8, 10, 21).

Tumor-infiltrating lymphocytes (TIL) were expanded from tumor fragments *ex vivo*. Briefly, fresh tumor was minced into 3 to 5 mm^2 fragments and cultured in media containing high concentration of IL2. Cells were divided into fresh culture media with expansion over 3 to 5 weeks. Subsequently, TILs were rapidly expanded per the Rapid Expansion Protocol using anti-CD3, irradiated feeder cells, and high-dose IL2. TILs were expanded to $\geq$1,000-fold in a 2-week period, recapitulating the product that would be infused into a patient in the context of adoptive TIL therapy.

T-cell proliferation

PBMCs from healthy donors were measured for proliferation using the carboxyfluorescein succinimidyl ester (CFSE, Life Technologies). Briefly, PBMCs were isolated by Ficoll gradient density 1.077 g/mL and labeled with CFSE (Invitrogen). T cells were cocultured with the indicated lung cancer cell line [that had or had not been pretreated with purified human NE (10 μg/mL; Athens Research) and washed with PBS] at a 1:5 PBMC:lung cancer cell ratio and/or stimulated with the addition of mAbs to CD3 and CD28 (3μg/mL; BD Biosciences). After 4 days in culture, cells were stained with PE-conjugated anti-CD4 and APC-conjugated anti-CD8 (BioLegend) and Ghost Dye Violet 510 viability dye and analyzed by flow cytometry.

Computational analysis and statistics

Bar and line graphs display the mean, with error bars denoting the standard deviation, unless otherwise indicated. As appropriate, two-way ANOVA two-tailed and Mann–Whitney unpaired *t* test was used for statistical analysis, with the Dunnett correction applied to the former as needed; the criterion for significance in all studies was $P = 0.05$.

Provisional lung adenocarcinoma patient data from TCGA Research Network (http://cancergenome.nih.gov/) was retrieved from the online portal www.cbioportal.org. Patients were grouped as "high" or "low" based on quartile expression of the macrophage marker CD68 or the CTL marker CD8 (*CD8A* and *CD8B*). Gene enrichment analysis was performed on mRNA expression (RNA Seq V2 RSEM) in the TCGA web interface; *P* values from the two-tailed, Fisher exact test are reported. Patient survival was analyzed in Prism v 7.0b; the log-rank (Mantel–Cox) test *P* value is reported. Hierarchal clustering of immunoproteasome core components in lung cancer cell lines was performed in RStudio version 3.3.0 (May 3, 2016) using the heatmap.2 function from the gplots package version 3.0.1.

Human subjects

All investigations involving humans were performed after approval by The MD Anderson Cancer Center Institutional Review Board. Lung adenocarcinoma PBMCs and TILs were obtained from genotyped HLA-A2 patients enrolled in the immunogenomic profiling of non–small cell lung cancer (ICON) project after informed consent was obtained from each patient or patient's guardian. Patients had no evidence of metastasis and were diagnosed with adenocarcinoma ($n = 4$) or squamous cell carcinoma ($n = 2$).

Results

Lung cancer cells internalize NE and P3 from multiple sources

Uptake of NE and P3 by lung cancer cell lines has been previously reported (23, 24). We first confirmed that the full-length transcripts of NE and P3 were not endogenously expressed in a panel of lung cancer cell lines (Fig. 1A), in agreement with publicly available data of cell lines from the Cancer Cell Line Encyclopedia (Supplementary Fig. S1; ref. 25). However, lung cancer cells can contain NE and P3 protein after coincubation with exogenous sources; the H2023 cell line internalized NE and P3 from soluble, purified proteins (Fig. 1B) and healthy donor-PMN (Fig. 1C). We extended these findings in a panel of lung cancer cell lines with purified NE (Fig. 1D). These data demonstrate that lung cancer cells lack endogenous NE and P3 but can take up NE and P3 from exogenous sources.

Serine proteases enhance antigen presentation via elevated HLA class I surface expression

The serine proteases NE and P3 contain the immunogenic, HLA-A2–restricted leukemia-associated antigen PR1, which was shown to be cross-presented by breast cancer and other solid tumors. Although NE was shown to be internalized by lung cancer (24), there are no reports of lung cancer cells presenting the

