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Influenza virus infection elicits protective antibodies and T cells specific for host cell antigens also expressed as tumor associated antigens: a new view of cancer immunosurveillance

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Abstract

Most tumor-associated antigens (TAA) are self-molecules that are abnormally expressed in cancer cells and become targets of antitumor immune responses. Antibodies and T cells specific for some TAA have been found in healthy individuals and are associated with lowered lifetime risk for developing cancer. Lower risk for cancer has also been associated with a history of febrile viral diseases. We hypothesized that virus infections could lead to transient expression of abnormal forms of self-molecules, some of which are TAA; facilitated by the adjuvant effects of infection and inflammation, these molecules could elicit specific antibodies, T cells and lasting immune memory simultaneously with immunity against viral antigens. Such infection-induced immune memory for TAA would be expected to provide life-long immune surveillance of cancer. Using influenza virus infection in mice as a model system, we tested this hypothesis and demonstrated that influenza-experienced mice control 3LL mouse lung tumor challenge better than infection-naive control mice. Using 2D-Difference Gel Electrophoresis (2D-DIGE) and mass spectrometry, we identified numerous molecules, some of which are known TAA, on the 3LL tumor cells recognized by antibodies elicited by two successive influenza infections. We studied in detail immune responses against GAPDH, Histone H4, HSP90, Malate Dehydrogenase 2 and Annexin A2, all of which were overexpressed in influenza-infected lungs and in tumor cells. Lastly, we show that immune responses generated through vaccination against peptides derived from these antigens correlated with improved tumor control.

Keywords

tumor antigens; cancer vaccines; flu; DIGE

Introduction

Genetic mutations and epigenetic modifications can lead to cellular transformation and cancer (1). Tumor immunosurveillance is the mechanism by which the immune system recognizes and protects against abnormal cells (2). Successful tumor immunosurveillance leads to tumor elimination, which involves the recognition of tumor-specific or tumor-associated antigens (TAA) by antibodies and immune cells. T cells can recognize tumor antigens presented by the major histocompatibility complex (MHC) class I and II molecules and kill the tumor cells via lytic granule release (CD8⁺ T cells) or promote cellular and humoral responses through the production of cytokines (CD4⁺ T cells). TAA-specific antibodies can bind to and lyse tumor cells with the help of complement or facilitate the killing of tumor cells by T and natural killer (NK) cells through antibody-dependent cellular cytotoxicity (ADCC) (3, 4). Molecular and biochemical characterization of tumor antigens have yielded targets for immunosurveillance and for the development of immunotherapeutic strategies.

Cancer patients have circulating tumor-specific antibodies and T cells that have been used as reagents to characterize the individual's tumor antigens (5-8). Immune responses to several TAA have been correlated with favorable prognoses. Even when target antigens are not known, infiltration of tumors by activated T cells has been correlated with better prognosis and longer disease-free and overall survival (9). The promising new approaches in cancer treatment include immunotherapies that are directed towards regaining immune control by targeting both the cancer and the immune system (10).

DNA sequencing has shown that tumors have on average a dozen or more mutations that could generate new epitopes known as tumor-specific antigens (11). While tumors could express these epitopes as targets and adoptive transfer of T cells or antibodies could lead to their recognition and elimination, spontaneous immune responses to such epitopes (e.g. mutated Kras, EGFR or p53) have not been found in cancer patients as often as could be expected from the frequency of these mutations (12). Instead, the majority of the spontaneous antitumor immune responses are directed against the non-mutated self-antigens, which are expressed on tumor cells and are named TAA. They include molecules that are overexpressed on tumor cells [e.g. Her-2neu (13), MUC1 (14), CEA (15), Cyclin B1 (5)], molecules with dysregulated stage- or tissue-specific expression [e.g. oncofetal antigens α -fetoprotein (16), cancer-testis antigens NY-ESO-1 (17), Mage 1-7 (18)], or molecules with altered posttranslational modifications (glycosylation or phosphorylation)[e.g. hypoglycosylation of MUC1 (14) or aberrant phosphorylation of β -catenin (19)]. The aberrant expression of many of these antigens can be detected on premalignant precursors of various cancers early in tumor development (6, 20).

Results from several large epidemiological studies have indicated that individuals with a history of febrile childhood infections had a reduced life-time risk of various cancers (21-23). The mechanisms underlying this protective function are unknown. Healthy individuals with no previous history of cancer have been shown to have antibodies and/or T cells specific for several TAA (24, 25). For example, we found that the TAA MUC1 was expressed in the tumor form (overexpressed and hypoglycosylated) on salivary gland ducts

during mumps parotitis infection (26), on breast ducts during lactation and in lactational mastitis (27), and in inflammatory bowel disease (20). Furthermore, we showed that the presence of anti-MUC1 IgG in women, who experienced early in life one or more of these events, correlated with a significantly lower risk for ovarian cancer (28).

