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RESEARCH ARTICLE

Investigation of Cellular and Humoral Immune Responses to Tumor-Associated Antigens and Survival of Patients with Advanced Cancers Treated with Combined Radiation and Immunotherapy

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ABSTRACT:
Immunotherapy is effective in treating patients with a variety of cancers. We developed and employed an immunotherapeutic protocol that combined intra-tumor injection of autologous immature dendritic cells with standard of care radiation to treat patients with a variety of different advanced solid tumors. To assess patients' cellular immune response to their tumors,
pre and post treatment peripheral blood mononuclear cells (PBMC) were assayed for their capacity to kill autologous tumor cell lines that had been established from patients with different advanced solid tumors. Peripheral blood lymphocytes obtained post treatment were cytotoxic to autologous but not allogeneic tumor cell lines. Cytotoxic activity increased as relative numbers of CD8+CD56+ dual positive lymphoid cells increased in PBMC obtained post treatment. The autologous tumor cell line directed cytotoxicity was shown to be mediated by both innate and adaptive immune mechanisms. The cytotoxicity of PBMC obtained post treatment was enhanced after co-culture with autologous and not allogeneic tumor cell line lysates.

To assess a humoral immune to an autologous tumor antigen, antibody activity to mesothelin, a tumor associated antigen expressed in a variety of solid tumors, increased in post treatment serum samples from lung cancer patients. Sera containing these antibodies mediated antibody directed cellular cytotoxicity to mesothelin expressing tumor cell lines.

The objective of cancer therapy is to enhance quality of life and increase survival. Survival of 96 patients with a variety different advanced cancer, treated on this protocol over a 3-year period and followed for 1000+ days was significantly increased in patients with low tumor burden (lesion at <5 sites with diameters < 3cm) (Km= <,0.0001) regardless of malignancy. These findings support the application of this combination of immunotherapy and radiation in the treatment of cancer patients with advanced disease.

Keywords: Immunotherapy, Radiation, Dendritic cells, Tumor antigens, Immune Response, Clinical response, Overall survival

Data Access Statement
Research data supporting this publication are available from the Japanese and University of Maryland coauthors.

Introduction

Over the past 20 years, various immune-based modalities have been developed for cancer treatment. A major focus has been on exploiting the adaptive cell T mediated immune response that develops against tumor-specific products that are viewed by the host immune system as non-self. Initial studies utilized in vitro expanded T cells harvested from the tumor microenvironment.[1] Another approach was modification of the T cell receptor (TCR) on CD8+ cytotoxic cells in order for these effector cells to recognize tumor derived peptides presented by HLA class I molecules expressed on the surface of the malignant cell[2,3]. With the recognition that cellular immune responses, developed over the course of malignant transformation, were held in check by interaction of surface receptors on effector cells with ligands on tumor cell surfaces, inhibitors of these pathways were develop and are employed in treatment of a variety of hematologic malignancies and melanoma.[4,5] More recently autologous peripheral blood lymphocytes from cancer patients have been transfected with a single chain multiple variable receptor complex (CAR-T) that on recognition structures on the tumor cell activates the transfected cell to produce products (ie. cytokines perforins, granzymes etc.) that kill the tumor cell.[6,7] Treatment with checkpoint inhibitors and CAR-T cells have been shown to be highly effective in hematologic malignancies, less so in solid tumors. However, treatment with check point inhibitors and CAR-T cell have substantial systemic adverse side effects due to the abundance of cytokines that are produced by cytotoxic effector cells on interacting with the targeted cancer cells.[8]

In contrast to the abundance of studies on cell-mediated immune responses to tumors, adaptive antibody responses have received little attention. Antibodies against tumor-associated antigens have been reported to be present in the serum of cancer patients, even in some cases, before the clinical manifestation of malignancy.[9,10.] The role this arm of the adaptive immune response may play in controlling malignancy is not clear.

Anti-tumor cell antibodies have been developed *in-vitro* using recombinant technology and have been shown to be highly effective in the treatment of some hematopoietic malignancies, either by direct binding to malignant cells, thus inhibiting their growth, or as mediators of antibody directed cellular cytotoxicity (ADCC).[11]

There is a need for an immunotherapeutic approach that expands the existing antitumor immune response and/or induces a response to neoantigens generated by genetic changes that occur in tumor cells as they expand and/or metastasize.

A significantly less toxic approach to generate a primary response and/or boost an existing response to tumor-associated antigens has been the development of vaccines that incorporate products of tumor cells and administered with therapeutic intent. Various platforms have been used to develop and deliver tumor antigens, including vaccination with autologous dendritic cells (DC) that have been armed in vitro by the following procedures: fusion of DC with autologous tumors, transfection with tumor-derived DNA or RNA, and pulsing DC with tumor lysates, or peptides.[12-16] Arming DC *in vitro* using these procedures may not generate an effective immune response, given the restriction of peptide recognition imposed by major histocompatibility gene products (HLA). Thus, what the investigator chooses may not be what the host immune system recognizes.