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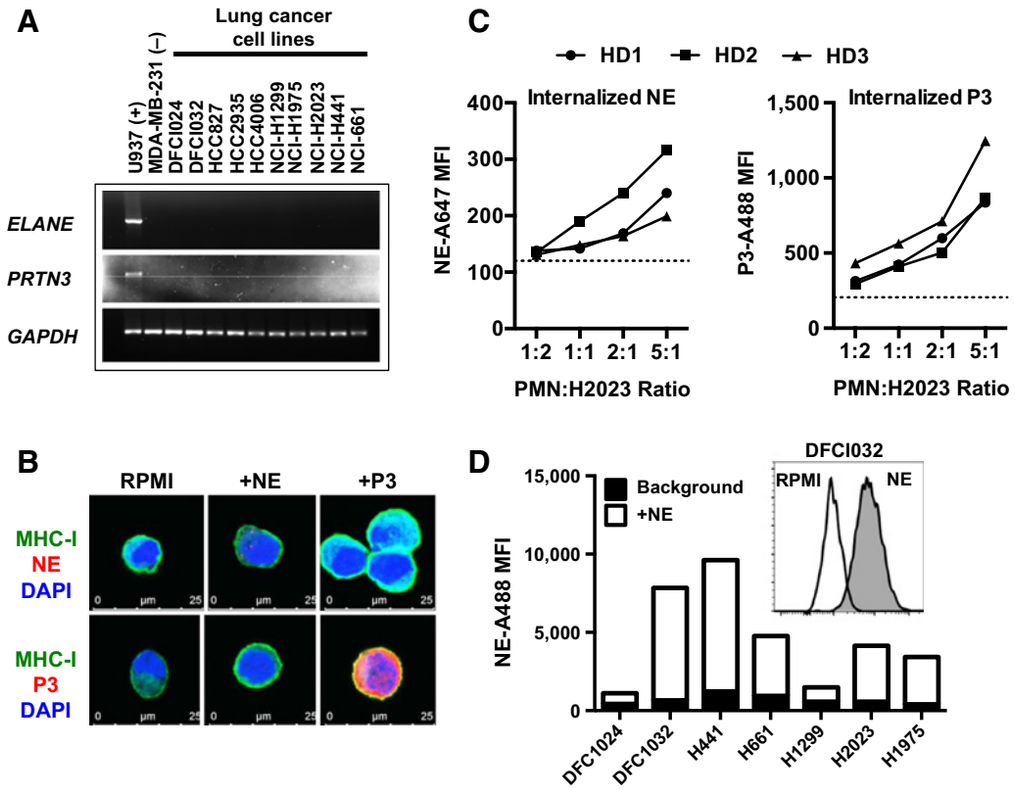

Figure 1.

Exogenous serine proteases are internalized by lung cancer cells. **A,** Lung cancer cells do not express full-length NE (*ELANE*) or P3 (*PRTN3*) transcripts, as determined by RT-PCR. **B,** Confocal imaging of internalized proteins containing the PR1 epitope, NE, and P3 in H2023 lung cancer cells. **C,** Flow cytometry of internalized NE and P3 from three separate healthy donor (HD) peripheral mononuclear (PMN) lysates by H2023; dashed line indicates staining of unpulsed cells. **D,** Uptake of purified NE detected by flow cytometry in a panel of lung cancer cells with autofluorescence removed; the inset shows DFCI032 as a representative histogram with NE (gray) and without (white, RPMI).

exogenous antigen PR1. Using the PR1/HLA-A2–specific monoclonal antibody 8F4 (5, 26), we detected cross-presentation of PR1 from NE by HLA-A2$^+$ lung cancer cells via flow cytometry (Fig. 2A), as previously shown in antigen-presenting cells, breast cancer, and melanoma (10, 27). Moreover, lung cancer cells exposed to NE had increased susceptibility to destruction by CTLs bearing the PR1-specific 8F4–chimeric antigen-receptor (CAR; ref. 28; Fig. 2B). Thus, lung cancer cells could cross-present PR1 in the presence of exogenous NE and were susceptible to PR1-targeted immunotherapies.

We have demonstrated an increase in surface HLA class I molecules on breast cancer cell lines and one lung cancer cell line after coculture with NE (12). We expanded these findings using an antibody specific for folded, pan–HLA-A,B,C (mAb W6/32) to detect expression of HLA class I molecules, which increased after exposure to NE, on all lung cancer cell lines examined (Fig. 2C). In the context of the lung cancer tumor microenvironment, NE is packaged in azurophilic granules and delivered to the tumor via TAN and TAM, which release the granule-contained serine proteases upon activation. The elevation in surface HLA class I expression was validated by a dose-dependent increase in surface HLA class I expression as the ratios of PMN to tumor cells was increased (Fig. 2D). Furthermore, our studies demonstrate that in lung cancer, NE contributes to a greater upregulation of HLA class I surface expression than P3 (Fig. 2E) and synergizes with the proinflammatory cytokine IFNγ to increase MHC class I on the surface of lung cancer cells (Fig. 2F).

Next, we validated these findings in tissue from primary lung cancer patients, by dividing TCGA lung adenocarcinoma provisional dataset into "high" and "low" TAM infiltration, based on quartile expression of the TAM marker CD68 (Fig. 3). Significantly higher expression of MHC class I heavy chain (HLA-A,B,C) and light chain (β_2M) genes was present in CD68^{HI} patient tissue (Fig. 3A), consistent with our findings in lung cancer cell lines that PMN contribute to enhanced MHC class I surface presentation on cancer cells. In order for TAM-enhanced MHC class I molecules to contribute to antitumor immunity, T cells must be present to recognize peptide/MHC class I molecule complexes. CD68^{HI} patient tissues were enriched for CTL marker CD8 (*CD8A* and *CD8B*) in addition to the T helper marker CD4 (Fig. 3B). Lung cancer cell lines demonstrated a suppressive effect on healthy donor CTL proliferation (Supplementary Fig. S2). However, we determined that prior exposure to NE by lung cancer cells did not augment suppression of CTL proliferation; in some instances, a slight increase in CTL proliferation was observed when incubated with lung cancer cell lines previously exposed to NE (Supplementary Fig. S2). CD68^{HI} lung cancer patient tissue was then stratified into CTL^{HI} and CTL^{LO} based on quartile expression of