In this study we present the first attempt to recapitulate these observations in an animal model. This experimental mouse model of influenza infection allows us to test the hypothesis that immunity and immune memory against abnormal self-antigens, known as TAA, is not elicited in response to their *de novo* expression on tumor cells or premalignant lesions, but rather it is elicited earlier in life in response to their expression during acute inflammations accompanying viral and other infections. When some of the same self-antigens are aberrantly expressed on premalignant lesions or tumor cells, they can be recognized by the infection-primed immune memory responses leading to tumor elimination or enhanced tumor control. We show that mice, which experienced two infections with two different influenza viruses, and which develop immunity to self-antigens abnormally expressed on infected lungs, have improved ability to control the growth of transplantable lung tumors expressing those same self-antigens. We analyzed in detail the infection-elicited immune responses to five such antigens: Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Histone H4, Malate Dehydrogenase 2 (MDH2), Annexin A2, and Heat Shock Protein 90 (HSP90). These antigens were all recognized in tumor cell lysates by post-infection sera. We show that they were overexpressed in tumor cells, as well as in influenza virus-infected lungs compared to healthy lungs, and that influenza virus infection induced antibody and CD8⁺T cells specific for these antigens. We demonstrate that immunization of mice with peptides derived from these antigens effectively protects them against tumor challenge.

Materials and Methods

Mice, tumor cell lines, and influenza virus

6-8 week old female C57BL/6 wildtype (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the University of Pittsburgh Animal Facility. All animal protocols were in accordance with IUCAC guidelines at the University of Pittsburgh. Lewis Lung Carcinoma cell line (3LL) derived from a murine lung epithelial tumor, was maintained in c-DMEM media containing 10% heat inactivated fetal calf serum (FCS), 1% Non-essential Amino Acid, 1% Penicillin/Streptomycin, 1% Sodium Pyruvate, 1% L-glutamine, 0.1% 2-mercaptoethanol. IG10, an epithelial tumor cell line derived from mouse ovarian epithelium, was cultured as described (29).

Influenza Virus Infection and Tumor Challenge

All mice were anesthetized with Ketamine (100mg/mL)/Xylazine (20mg/mL) solution. Mice were infected intranasally with 1.25×10^3 pfu of H1N1 Influenza A/Puerto Rico/8/34 (PR8) virus and re-infected 35 days later with 1.25×10^3 pfu of H3N2 Influenza A/Aichi/2/68 (Aichi) X-31 virus. Percent weight loss was used as a measure of successful infection, and mice were weighed at two-day intervals. On day 60 following the first infection, mice were injected subcutaneously in the right hind flank with 1×10^5 3LL tumor cells. Tumor length

and width were measured every 2 days using calipers. Mice were sacrificed when the tumor diameter reached 20 mm, or the tumors became severely ulcerated, or otherwise advised by the University of Pittsburgh animal facility.

Staining of tumor cells with pre- and post-infection sera

Four days prior to primary influenza infection, mice were bled to obtain their pre-infection sera antibody repertoire. Ten days following the second infection, mice were bled to obtain post-infection sera antibodies. Prior to staining, both sets of sera were diluted 1:62.5 in PBS. 2×10^5 3LL and IG10 tumor cells were plated in a 96-well plate and stained on ice for 1 hour with 100uL of the pre- or post-infection sera. Cells were then stained on ice for 30 minutes with FITC-conjugated Rat anti-mouse IgG2a (BD Bioscience) as the secondary antibody. Cells were fixed in 1.6% paraformaldehyde and samples were run on a LSRII flow cytometer.