Another approach for arming DC with resident tumor products is to inject autologous DC directly into primary tumors and/or metastatic sites, thus allowing tumor antigen capture *in-situ*. With this intent, a protocol to treat patients with advanced cancers was developed in which autologous immature dendritic cells, generated in vitro from peripheral blood monocytes, were administered parentally or by direct intra tumoral injection into primary and/or metastatic lesions, followed by standard-of-care radiation to the injected site.[17-19] Intra tumoral DC injection was repeated for new lesions identified on follow-up PET-CT. This protocol was designed to generate an autologous antitumor vaccine in vivo and was designated as a human-initiated tumor vaccine (HITV).

Herein we report the results of in-vitro studies conducted to identify cellular and antibody responses to tumor-associated antigens in patients with stage 4 and/or recurrent cancer that were treated on this protocol. The targets for determining the development of cytotoxic T cell response to autologous tumors were cell lines that had been established from patients with renal, lung, and gastric cancers and the effector response, PBMC obtained from these patients before treatment and at post-treatment intervals.

To evaluate induction of or augmentation of existing antibody activity, we expanded on previously reported observations that lung cancer patients, treated on this protocol, developed an expanded response to mesothelin, a tumor associated antigen.[18]

Materials and Methods

Patients: Informed consent was obtained from each patient before treatment. The consent form was approved by the ICVS Tokyo Clinic and Research Institute Institutional Review Board.

Treatment: The detailed treatment protocol has been reported in previous publications.[17-19] Briefly, tumors (primary and metastatic) in patients with advanced malignancy were directly injected with autologous dendritic cells prepared from peripheral blood monocytes. The injection sites were irradiated with fractionated doses and the tumors reinjected with dendritic cells. Responses were monitored using PET-CT, and new or recurrent tumors were reinjected with DC. The patients treated with this protocol and those reported herein are listed in Table 1.

Table 1. Baseline characteristics of participants.

Variables	**Participants (n = 96)**	
Median Age in years, (range)	63, (26-86)	
Sex, n (%)		
Male	50 (52%)	
Female	46 (48%)	
Tumor type	**n**	**Percent of Total**
Appendix	1	1.0%
Bile Duct	1	1.0%
Bladder	1	1.0%
Brain	1	1.0%
Breast	15	15.6%
Colon	11	11.5%
Duodenal	1	1.0%
Esophageal	3	3.1%
Gall bladder	1	1.0%
Gastric/Stomach	4	4.2%
Head and Neck	5	5.2%
Hepatocellular	2	2.1%
Liposarcoma	1	1.0%
Lung	14	14.6%
Lymphoma	2	2.1%
Ovarian	3	3.1%
Pancreatic	2	2.1%
Prostate	2	2.1%
Rectal	6	6.3%
Renal	4	4.2%
Thymoma	1	1.0%
Unknown	3	3.1%
Urothelial	1	1.0%
Uterine	10	10.4%
Vaginal	1	1.0%

Cells and Cell lines: Autologous tumor cell lines were established from tumor biopsies of patients with lung, renal, or gastric cancer and employed as targets. in cytotoxicity assays. The cell lines used as allogeneic controls were obtained from ATCC. The cell lines were cultured in cRPMI [RPMI (Corning) containing 10% FBS (Gemini), 1% penicillin/ streptomycin (Gibco), and 1% glutamine (Gibco)]. Peripheral blood mononuclear cells (PBMC) and serum were prepared from whole blood samples obtained before and at intervals after treatment and cryopreserved until use.

Tumor Cell Lysates: Tumor cell lines were suspended in distilled water, subjected to three freeze-thaw cycles, centrifuged, and lysates collected. The protein concentration in the supernatant was measured using the Pierce Coomassie Plus (Bradford) assay. The lysates were stored at -80°C until use.

Stimulation of PBMC with tumor cell lysates: PBMC were thawed, suspended in cRPMI, and

cultured with 100 ug/ml tumor cell lysates or media alone for 72 hours at 37°C/5% CO_2. Additional autologous T cells were added, and the combination was cultured for an additional five days in cRPMI supplemented with 1 ng/ml IL-7 and 10 ng/ml IL-15. Culture supernatants were collected and tested for cytokine levels by ELISA. Tumor cell lysate-exposed cells were used as effector cells in cytotoxicity assays.

Cytotoxicity Assays: cytolytic activity of the stimulated PBMC was measured by 4-hour chromium 51 (^{51}Cr)-release assays following previously described protocols.[11] Briefly, tumor cell lines were labeled with chromium 51, combined with stimulated PBMC, incubated at 37°C °C with 5% CO_2 for 4 hrs, and released chromium 51 was measured using a gamma counter.