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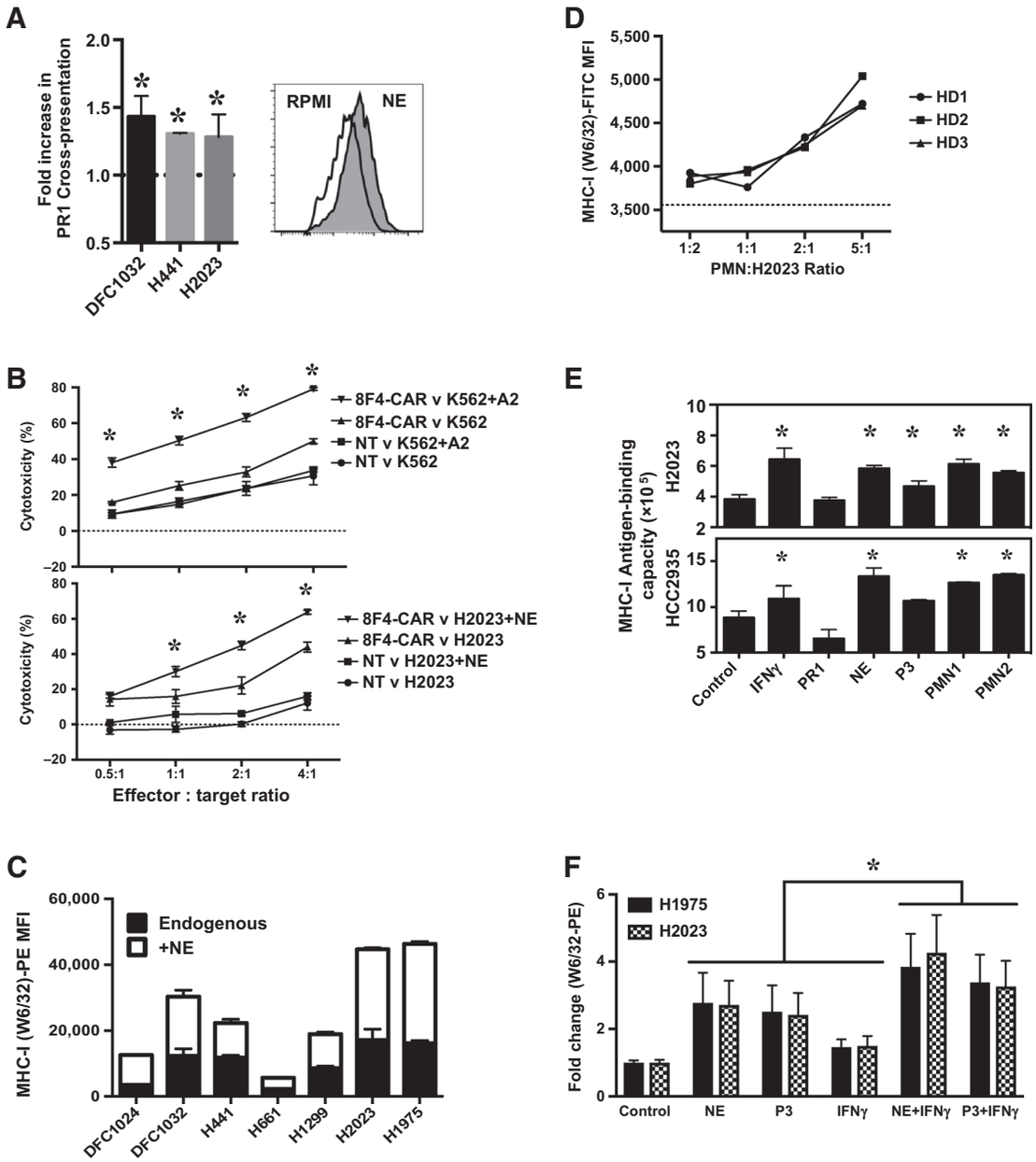

Figure 2.