Affinity purification of 3LL antigens

Total cell lysates were generated from 50×10^6 3LL cells in 300uL NP-40 lysis buffer (0.5% NP40, 0.5% Mega 9 (octylglucoside), 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.5, 2 mM PMSF, 5 mM iodoacetamide, and Protease Inhibitor (Roche)). Lysates were pre-cleared with the addition of Protein G Sepharose beads (Sigma-Aldrich, Inc, St. Louis, MO) and the mixture incubated for 1 hour at 4° C on an orbital shaker. Protein G beads were removed by centrifugation at 1200 rpm prior to affinity purification. Protein G HP Spin Trap Columns and Buffer Kits (GE Healthcare UK) were used following the manufacturer's protocol with the following modifications. Pre-infection and post-infection sera were pooled separately from mice (n=6) and each set of sera was poured over multiple protein G columns (100 μ L per column). Columns were washed with the wash buffer and 50 mM dimethyl pimelimidate dihydrochloride (DMP) was added to covalently cross-link the bound antibodies from each set of sera to the protein G columns, as described in the manufacturer's protocol. This was done to ensure that only the bound protein fractions were eluted from the columns and not the antibodies. 400 μ L of 3LL tumor lysate was then added to both the pre- and post-infection sera columns and incubated overnight at 4° C on an orbital shaker. The following day the columns were washed with TBS (50 mM Tris, 150 mM NaCl, pH 7.5) and the bound proteins were eluted off the columns with 0.1 M glycine provided from the kit with 2 M urea, pH 2.9. Pooled proteins from the pre-infection and post-infection antibody columns were concentrated and the elution buffers were changed to 2D-gel buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT, 10m M HEPES, pH 8.0) using 5000 MWCO Vivaspin columns (Sartorius Stedim Biotech, Goettingen, Germany).

2D-DIGE and Liquid Chromatography/Mass Spectrometry (LC/MS) analysis

The immunoprecipitated proteins were subjected to Difference Gel Electrophoresis (DIGE) (30) to identify proteins largely or uniquely precipitated by post-infection sera. Protein labeling, Isoelectric Point Focusing (IEF), and 2nd dimension SDS-PAGE were conducted as described (31) with the following modifications. 2.5 μ g of pre-infection proteins and post-infection proteins were reduced in 10 mM Tris(2-carboxyethyl)phosphine (TCEP, Sigma) for 60 minutes in the dark at 37°C. 10 mM CyDye DIGE Fluor Cy3 or Cy5-maleimide saturation dyes (GE Healthcare, Uppsala, Sweden) diluted in Dimethylformamide (DMF)

(Sigma), which label all available TCEP reduced cysteines on all proteins, were added to each sample for 30 minutes at 37° C. Labeling was quenched with 7M Dithiothreitol (DTT). Samples were then combined and immobilized pH gradient (IPG) buffer (GE Healthcare) was added at 1μL/40μL of sample. Labeling of the two samples was reversed (reciprocal labeling) and run concurrently on a 2nd gel to eliminate dye-dependent differences. Proteins were separated in the 1st dimension on 13cm pH3-10NL IPG strips on an IPGphor apparatus (GE Healthcare) for 35000 Volt-hours. The samples were then separated on the 2nd dimension SDS-PAGE in pre-cast 10-20% gradient polyacrylamide gels encased in low fluorescent glass (www.precastgels.com) in standard Tris-Glycine-SDS running buffer. Fluorescent images of reciprocal gels were taken as described (31). The Bioinformatics Analysis Core of the University of Pittsburgh Genomics and Proteomics Core Laboratories analyzed the resultant fluorescent images and selected spots that were then cut from the gels and identified via Nano LC-ESI-MS/MS, as described (32).

Western blot and densitometry analysis

Lung tissues were homogenized with a 2 mL dounce homogenizer and total lysates were obtained in NP-40 lysis buffer. The same procedure was applied to generate total cell lysates from 3LL and IG-10 tumor cells. Prior to Western blotting, protein concentrations were determined via Bradford assay. 50 μg of protein from various groups were separated on 10% TGX pre-cast gels (Bio-Rad) and immunoblotted onto PVDF membranes. The following antibodies were used to probe for their respective proteins on separate blots: anti-HSP 90α/β (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Annexin II (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Histone H4 (1:1000, Abcam, Cambridge, MA), anti-GAPDH (1:1000, Abcam, Cambridge, MA), anti-Malate Dehydrogenase 2 (1:100, Abcam, Cambridge, MA), anti-Actin (1:15000, Sigma-Aldrich, Inc, St. Louis, MO), goat anti-mouse HRP (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-rabbit HRP (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). All Western blots were scanned on Kodak Image Station 4000MM and band densitometry analysis was performed on all blots using Image J (NIH, Bethesda, MD). All bands were normalized according to their actin control. Once normalized, all experimental bands and lanes were compared to a normal uninfected mouse lung.