Expanded NK Cells: PBMC (1.5 x10^6) were cultured for six days with irradiated K562-mb-IL15-41BBL cells (10^6) in cRPMI containing 200 ul/ml human IL-2 (R&D Systems), harvested, and cultured for an additional 14 days in cRPMI supplemented with IL-2.[14]

Antibody Directed Cellular Cytotoxicity (ADCC): Patient serum-mediated NK cell cytotoxic activity was measured using a ^{51}Cr-release assay, as described above. To assess ADCC, sera were added to ^{51}Cr labeled target cells (A549 or PC-3), followed by IL-2 activated PBMC. The mixture was incubated for four hours at 37°C °C with 5% CO_2, the supernatants were harvested, and ^{51}Cr release was measured.

Mesothelin antibody detection: Mesothelin antibody responses were detected using ELISA, as previously reported.[18] 96-well flat-bottom plates were coated overnight at 4°C with 1 u/ml rHu mesothelin (R&D) in 0.1 M carbonate buffer (BioLegend). Pre and post treatment serum samples were added to the wells, the plates were developed, and the absorbance was read at 450 nm using a VersaMax plate reader.

Flow Cytometry: A standard fluorescence cytometry labeling protocol was used to determine the surface phenotypes of PBMC and tumor cell lines. A minimum of 10000 events were acquired using FACSCanto II (BD Biosciences), and the results were analyzed using FlowJo 10.4.0 software as previously described.[17-19] The following chromophore-labeled antibodies were used for flow cytometry to detect cell surface markers. Unless otherwise noted, all the antibodies were obtained from BD Biosciences. FITC-conjugated CD8, CD19, CD14, HLA-DR, CD83, CD86, CD209, CD86, isotype controls IgG1, IgG2α, IgG2β, PE-conjugated CD4, CD16, PD-1 (CD279), CD40, TLR4 (CD284), CD80, isotype controls IgG1, IgG2α, IgG2β; PE-CY7-conjugated CD56, CD11c; APC-conjugated CD3, TLR9 (CD289, biosciences); isotype controls IgG1 and IgG2α; APC-CY7 FVS780; 7AAD; CD45 conjugated to FITC, PE, PE-CY7, APC, and APC-Cy7 served as controls.

Intracellular cytokine and Fox-P3 gene expression: intracellular cytokine and Fox-P3 gene expression: PBMCs stimulated with tumor cell lysates were incubated on ice for 4-6 hours in PBS containing 5% FBS and 1 µL GolgiStop™ (BD Biosciences). The cells were washed, antibodies for surface markers (i.e., CD4 or CD8) were added, the mixture was incubated on ice for 15 min, and the cells were washed and fixed in 4% PFA. Permeabilization wash buffer (BD Biosciences) together with anti-cytokine or anti-Fox p3 antibodies was added, and the cells were incubated for 30 min at RT. Flow cytometry data were collected using a FACSCanto II (BD Biosciences) and analyzed using FlowJo 10.4.0. Antibodies were purchased from BD unless otherwise noted. Isotype control: mouse IgG1κ (MOPC-21). Anti-human: CD45 (HI30), CD3 (UCHT1), CD4 (L200), CD8 (RPA-T8), IFNγ (B27), Foxp3 (236A/E7;(bioscience). The cells were washed and fixed in 4% PFA. Permeabilization wash buffer (BD Biosciences) and anti-cytokine or anti-Fox p3 antibodies were added, and the cells were incubated for 30 min at room temperature. Flow cytometry data were collected using a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo 10.4.0. Antibodies were purchased from BD Pharmingen, unless otherwise noted. Isotype control: mouse IgG1κ (MOPC-21). Anti-human: CD45 (HI30), CD3 (UCHT1), CD4 (L200), CD8 (RPA-T8), IFNγ (B27), and Foxp3 (236A/E7; bioscience).

Statistical analysis: Descriptive statistics were calculated and analyzed by Student *t* t-test using GraphPad Prism 9 software. $P < 0.05$.

Results

Increased numbers of CD8+CD56+ cells in PBMC obtained post-treatment correlate with in vitro cytotoxicity to autologous tumor cell lines. We previously reported that patients with a variety of recurrent and advanced cancers treated with this protocol had increased numbers of CD56+ cells and CD8+CD56+ dual-positive cells in peripheral blood samples obtained after treatment.[17,18] Cells with this phenotype have been reported to have the ability to kill targets via both innate and acquired immune mechanisms.[20] The availability of autologous tumor cell lines provided the opportunity to test both mechanisms. Cytokine-activated PBMC from patients with the three cancers, obtained