NE and P3 enhance antigen presentation. **A,** Increase of the immunogenic PR1 peptide on HLA-A2$^+$ lung cancer cells after 24 hours coincubation with NE, displayed as the fold increase in PR1 cross-presentation over baseline, determined with flow cytometry by binding of 8F4 antibody (anti-PR1/HLA-A2) directly conjugated to AlexaFluor647. The inset is a representative histogram of DFCI032 with NE (gray) and without (white, RPMI). **B,** CAR-T cells recognizing PR1/HLA-A2 from healthy donors lyse NE-exposed H2023 lung cancer cells. The NE- and P3-endogenously expressing K562 cell line transduced with HLA-A2 (to allow PR1 presentation) and nontransduced T cells (NT) were used as effector controls. Graph displays the mean of 3 technical replicates representative of 2 independent experiments; asterisks indicate significant difference between target cell populations of 8F4-CAR–transduced effectors by the two-way ANOVA with $P = 0.05$ as the criterion for significance. **C,** Purified NE, and **D,** NE supplied by PMNs from healthy donors, enhanced total HLA class I surface expression, as detected by flow cytometry with W6/32, the antibody to folded, HLA-A,B,C. **E**, NE contributes to enhanced surface HLA class I. Lung cancer cells were incubated for 24 hours with IFNγ (100 U/mL, positive control), PR1 peptide (10 μg/mL, negative control), purified NE or P3 (10 μg/mL), or lysates from healthy-donor PMN at a 2:1 PMN:target cell ratio. Asterisks denote significant elevation over baseline HLA class I antigen binding capacity via two-way ANOVA with $P = 0.05$ as the criterion for significance. **F,** Serine proteases NE and P3 synergize with IFNγ to upregulate surface MHC-I. Cells treated as in **E**; the mean and standard deviation from 5 independent experiments performed in triplicate is shown. Asterisk indicates significant increase in surface MHC-I of combined treatments compared with respective single treatments alone by the two-way ANOVA with $P = 0.05$ as the criterion for significance.

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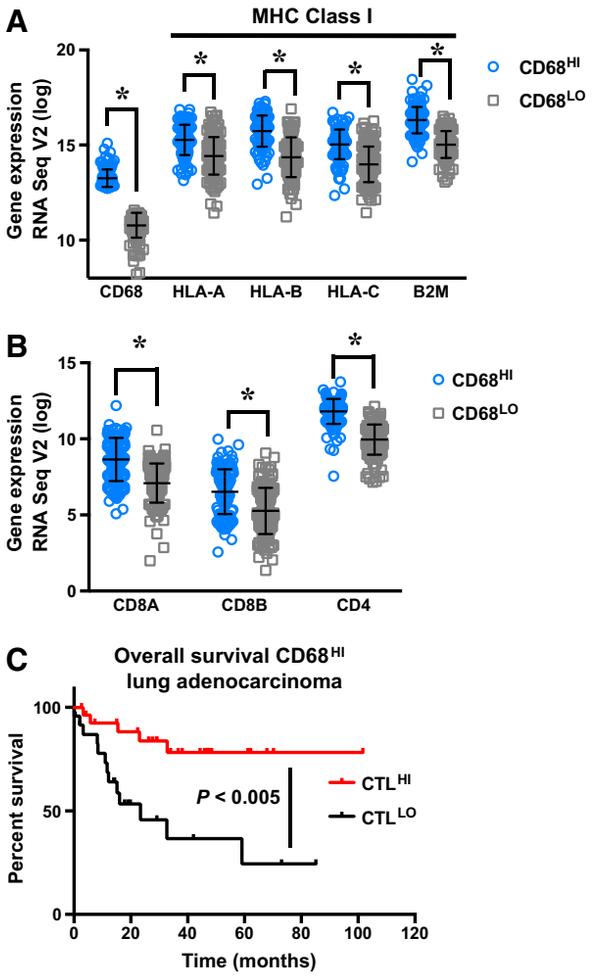

Figure 3.

Enriched expression of T-cell markers and MHC class I genes with CD68 expression in lung adenocarcinoma patient tissues. **A** and **B,** TCGA lung adenocarcinoma provisional dataset was divided into $CD68^{HI}$ and $CD68^{LO}$ based on upper and lower quartiles. **A,** Patient tissue with high *CD68* expression had higher expression of the MHC class I heavy chains (*HLA-A,B,C*) and light chain (*B2M*) genes. **B,** Enriched expression of CTL marker CD8 (*CD8A* and *CD8B*) as well as the helper T-cell marker CD4 was observed in $CD68^{HI}$ patient tissue. Asterisks indicate significance derived from the Fisher exact test. **C,** $CD68^{HI}$ patients were divided into two groups based on quartile expression of the CTL genes *CD8A* and *CD8B*. CTL^{HI} patients had significantly improved overall survival, compared with CTL^{LO} patients ($P = 0.005$).

CD8A and *CD8B*. A significant improvement in overall survival was observed in patients with greater CTL gene expression (Fig. 3C). Independently, the upper quartile for TAM or CTL gene expression did not select patients with significantly improved overall survival over that of patients within the respective lower quartile (data not show). Thus, higher coexpression of both the TAM marker CD68 and CTL marker CD8 selected patients with an improved overall survival, in agreement with our hypothesis that innate immune cells, including PMN and macrophages, contribute to CTL antitumor immunity. Thus, the observed *in vitro* enrichment of MHC class I genes and T-cell markers in lung cancer tumors with higher TAM gene marker expression, in addition to improved overall survival of $CD68^{HI}CTL^{HI}$ patients, supports our studies in cell lines that TAN and TAM contributed to antitumor immunity via enhanced MHC class I surface expression of immunogenic antigens on lung cancer cells.