ELISA

15 μg/mL of one of the following proteins were coated on Immulon 4HBX ELISA plates (Thermo scientific) in duplicate wells to examine the differences in antibody recognition between pre- and post-infection sera: MDH2 (Novus Biologicals, Littleton, CO), GAPDH (Abcam, Cambridge, MA), Histone H4 (New England Biolabs, Ipswich, MA), HSP90α (Abcam, Cambridge, MA), Annexin A2 (Novoprotein, Summit, NJ). Human proteins were used due to their high conservation between mouse and human. Duplicate wells that were not coated with antigen served as controls for non-specific binding. Plates were then placed on an orbital shaker overnight at 4° C. The next day pre- and post-infection sera were diluted (1:62.5) in PBS, added to ELISA plates, and placed on an orbital shaker for 2 hours at room temperature. Plates were washed and 0.3% Hydrogen Peroxide was added to the wells to block background peroxidase activity. Plates were washed and rat anti-mouse IgG-HRP (1:500) was added. Plates were again washed and TMB substrate was added for 15 minutes

and 2N Sulfuric Acid was added to stop the developing signal. ELISA plates were then read at 450 nm on a Gen 5 plate reader. Data were represented using the average of duplicate antigen-coated wells after subtracting the value from the no antigen control wells.

Peptide identification and MHC-I binding assays

Candidate peptide sequences were identified using the Immune Epitope Database (IEDB) MHC-I binding predictor program with a percentile rank of 5 or less (33). MDH₂₅₁₋₂₆₀ (MAYAGARFVF), GAPDH₃₀₀₋₃₁₀ (ALNDNFVKLIS), Annexin A2₁₈₄₋₁₉₁ (SVIDYELI), H4₈₇₋₉₅ (VVYALKRQG), Histone H4 with an amino acid substitution (H4-sub VVYAFKRQG) peptides were synthesized by the Peptide Synthesis Core of the University of Pittsburgh Genomics and Proteomics Core Laboratories as described (34). MHC-I binding was verified performing RMA-S, a TAP-deficient cell line, MHC-Class I stabilization assays. In short, 2×10^5 RMA-S cells were plated in a 96-well plate. The cells were cultured overnight at 29° C. Unloaded RMA-S cells served as controls. Each peptide candidate was added in triplicates to the plate from 10^{-4} M to 10^{-7} M for 2 hours and 30 minutes in a 29° C incubator. Cells were placed in a 37° C incubator for 1 hour and 30 minutes. RMA-S cells were fixed in 1.6% Paraformaldehyde and then stained with anti-H2-K^b or anti-H2-D^b (BD Bioscience). Samples were run on the LSR II Flow Cytometer (BD Bioscience) and analyzed using FACS Diva Software (Supplementary Figure S1).

Antigen-specific T cell detection

Animals were sacrificed six days following the second influenza infection. Lungs and spleens were harvested and cells isolated as described (35). 1×10^6 cells from each set of tissues were stained with anti-CD3, anti-CD4 and anti-CD8 antibodies (BD Bioscience). Dimer-X soluble dimeric mouse H-2K^b:Ig Fusion Protein and H-2D^b:Ig Fusion Protein (BD Bioscience) were used according to the manufacturer's protocol to detect MDH₂₅₁₋₂₆₀, GAPDH₃₀₀₋₃₁₀, Annexin A2₁₈₄₋₁₉₁ and H4₈₇₋₉₅ peptide-specific CD8⁺ T cells. In addition, the presence of flu-specific T cells was evaluated using peptides PA₂₂₄₋₂₃₃ and NP₁₄₇₋₁₅₅ purchased from GenScript USA, Piscataway, NJ. 100,000 events were collected and samples were run on the LSR II Flow Cytometer (BD Bioscience), gated (example in Supplementary Figure S2) and analyzed using FACS Diva Software.

Vaccination and Tumor Challenge

D1 dendritic cells (DC) are an established growth factor-dependent immature dendritic cell line. D1 DC were grown and maintained as described and used in all vaccinations (36). 1.25×10^6 D1 DCs/ mouse were cultured in 6-well plates, loaded separately with 100 µg of MDH₂₅₁₋₂₆₀, GAPDH₃₀₀₋₃₁₀, Annexin A2₁₈₄₋₁₉₁, or H4₈₇₋₉₅, and matured with 12.5 µg/mL of Poly IC:LC adjuvant. 0.25×10^6 D1 DCs loaded with individual peptides were pooled together for a total number of 1×10^6 D1 DCs. An additional 50 µg of each soluble peptide per mouse was added to the mixture and injected into mice in the right hind flank. Unloaded, Poly IC:LC matured D1 DCs were injected into control mice. Animals were vaccinated at weeks 0, 2, and 6, and challenged with 1×10^5 3LL cells in the right hind flank two days following the week 6 vaccination. Tumor length and width were measured at two-day intervals using calipers.