before and after treatment, were tested in vitro for their ability to kill autologous and allogeneic tumor cell lines. The results are shown in Figures 1A and 1B. Autologous target cell killing increased as the number of dual-positive cells increased in the peripheral blood samples obtained post-treatment. The a/b T-cell receptor (TCR) on CD8+ cytotoxic T cells are primed to recognize tumor-derived peptides bound to HLA class I molecules expressed on the target cell surface. In contrast, NK CD56+ innate immune effector cells express are activated on recognition of ligands expressed on tumor cells. To determine whether blocking effector cell-target cell interactions affected target cell killing, antibodies that potentially interrupted CD8+ T cell killing (HLA class I and TCR a/b) or antibodies to NK cell-activating receptors were added to the cytotoxicity assays. The results are shown in Figure 1C. HLA class I and TCR a/b antibodies reduced cytotoxic activity in autologous tumor cell lines, whereas antibodies to structures expressed on both NK and CD8+ cytotoxic effector cells (NK2D and DNAM-1) decreased the killing of both targets. This pattern was replicated in studies with PBMC and autologous cancer cell lines from 3 patients with different cancers (lung, renal and gastric). These results are consistent with the conclusion that both innate and adaptive immune responses to autologous tumors are either induced de novo and/or boosted in patients with advanced cancers treated on this protocol. While these results are consistent with the interpretation that the dual positive cells that expanded in number post-treatment have the capacity to kill autologous tumors, we cannot exclude the possibility that CD8+ T cells and/or CD56+ natural killer cells present in the PBMCs also kill cancer cell targets.

Characterization of the adaptive immune response: Additional in vitro studies were conducted to further characterize the adaptive immune responses to autologous tumors. Lysates prepared from autologous and allogeneic cancer cell lines were added to PBMC obtained before and after treatment from patients with the different cancers. Autologous but not allogeneic target cell killing increased in lysate cultured cells drawn post treatment (Figure 2A).

Cytokine production by lymphocytes in response to antigen recognition is characteristic of adaptive immune memory. To determine if the HITV treatment protocol induced immune recognition of antigen(s) in autologous cancer cell lines, supernatants from lysate exposed to PBMC obtained before and after treatment were tested for Il1b, IFNg and IL10 by ELISA (Figure 2B). PBMC obtained after treatment from patients with the different cancers and cultured with autologous tumor cell lysates showed increases in IL1b and IFNg, cytokines associated with effector activity, but did not produce increased amounts of IL10, a cytokine associated with suppressor responses

Production of these cytokines did not increase in cells cultured with allogeneic tumor cell lysates. These studies support the conclusion that HITV-treated patients with different cancers develop an adaptive immune response to constituents of their resident cancers.

Cytokine production in T cell subsets: We tested lysate exposed PBMC, obtained before and after treatment from the renal and lung cancer patients for production of intra-cellular cytokines in CD4+ and CD8+ T cell subsets after culturing with autologous or allogeneic cell lysates (Figure 2C). Increased numbers of CD4 + and CD8+ T cells producing cytokines were found in PBMC obtained after treatment in cultures containing autologous lysates, but not in cultures with lysates of allogeneic cancer cell lines. in the post-treatment follow-up sample from this patient. The intracellular production of FOXP3 (found in Treg) was not observed in any of the post treatment samples. These studies support the conclusion that HITV-treated patients with different cancers develop an adaptive immune response to the constituents of their resident cancers. Interestingly. there was no evidence of suppressor response. These studies support the conclusion that HITV-treated patients with different cancers develop an adaptive cytotoxic immune response to constituents of resident cancers.

Mesothelin antibody response in HITV-treated lung cancer patients: Mesothelin is a glycoprotein expressed on the surface of lung cancer cells and in a variety of other cancers and is considered a tumor-associated antigen. Serum samples drawn before treatment and periodically thereafter were tested for anti-mesothelin antibody (Figure 3A). Significant increases in mesothelin antibody reactivity were found in sera from 4 of the 6 lung cancer cases.

Serum samples from 4 lung cancer patients were tested for their capacity to mediate activated NK cell killing of target cells (ADCC) (Figure 3B). The target cells tested in this study were A549, a lung cancer cell line expressing mesothelin, and PC3, a prostate cancer cell line that does not express mesothelin. Increased levels of lung cancer target cell death were found in cultures containing sera obtained post-treatment compared to those containing sera obtained before treatment. No increase in cytotoxicity to the prostate cancer cell

line was identified. We cannot claim that the anti-mesothelin antibodies detected in these serum samples are solely responsible for mediating NK cell death. Antibodies to unidentified antigens shared by the patient's tumor and the target cell may have been produced by the patients, thus accounting for the observed results. However, these results confirm the functional capability of an antibody response to a tumor-associated antigen induced by treatment with the HITV protocol.

Overall Survival (OS) duration in response to treatment: 96 patients with advanced cancer of various types were treated within this protocol over 3 years and followed for 1000+ days (see methods). During this period, 27 patients were lost to follow-up, and the remaining patients were analyzed for factors associated with survival duration.