NE and P3 induce unique, endogenous peptides that are novel antigens

We then sought to identify if NE and P3 affected the antigenic landscape and used mass spectrometry to identify unique, endogenous antigens presented on lung cancer cells after exposure to NE or P3. Two $HLA\text{-}A2^{+}$ lung cancer cell lines were selected for investigation of the immunopeptidome repertoire (Fig. 4A). Briefly, lung cancer cells were incubated in serum-free RPMI with or without NE and P3 for 24 hours. Immunoprecipitation was performed to capture HLA class I molecules, and peptides were detected by mass spectrometry after size exclusion filtration and reversed-phase HPLC fractionation. By quantifying detected eluted peptides by PSM, we determined that the examined serine proteases increased peptides with 25 PSM or fewer (H2023: 81% NE, 80% P3; H441: 86% NE, 90% P3) to a far greater extent than peptides with more than 25 PSM (H2023: 52% NE, 66% P3; H441: 55% NE, 50% P3; Fig. 4B). Additionally, a slight enrichment of mutant neoantigens was observed after NE or P3 exposure (Supplementary Fig. S3; ref. 19). Thus, NE and P3 had the greatest impact on the lung cancer immunopeptidome by increasing low-abundance, endogenous peptides, with "low" indicating 25 or fewer events detected by MS.

We then interrogated the immunogenicity of unique, endogenous peptides detected after coculture with NE or P3. The strongest, predicted HLA-A2 binders based on affinity were selected (Table 1), because greater binding to HLA class I correlates with immunogenicity (29, 30). Many of the eluted peptides were encoded by genes that were implicated in biological processes altered by serine proteases such as cell adhesion (*PCDH20*), proliferation (24, 31, 32; *ALK*), transcription (33, 34; *INSM2*, *ZFHX3*), signal transduction (*ABCA1*), and protein transport (*CLPB*, *SLC28A1*, *SLC32A1*, *SLC41A3*, and *SNX14*).

We experimentally confirmed HLA-A2 binding of selected, unique, endogenous peptides in Table 1 using the T2 binding assay (Fig. 4C). In order to interrogate immunogenicity, peptides were pooled into groups of five, and healthy donor CTLs were expanded. After expansion, T2 cells were loaded with individual peptides to confirm the presence of successfully expanded CTLs (Fig. 4D, top). We detected CTLs with cytotoxic activity against unique peptide/HLA-A2 complexes in 10 out of 11 different $HLA\text{-}A2^{+}$ healthy donors post expansion (represented by row in Fig. 4E). Lung cancer cell lines were then tested as targets (Fig. 4D, bottom); enhanced CTL lysis of lung cancer cells with serine protease coincubation was observed in 9 out of 10 confirmed expansions (Fig. 4E). This therefore demonstrates that the identified unique, endogenous, self-antigens are indeed valid immunogenic tumor antigens.

Enrichment of CTLs reactive to induced endogenous peptides and PR1 in lung cancer patients

Having identified that CTLs from healthy donors were present and functional against serine protease–induced endogenous peptides in the immunopeptidome of lung cancer cells, we then expanded our studies into lung cancer patients via peptide/HLA-A2 tetramer staining of patient circulating PBMCs as well as TILs. Building upon our findings from healthy donors, peptides were

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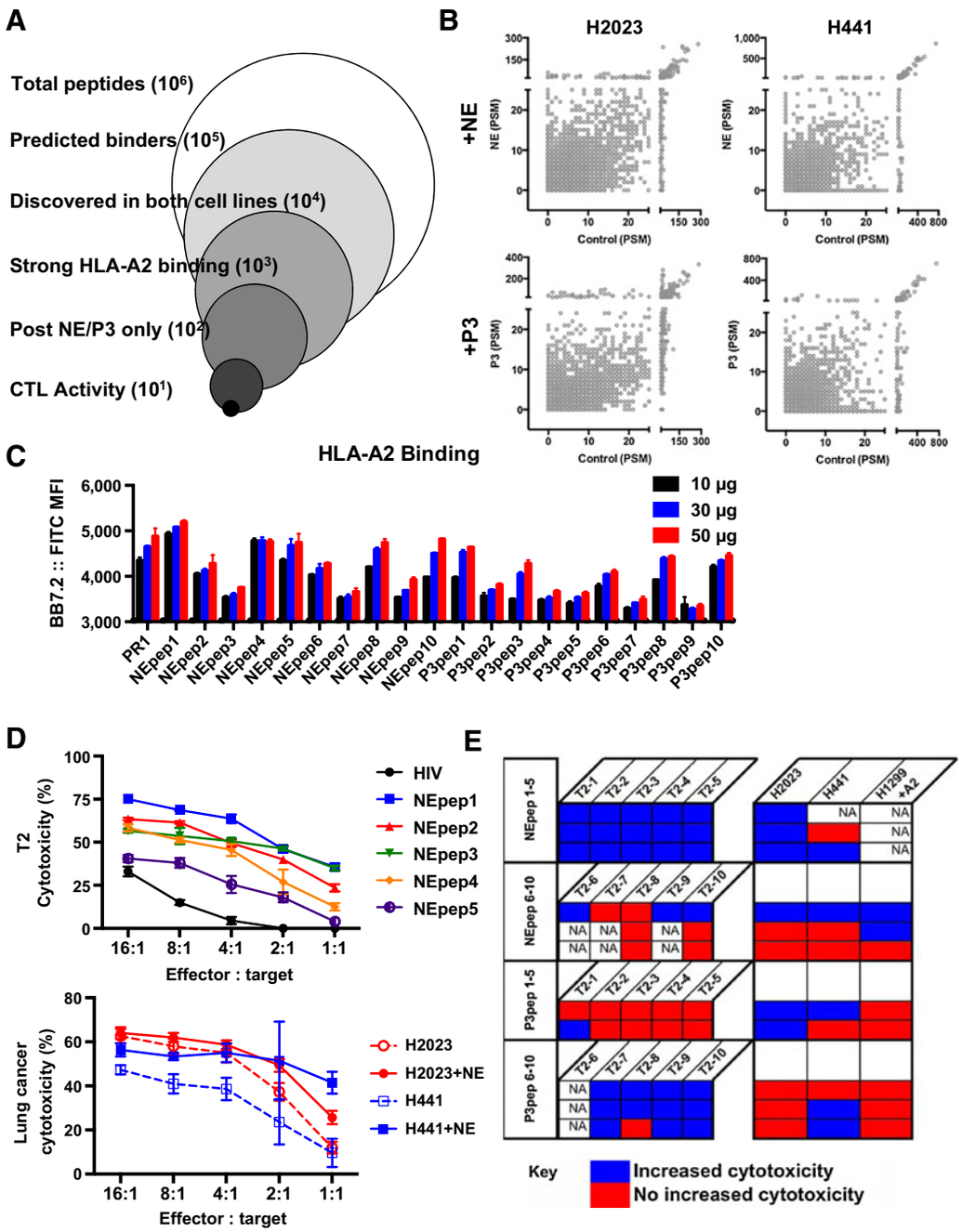