Data Analysis

Statistical analysis was performed using GraphPad Prism v6.0 software (GraphPad Inc. San Diego, CA). Results were represented as means $\pm$ standard error of the mean (SEM). Statistical means and significance were analyzed using unpaired two-tailed student's t test. Kaplan-Meier survival curves were analyzed with the log rank test. Significance for all experiments was defined as the following: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Influenza virus infection induces antibodies to multiple host cell antigens, some of which are known TAA

Mice were infected with influenza virus PR8 and then 35 days later with the second influenza strain Aichi, as described in Materials and Methods. Mice experienced the general signs of malaise and lost close to 20% of their starting body weight in the first week following each infection. By 7-8 days post infection, mice began to recover and by days 16-18 their weight returned to baseline (Figure 1A). Day 25 post second infection, animals were injected subcutaneously with 3LL tumor cells. Tumors became palpable eight days post injection (Figure 1B). At day 14, tumor growth kinetics between the influenza-experienced animals and the naïve mock-infected group began to diverge. Tumors in the influenza-experienced animals grew slower from days 14 to 22. On day 16 the average tumor size in the influenza-experienced mice was 20.67 mm² versus 51.63 mm² in the control group. The size difference was still significant at day 18 when the average tumor size in the influenza-experienced group was 31.78 mm² compared to 73.25 mm² in the control mice.

We examined the post-infection sera for the ability to stain 3LL tumor cells, which would suggest that flu infections elicited antibodies against tumor cell surface proteins. In the experiment illustrated in Figure 1C, we obtained sera from mice (n=7) pre- and post-influenza infection. Tumor cell surface staining was performed with individual sera and the results were pooled into pre- and post-infection groups and represented as an average value. Average Mean Fluorescence Intensity (MFI) of staining of 3LL tumor cells with post-infection sera was significantly higher compared to that with pre-infection sera. The same result was obtained staining another mouse epithelial tumor cell line IG10 with the same sera.

To identify molecules specifically recognized by post-infection sera, pre- and post-infection antibodies were bound to Protein G columns for affinity purification of proteins from 3LL tumor cell lysates. Tumor proteins bound to the antibody columns were eluted, labeled with two different Cyanine-based saturation dyes and resolved by 2D-DIGE as described in Materials and Methods. In a concurrently-run reciprocal gel, labeling was reversed such that the sample previously labeled with Cy3 was labeled with Cy5 and *vice versa*. Gel images were false-colored for analysis: green for Cy3, red for Cy5. Overlays were then created using Delta2D software, where proteins unique to one sample appeared either green or red, while proteins common to both samples appeared yellow (green and red combined). Figure 2 shows the gel used to identify antigens described below. Green dots mark proteins eluted

from the pre-infection antibody columns while red dots mark proteins eluted from the post-infection antibody columns. Spots that were most remarkable (yellow circles) were cut out and subjected to mass spectrometry analysis and protein sequencing. Many proteins with a wide variety of functions were identified. They included voltage-dependent ion channels, proteasome subunits, mitochondrial and cytosolic enzymes, heat shock proteins and structural proteins (data not shown). We selected five identified proteins: Histone H4, Malate Dehydrogenase 2 (MDH2), Annexin A2, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and Heat Shock Protein 90 (HSP90) for further study (Table 1). Several reports based on methods and approaches unrelated to ours had already identified these molecules as TAA in tumor-bearing mice or in cancer patients (37-41).

Overexpression of GAPDH, Histone H4, MDH2, Annexin A2, and HSP90 in tumor cells and influenza virus-infected lungs leads to specific immunity

We hypothesized that post-infection antibodies against these proteins were elicited due to differences in their expression in infected versus normal lungs, analogous to their abnormal expression in tumors. Western blot analysis showed that these proteins were constitutively overexpressed in both epithelial tumor cell lines, 3LL and IG10, and also at various time points in the flu-infected lungs (2-7.5-fold higher compared to healthy lungs) (Figure 3). Expression of GAPDH in influenza-infected lungs appeared to be the highest at day 3 post-infection. Histone H4 protein levels were constitutively elevated in tumor cells and at all time points post flu-infection by 5 to 7-fold higher than in normal lung. MDH2 levels were elevated 12 hours post-infection and decreased to normal levels at day 3 post-infection. Annexin A2 remained 3-fold higher than in normal lung at all time points. HSP90 protein level was the highest at day 2 post-infection.