Age at treatment initiation ranged from 45 to over 65 years and did not affect survival probability (Figure 4A). In contrast, patients who did not receive chemotherapy before treatment in this protocol had a significantly longer survival duration than those who received prior chemotherapy (Figure 4B, $p = 0.0145$).

Patients were stratified based on estimated tumor burden: low tumor burden, defined as PET-CT-detected lesions at < 5 sites with a diameter of 3 cm or less, and high tumor burden, defined as lesions at > 5 sites with a diameter > 3cm. The survival duration of patients with the most frequently treated cancers (lung, breast, and uterine) with a low tumor burden were significantly longer than that of patients with a high tumor burden. This has also been observed in patients treated for other malignancies. (Figure 4C)

Overall survival was significantly extended in treated patients with a low tumor burden compared to those with a high tumor burden (Figure 4D, $p = 0.0001$)

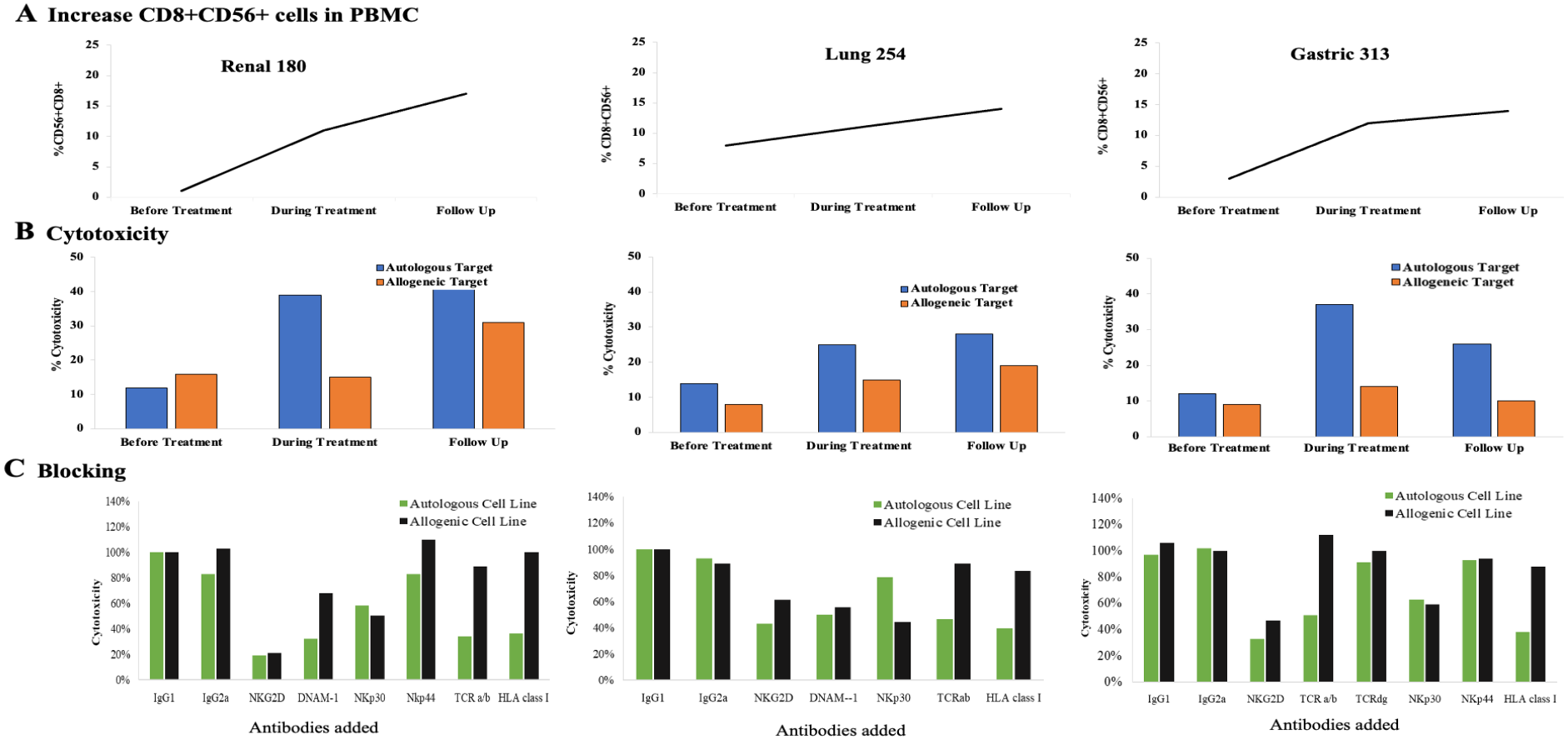


Figure 1. Cellular immune response

Panel A. CD8+ CD56+ duel positive cells in PBMC increased post treatment B. PBMC obtained at these time points were activated in –vitro (See materials and methods) and tested for their ability to kill autologous cancer cell line targets. Killing was concomitant with the increase in numbers of CD8+CD56+ dual positive cells in post treatment samples. C. Antibodies reacting with activation receptors on NK cells (NKG2D, DNAM-1) decreased cytotoxicity to both autologous and allogeneic targets while blocking structures on CD 8+ T cells (T cell receptors) and antibodies to HLA class I reduced cytotoxicity to autologous targets but not allogeneic tumor cell lines. Cytotoxicity data represents the percentage of target cells killed in the presence of antibodies expressing each marker and is based on total target cell killing in the presence of isotype antibodies.