Figure 4.

Strategy and profiling of HLA class I–bound antigens on lung cancer cells after NE or P3. Two HLA-A2$^+$ lung cancer cell lines, H2023 and H441, were incubated for 24 hours with NE or P3. **A,** HLA class I molecules were captured from lysed cells by the anti–HLA-A,B,C antibody clone W6/32, peptides were eluted and separated with a 3-kDa ultraspin filter and discovered by mass spectrometry. HLARestrictor 1.2 was used to determine *in silico* binding to HLA-A and HLA-B. Peptides predicted to bind to HLA-A2 (the only conserved HLA allele) were then analyzed, and peptides discovered within untreated cells were removed to retain unique peptides only. **B,** Scatter plot of eluted peptides from control (*x*-axis) and NE/P3-treated lung cancer cells (*y*-axis) quantified by peptide spectrum matches (PSM). A shift toward the upper-left indicates upregulated or novel peptides. **C–E,** Ten peptides per treatment were selected for immunogenicity testing with CTL expansion. **C**, Predicted HLA-A2 binding was validated by the T2 binding assay after 2 hours; stabilized surface HLA class I was detected by flow cytometry with the anti–HLA-A2 antibody BB7.2. **D–E,** CTLs from healthy donors were expanded against pools of five peptides for 2.5 weeks, then examined for cytotoxic capacity against T2 cells loaded with individual peptide. An HLA-A2–binding peptide from HIV served as a negative control. Expanded CTLs demonstrated enhanced cytotoxicity against lung cancer cell lines incubated with serine protease (compare solid and dash lines of similar color). Representative cytotoxicity assays are shown in **D**. **E,** Table summarizing cytotoxicity results from 11 different HLA-A2$^+$ healthy donor CTL expansions, denoted by each row. Blue boxes denote expansion against specific peptide/HLA-A2 complexes and statistically improved CTL cytotoxicity against peptide-loaded T2 cells compared with HIV or unpulsed T2 cells and greater CTL lysis of serine protease-treated lung cancer cells compared with unexposed cells. Red indicates statistically enhanced CTL killing of targets was not achieved. Peptide loading was confirmed by flow cytometry for each T2 target cell set; not assessed (NA) denotes no peptide loading for T2 controls or the lung cancer cell line was not assayed. H2023 and H441 were discovery cell lines, and the HLA-A2–transduced H1299 cell served as a validation cell line.

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Table 1. Peptides in lung cancer immunopeptidome