Even though these antigens were identified by affinity purification on post-infection sera bound to Protein G columns, we wanted to confirm in another assay the specificity of post-infection antibodies for each of these individual molecules. Figure 4 shows that in most mice there was an increase post-infection in IgG specific for GAPDH, Histone H4, MDH2, Annexin A2 and HSP90 α as determined by ELISA.

Influenza virus infection also induced an increase in CD8⁺ T cells specific for all of these antigens. Spleens and lungs were harvested from mice six days after the second influenza infection and from uninfected control mice. Peptides GAPDH₃₀₀₋₃₁₀ (ALNDNFVKLIS), Annexin A2₁₈₄₋₁₉₁ (SVIDYELI), MDH₂₅₁₋₂₆₀ (MAYAGARFVF), Histone H4₈₇₋₉₅ (VYALKRQG), Histone H4 with an amino acid substitution (H4-sub VVYAFKRQG) (38), were selected from the Immune Epitope Database (IEDB) and confirmed to bind to MHC-I in RMA-S stabilization assays (supplementary figure 1). Each peptide was loaded onto DimerX H-2K^b or H-2D^b molecules and used to detect specific CD3⁺CD8⁺ T cells. There were no CD8 T cells specific for these peptides in the lungs and spleens of uninfected control mice but in flu-experienced animals they were present in similar numbers to the flu-specific T cells (Figure 5). The highest numbers both in the lungs and in the spleens were H2-K^b-restricted GAPDH-specific and H2-D^b-restricted MDH2-specific T-cells.

Vaccination with DC loaded with the new TAA peptides delays tumor growth and promotes survival

We loaded the D1 dendritic cells with MDH2₂₅₁₋₂₆₀, GAPDH₃₀₀₋₃₁₀, Annexin A2₁₈₄₋₁₉₁, and H4₈₇₋₉₅ and vaccinated mice as described in Materials and Methods. Control mice were vaccinated with unloaded D1 cells. Two days following the second boost, mice were challenged subcutaneously with 3LL tumor cells. In vaccinated animals tumor growth began to slow down significantly by day 12 (Figure 6A) resulting in all vaccinated animals still surviving at day 40, compared to only 2 animals surviving in the unloaded DC vaccinated controls (Figure 6B). On day 12, the average tumor size in peptide-loaded DC-vaccinated animals was 10.67 mm² compared to 30.67 mm² for the controls.

Discussion

The data we present here, add a new dimension to our understanding of the process of cancer immunosurveillance and its targets. We show that immune responses against abnormally expressed self-antigens, many of which have been characterized as TAAs, are generated during non-malignant infectious inflammatory events that occur much earlier in life than malignancies. We propose that immune memory for these antigens is later recruited for cancer immunosurveillance.

We used a mouse model to demonstrate that two bouts with influenza virus infection led to the ability of the host's immune system to slow down tumor growth. This effect was small and transient, which may be all that could be expected from a limited exposure of the mice to this one type of infection and to no other pathogens prior to influenza infection as the mice are kept in pathogen-free conditions. The significance of this small delay in tumor growth was confirmed by the induction of antibodies against multiple molecules in the tumor cell lysate. Focusing on five antigens identified by infection-elicited antibodies, GAPDH, Histone H4, MDH2, Annexin A2, and HSP90, we showed that these antigens were abnormally expressed (overexpressed) in flu-infected lungs and in mouse epithelial tumor cell lines, and that in addition to IgG the flu-infection induced antigen-specific CD8 T cells against these molecules. As predicted by our hypothesis, vaccination with peptides derived from GAPDH, H4, MDH2, and Annexin A2 led to a much more profound slowing down of tumor growth compared to that elicited by the virus infection, and to a prolonged survival of tumor-bearing animals.

Other viral infections may be capable of inducing TAA specific antibodies and T cells. Vaccinia Virus- (VV) and Lymphocytic Choriomeningitis Virus (LCMV)-infected mice were reported to have developed antibodies against many host cell antigens, some of which are orthologues of human TAAs (42). Human fibroblasts infected with varicella-zoster virus (VZV) or human cytomegalovirus (CMV) overexpress cyclin B1 in the cytoplasm in a similar fashion to tumor cells where cyclin B1 was identified as a TAA (43, 44). Cyclin B1 has been found in VZV virions (45). Many healthy individuals, presumably having experienced these infections, have cyclin B1-specific IgG and memory T cells (24). It has been reported that GAPDH and Annexin A2 are found in influenza virions produced by infected epithelial cell lines Vero and A549 (46). HSP90, Annexin A2, and GAPDH were also found within human CMV particles (47). A study examining the measles virus effect on