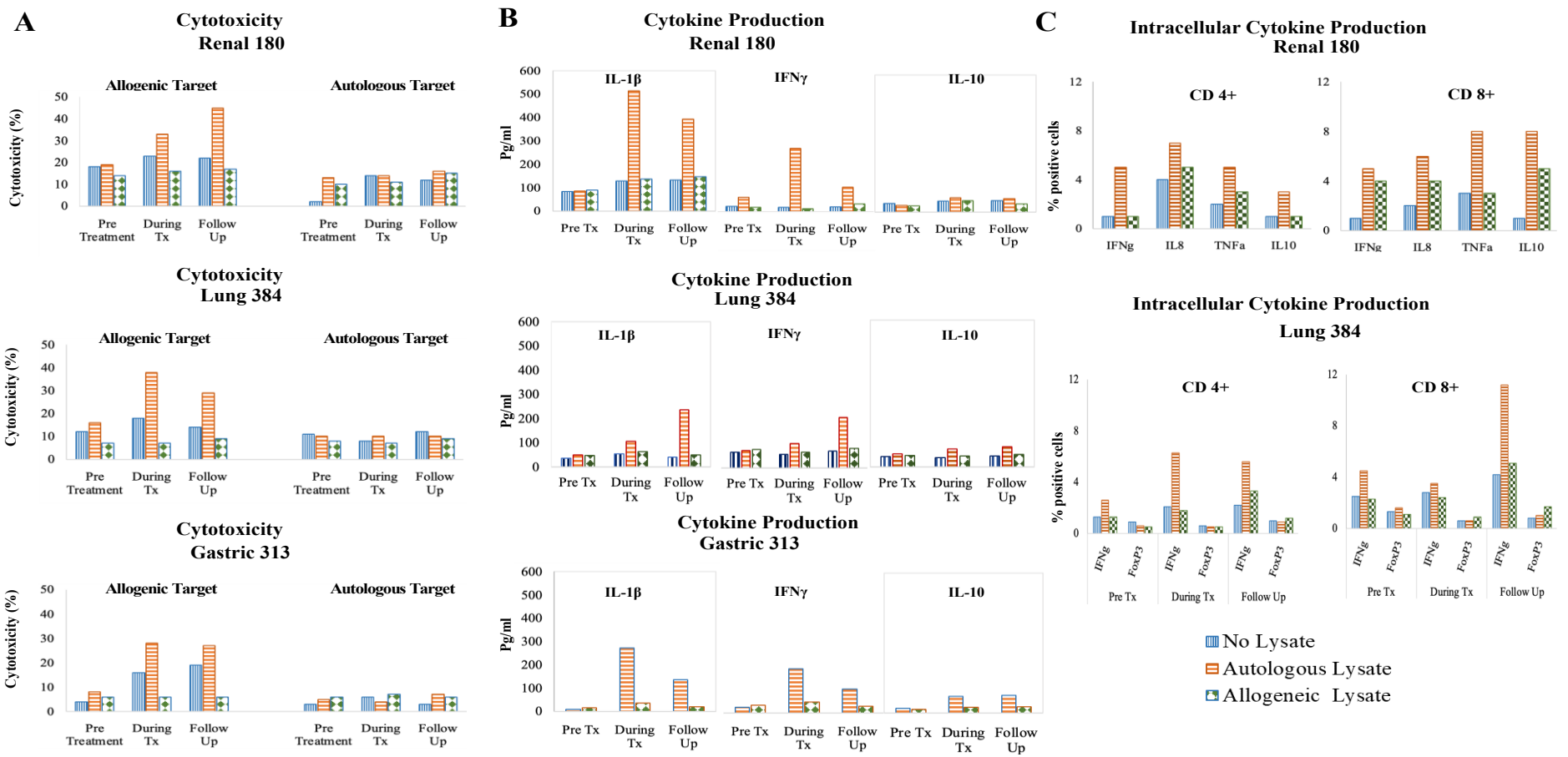
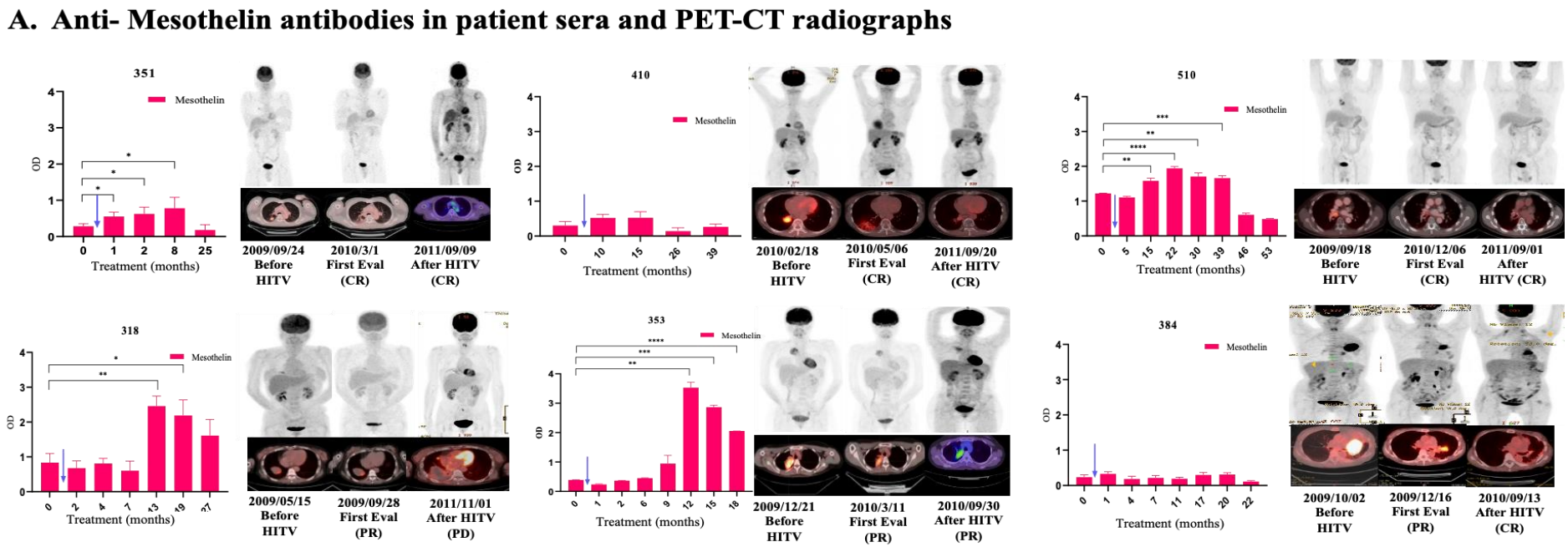