Name	Peptide	Affinity (nmol/L)	Gene name	Description
NE-Novel				
NEpep1	LLSAVLPSV	3	*MCMBP*	Mini-chromosome maintenance complex binding protein
NEpep2	ALAGLSPV	4	*ZFHX3*	Zinc finger homeobox protein 3
NEpep3	ALIGGPPV	5	*ABCA1*	ATP-binding cassette sub-family A member 1 family A member 1
NEpep4	LLLELAGVTHV	7	*SIRPD*	Signal-regulatory protein delta
NEpep5	SLLGLALLAV	13	*VSIG10L*	V-set and immunoglobulin domain-containing protein 10-like
NEpep6	LLAGPPGV	15	*SPATA5L1*	Spermatogenesis-associated protein 5-like
NEpep7	ALAAFLGL	20	*SLC28A1*	Sodium/nucleoside cotransporter 1 protein 1
NEpep8	LLGPAPGV	21	*ALK*	ALK tyrosine kinase receptor
NEpep9	LLLRSPAGV	24	*GLS*	Glutaminase kidney isoform, mitochondrial
NEpep10	GLGPVAAV	26	*CELF6*	CUGBP Elav-like family member 6
P3-Novel				
P3pep1	LLLPALAGA	9	*INSM2*	Insulinoma-associated protein 2
P3pep2	ALAAALVV	12	*CLPB*	Caseinolytic peptidase B protein homolog
P3pep3	LLWRKLLWHQV	12	*SLC32A1*	Vesicular inhibitory amino acid transporter
P3pep4	ALLGIPKV	13	*BBS10*	Bardet-Biedl syndrome 10 protein
P3pep5	GLLAIVKV	16	*FAM222B*	Protein FAM222B
P3pep6	LLFPYILPPKA	17	*SNX14*	Sorting nexin-14
P3pep7	ALVSIIKV	18	*PCDH20*	Protocadherin-20
P3pep8	LLAAVAALL	18	*SLC41A3*	Solute carrier family 41 member 3
P3pep9	ALISKNPV	19	*AMZ2*	Archaemetzincin-2
P3pep10	LLGCPVPLGV	19	*NLRP5*	NACHT, LRR and PYD domains-containing protein 5

selected based on their ability to form peptide/HLA-A2 complexes *in vitro* (used for the tetramer staining), and their capacity to elicit potent T-cell expansions with the greatest cytotoxic activity against lung cancer cells (Fig. 4), as well as correlation of the encoding gene with T cells, neutrophils, and/or macrophage gene markers in the non–small cell lung adenocarcinoma provisional dataset from TCGA. Endogenous peptides folded into HLA-A2 tetramers were NEpep1 (*MCMBP*), NEpep3 (*ABCA1*), and NEpep4 (*SIPRD*). Attempts to generate peptide/HLA-A2 complexes for P3pep7 (*PCDH20*) and P3pep9 (*AMZ2*) were unsuccessful. The exogenous antigen PR1 was also included in our studies.

Circulating, peptide/HLA-A2 tetramer$^+$ PBMCs were detected in both healthy donor (white bars) and lung cancer patients (gray bars) at comparable frequencies (Fig. 5). The CTL populations specific for the three endogenous NE-induced peptides (NEpep1, NEpep3, and NEpep4) were enriched in the TIL populations. PR1/HLA-A2–specific CTLs were also detected in TIL populations. Therefore, CTLs specific for endogenous peptides induced by NE, in addition to the exogenous antigen PR1, are present in lung cancer patients and are enriched in their TIL populations.

Discussion

In these studies, we demonstrated that components of the tumor microenvironment, the serine proteases NE and P3, increased antigen presentation of immunogenic peptides by lung cancer cells. We found that healthy donor–derived PMN enhanced surface HLA class I expression and showed the upregulation of surface HLA class I molecules by serine proteases in a panel of lung cancer cell lines that encompassed a spectrum of the epithelial-to-mesenchymal phenotype (35), placing an emphasis on peptide discovery in an epithelial-like (H441) and mesenchymal-like (H2023) cell line.

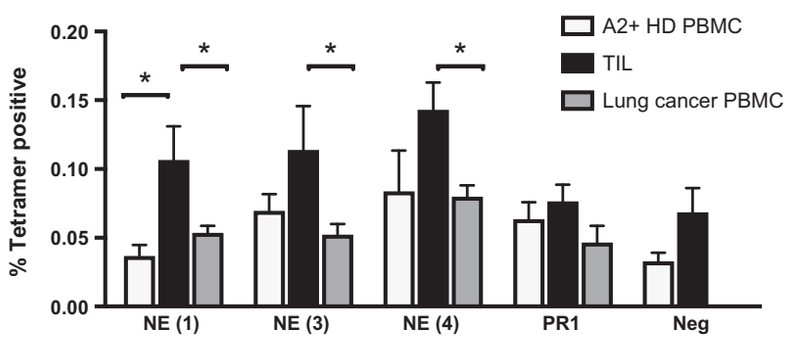

Figure 5.

CTLs specific for endogenous and exogenous antigens induced by and from serine proteases presented by HLA-A2 are enriched in lung cancer patients. The frequency of CTLs reactive to peptide/HLA-A2 complexes was determined by tetramer staining of healthy donor PBMCs (white bars, $n = 3$), circulating PBMCs from lung cancer patients (gray bars, $n = 5$) and TILs (black bars, $n = 5$). The following peptides were used: NE (1) peptide (LLSAVLPSV); NE (3) peptide (ALIGGPPV); NE (4) peptide (LLLELAGVTHV); PR1 (VLQELNVTV); negative peptide (ALIAPVHAV). Bar graph displays the mean frequency; error bars denote the standard error of the mean. Asterisks indicate statistical significance by the two-tailed, unpaired Mann–Whitney *t* test, with a $P = 0.05$ as the criterion for significance.