presentation of self-peptides on MHC class I during infection showed that two abundant self-peptides on HLA-A*0201 measles-infected cells could induce auto-reactive CD8⁺ T cells. One of the peptides identified was HSP90β₅₇₀₋₅₇₈ (ILDKKVEKV)(48). The HSP90β₅₇₀₋₅₇₈ peptide has been found in melanoma cell lines (49). It is possible that these “auto-reactive” T cells contribute to increased tumor immune surveillance. None of these observations were followed by experiments to test the potential antitumor effects of either the viral infections or the immune responses against the identified molecules, with the exception of cyclin B1 that we showed was a target of antitumor immune responses (24).

The same protective effect of influenza virus-primed immunity specific for abnormally expressed self-antigens that we showed here, could be a collateral benefit of other viral, bacterial and parasitic infection or various acute inflammatory conditions of unknown etiologies. Therefore, we propose that the molecules abnormally expressed in these different disease states and also in cancer cells, that are currently referred to as TAAs, should be renamed disease-associated antigens (DAA) (50). Pre-existing immune responses to several known tumor antigens that are candidate DAAs have been reported to increase the odds of successfully eliminating spontaneously arising tumors (28). The arrival of memory DAA-specific T cells to the site of the tumor as a secondary immune response could promote priming of tumor-specific responses directed against individual mutations and epitope-spreading, adding to the efficacy of immunosurveillance. If DAA-specific immune memory is lacking or weak due to limited early exposures to infections, this may lead to establishment of chronic inflammation at the tumor site due to unopposed innate immune responses, which is likely to promote tumor development. Therefore, better understanding of the events early in life that prepare the immune system to protect an individual against known and unknown pathogens as well as future malignancies, will help direct vaccines and other immune manipulation towards strengthening rather than impairing the establishment of life-long immunosurveillance. In addition, these findings support the use of vaccines based on DAAs/TAAs for cancer prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Influenza virus infection delays tumor growth at early time points

(A) Animals were intranasally infected with PR8 and Aichi influenza viruses on day 0 and day 35, respectively. Mice were weighed every two days. (B) Influenza-experienced animals and naïve animals were challenged subcutaneously with 1×10^5 3LL tumor cells in the right hind flank. Tumor length and width were measured every two days using calipers. Data are representative of two experiments with at least 8 mice per group and are expressed as means $\pm$ SEM. (C) Sera from animals ($n=7$) four days prior to PR8 infection (pre-infection) and ten days after Aichi infection (post-infection) were diluted 1:62.5 and used to stain 3LL and IG10 tumor cells. Cells were subsequently stained with FITC-conjugated goat anti-mouse IgG2a antibody and analyzed on the LSRII flow cytometer. Results are shown as Mean Fluorescent Intensity (MFI). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 2. Influenza infection induces antibodies against specific proteins of tumor cells

3LL tumor lysates were affinity purified on protein G columns to which post-infection and pre-infection antibodies were covalently bound and the eluted proteins compared by 2D-DIGE gels. Green spots mark proteins eluted from pre-infection antibody columns and red spots mark proteins from the post-infection antibody columns. Yellow color marks overlapping spots. Yellow circles indicate spots picked for sequencing. Spots were chosen according to image analysis provided by the University of Pittsburgh Bioinformatics Analysis Core (BAC) of the Genomics and Proteomics Core Laboratories.

Figure 3. Histone H4, MDH2, GAPDH and HSP90 are elevated in epithelial tumor cells lines and influenza-infected lungs
Whole cell lysates were generated from 3LL and IG10 cell lines and from normal and influenza-infected lungs. 50 μ g of protein was loaded on each gel, resolved by electrophoresis and transferred onto PVDF membranes. Membranes were blotted using antibodies against GAPDH, Histone H4, MDH2, Annexin A2, and HSP90. Actin loading controls were used to normalize each band. Densitometry analysis was performed using ImageJ. All lanes were compared to Normal Lung. Data are representative of two experiments.

Figure 4. Antibodies specific for GAPDH, Histone H4, MDH2, Annexin A2 and HSP90α increase following influenza infection
Pre-infection sera (four days prior to the first infection) and post-infection sera (ten days post second infection) were assayed on ELISA plates in duplicate wells coated with individual proteins. Uncoated wells served as non-specific binding controls and their values were subtracted from the values in matching antigen-coated wells. Results are represented as mean optical density (OD) $\pm$ SEM of two experimental repeats with an n=7.