Figure 2. Cellular immune recall response to autologous tumors

A. Autologous cancer cell lysate exposed PBMC obtained post treatment increased autologous target cell killing but not killing of allogenic tumor cell lines. Exposure of the PBMC to allogeneic tumor cell lysates did not increase killing

B. Supernatants from lysate stimulated PBMC were tested for selected cytokines by ELISA. Autologous lysate exposed post PBMC obtained post-therapy produced cytokines while cytokines was not increased when PBMC were cultured with allogeneic cancer cell lysates. C. Induction of cytokine production was documented in CD4+ and CD8 + PBMC obtained post treatment and cultured with autologous lysates. Lysate exposure did not induce production of FOX-P3.



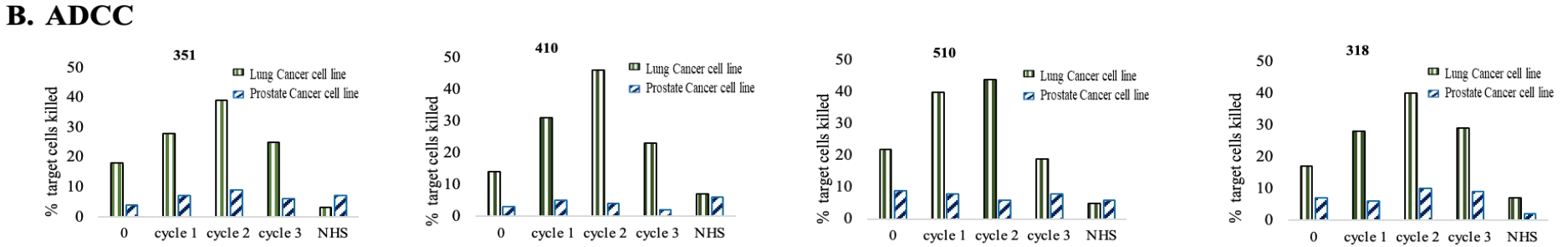


Figure 3. Anti-mesothelin antibodies in patient sera and PET-CT radiographs.
Anti-mesothelin antibody titers increased significantly in serum samples drawn post treatment from 4 of 6 patients. (Indicated by arrow). Serum samples from four HITV treated lung cancer patients containing anti-mesothelin antibodies were tested for their capacity to mediate ADCC to mesothelin expressing lung cancer cell line (A-549) or to (PC-3), a prostate cancer cell line. *p=0.05,**p=0.001****p=0001