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Lung cancer cells contain a high mutational burden, second only to melanoma (30, 36). Peptides resulting from mutations are ideal targets for T cell–based therapy, as they are highly immunogenic, due to lack of tolerance against these antigens in the TCR repertoire, and are sourced from the tumor cells specifically (1). Additionally, immunogenic mutations are associated with favorable patient survival (1, 37). Our detection here of predicted mutant antigens (19) and immunogenic endogenous antigens after exposure to NE and P3, and the presence of endogenous antigen/HLA-A2–specific CTLs in lung cancer patients, supports previous findings that neoantigen presentation may account for some of the benefits that have been observed in some patients who receive immune-checkpoint blockade (1), which enables T-cell activation in the presence of stimulating peptide/HLA class I complexes.

Specifically, we demonstrated that serine proteases delivered to the tumor by TAN and TAM enhanced immunogenic antigen presentation by lung cancer cells. Tumor tissues from lung cancer patients with high expression of the TAM marker CD68 were found to be enriched for MHC class I genes and T-cell markers. As evidence that TAN and TAM support T-cell recognition of tumor antigens in patients, patients with $CD68^{HI}$ tumor tissues, and concurrent high expression of the genes encoding the CTL marker CD8, had significantly improved survival compared with patients with high CD68 expression in tumor tissues, but low CTL infiltration. When TAM and CTL markers were evaluated independently, high expression did not confer a survival benefit (data not shown), suggestive that these two cell populations synergize to improve host antitumor immunity. In murine pancreatic cancer models, reduction of alternatively activated myeloid cell population (i.e., type II TAM or M2) improved antigen presentation, antitumor T-cell activity, and response to immune-checkpoint blockade therapy (38). Thus, in view of our data here and our previous reports (10–12), lung cancer patients with relatively large N1 TAN or M1 TAM populations may have a better prognosis and response to immunotherapies that activate T-cell responses, specifically immune-checkpoint blockade.

Antigen presentation impacts the survival of non–small cell lung cancer patients. Deficiency of immunoproteasome components correlating with poor patient outcomes was identified in mesenchymal-like cells (21), and cells undergoing an epithelial–mesenchymal transition are associated with an inflammatory microenvironment and increased immune-checkpoint molecules (39). Thus, mesenchymal-like lung cancer cells are specifically poised to escape CTL destruction. Our analysis here of 186 lung cancer cell lines available through the Cancer Cell Line Encyclopedia reaffirmed a diversity in expression of IFNγ-inducible immunoproteasome subunits within the core components PSMB9, PSMB8, and PSMB10, and ubiquitous expression of proteasome core subunits (Supplementary Fig. S4A–S4B). We tested whether NE and P3 altered the antigen repertoire via induction of the immunoproteasome; however, we did not find evidence to support this hypothesis (Supplementary Fig. S4C). Because NE-induced surface HLA class I enhancement on breast cancer cells results from stabilized surface molecules and is dependent upon serine protease activity (12), neoantigens are likely generated as a direct result of serine protease cleavage, as in the case of the E75 cyclin E antigen (11). This mechanism agrees with our increased surface HLA class I expression and neoantigens on epithelial- and mesenchymal-like lung cancer cells. Thus, the increase in antigen presentation on lung cancer cells induced by serine protease is not restricted by an epithelial–mesenchymal phenotype.

NE is internalized via a receptor-dependent mechanism (11, 23, 24), but P3 displays receptor-independent internalization despite reported receptor associations (40–42). Platelet-derived growth factor receptor (PDGFR) has been implicated in the proliferative effects of NE on lung cancer cells; however, the receptor responsible for directing internalized NE towards PR1 cross-presentation has yet to be reported. Elucidation of a receptor that modulates cross-presentation is beyond the scope of these studies, but underscores the foundation to understanding the tumor context in which cross-presentation occurs.

To summarize, we demonstrated that PMN/macrophage-associated NE enhanced antigen presentation by lung cancer cells, including the exogenous antigen PR1 and induced endogenous antigens, and that lung cancer cells were susceptible to lysis by CTLs that target these peptide/HLA-A2. We also showed that CTLs specific for NE-induced, endogenous antigens are present in lung cancer patients, and specifically in the TIL populations. Thus, lung cancer patients, particularly those with abundant TAN and TAM, could greatly benefit from immune-checkpoint blockade and PR1-based immunotherapies, such as CAR-T cells (28, 43), monoclonal antibodies (5, 26), and vaccines (9). Finally, PR1-based immunotherapies could synergize with immune-checkpoint blockade to achieve profound and long-term benefits for lung cancer patients.

Disclosure of Potential Conflicts of Interest

D.L. Gibbons is a consultant/advisory board member for AstraZeneca. No potential conflicts of interest were disclosed by the other authors. John. V. Heymach has disclosed consultant/advisory board member for AstraZeneca Lilly, BMS, Genetech, EMD Serono, Boehringer Ingelheim, Novartis.

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Serine Proteases Enhance Immunogenic Antigen Presentation on Lung Cancer Cells

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