Figure 5. Antigen-specific CD8⁺ T cells increase in the lungs and spleens following influenza infection

Mice were infected with PR8 and Aichi Influenza viruses or mock PBS infected on day 0 and day 35, respectively. Six days after the second infection spleens and lungs were harvested and analyzed by Flow Cytometry for the presence of T cells staining with the Dimer-X reagent (as described in Methods and Materials). Data are representative of two experimental repeats of least n=3 animals per group and are expressed as means $\pm$ SEM.

Figure 6. Vaccination with GAPDH, Histone H4, MDH2, and Annexin A2 peptides loaded on DC confers early protection and prolonged survival against 3LL tumor challenge

(A) Mice were vaccinated in the right hind flank with 1×10^6 D1 cells/mouse (a pool of 2.5×10^5 D1 dendritic cells/peptide) at week 0, week 2, and week 6. Control mice received the same number of unloaded D1 DC at the same time points. Two days after the week 6 boost, animals were challenged subcutaneously with 1×10^5 3LL tumor cells in the right hind flank. Tumor growth was measured with calipers at two-day intervals and expressed as length x width (mm²). (B) Survival post tumor challenge. Data are representative of two experiments with at least $n=8$ mice per group and are expressed as means $\pm$ SEM.

Table 1
Biochemical characterization of selected tumor antigens detected by post infection antibodies

Spot Number	Accession Number	Protein Name	Peptide Sequence	X-Corr	Score	Charge	M/Z [Da]	MH+ [Da]	M [ppm]	MW [kDa]	pI	% Sequence Coverage	Ions Matched
2	IP100885367.1	Annexin A2	TNQELQEINR QDIAFA YQR	2.74 2.55	0.36 1.00	2	622.81384 556.28143	1244.62041 1111.55559	-2.08 2.22	19.6	5.96	10.80	14/18 14/16
3	IP100468203.3	Annexin A2	TPAQYDASELK AEDGSVIDYELIDQDAR SLYYIQQDTK TNQELQEINR WISIMTER DHISDTSGDFFR QDIAFA YQR	3.52 3.46 3.30 3.08 2.67 2.53 2.41	0.65 0.68 0.47 0.47 1.00 1.00 0.60	2 2 2 2 2 2 2	611.80090 954.94586 711.35215 622.81384 518.26892 613.28820 556.28027	1222.59453 1908.88445 1421.69704 1244.62041 1035.53057 1225.56914 1111.55327	-0.43 1.16 1.62 -2.08 1.29 -0.36 0.13	38.7	7.69	22.71	15/20 17/32 16/20 15/18/ 13/14 16/20 15/16
4	IP100407339.7	Histone H4	ISGLIYEETR TVTAMDVVY ALK TVTAMDVVY ALK DAVTTYEHAK VFLENVIR	3.39 3.22 2.88 2.61 2.56	0.68 0.74 0.42 0.52 0.36	2 2 2 2 2	590.81536 655.85559 663.85327 567.77410 495.29345	1180.62346 1310.70391 1326.69927 1134.54094 989.57964	2.19 1.06 1.39 -1.47 1.76	11.4	11.36	38.83	16/18 16/22 14/22 15/18 12/14
4	IP100622795.2	GAPDH	LENPAKYDDIK	2.36	8.03	2	653.33881	1305.67035	0.20	35.8	8.03	3.30	10/20
4	IP100323592.2	Malate Dehydrogenase 2	IFGVTTLDIVR	2.82	0.41	2	617.36487	1233.72246	0.41	35.6	8.68	3.25	14/20
5	IP100885367.1	Annexin A2	TNQELQEINR	3.06	0.47	2	622.81384	1244.62041	-2.08	19.6	5.96	5.68	14/18

Protein Accessions- Protein Accessions in IPI mouse database

Protein- Protein name

Peptide sequences- Primary amino acid sequence for identified peptides

Xcorr- XCorr is the cross-correlation of the experimental and theoretical spectra from SEQUEST search

Score- A measure of the difference between the top two scores for the peptides identified by that spectrum

Charge- Charge stage of precursor ion

M/Z [Da]- Observed mass to charge ratio of precursor ion

MH+ [Da]- Monoisotopic mass for protonated molecular ion MH calculated based on observed m/z

M [ppm]- Deviation from expected MH

MW-Molecular Weight

pi- Isoelectric Point

% Sequence Coverage- percentage of the database protein sequence covered by matching peptides

Ions Matched- The number of fragment ions matched out of the total number of fragment masses for the peptide