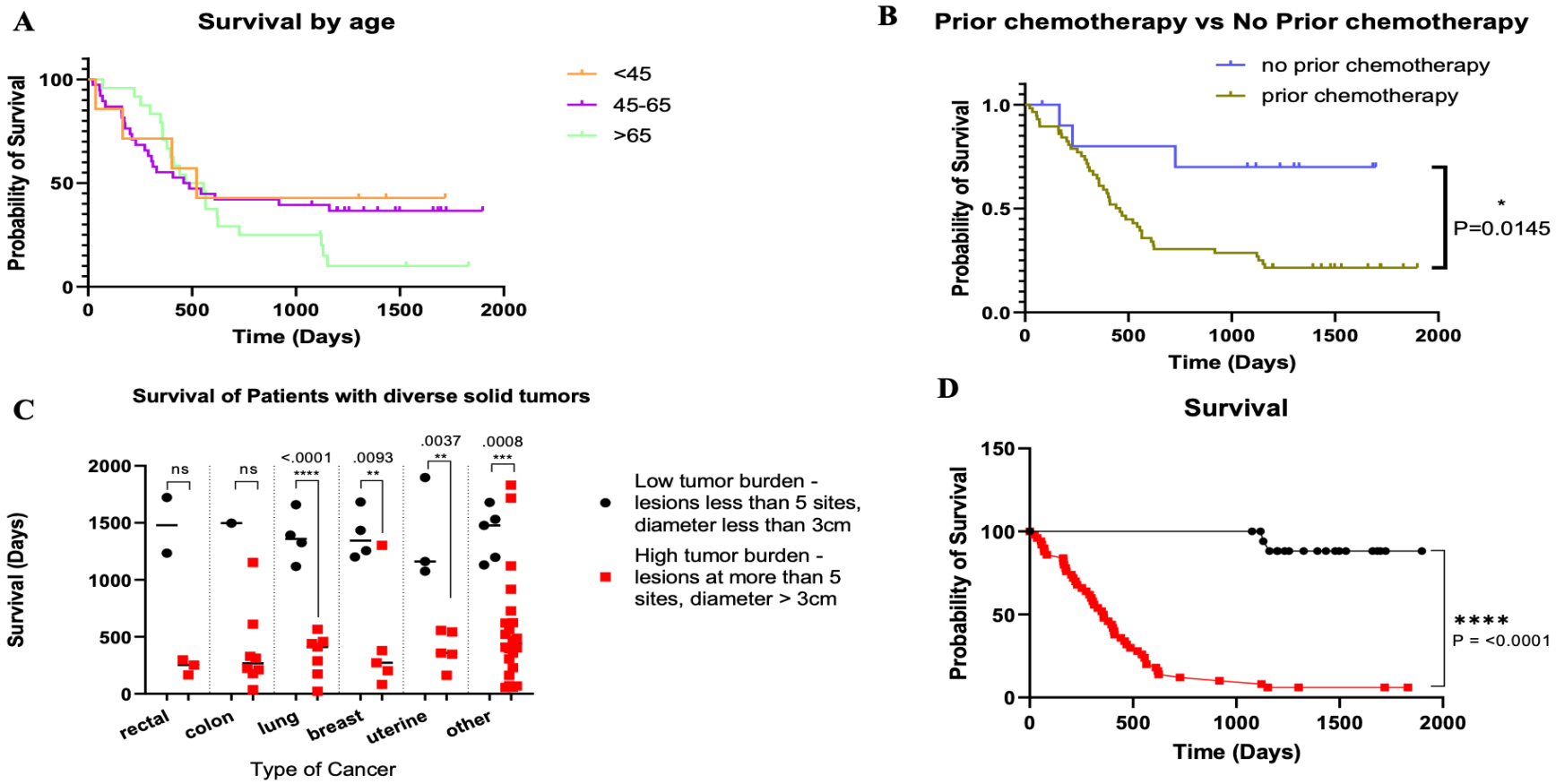


Figure 4. Factors associated with survival

Figure 4. Factors that influence survival of patients with solid tumors treated with HITV. A. Age of patients at initiation of treatment was not a significant factor in survival. (p= .2948) B. Patients who had not received chemotherapy prior to treatment had significantly increased survival p =0.0145. C. Survival of patients with different malignancies stratified based on tumor burden. D. Overall survival at follow up of all patients grouped by low and high tumor burden.

Discussion

A protocol combining intra -tumoral dendritic cell injection with radiation (HITV) was used to treat patients with advanced malignancies. We previously reported the feasibility and safety of this treatment protocol as well as the clinical responses observed in patients with an array of different solid tumors.[17-19] Herein, we report the results of in-vitro studies that document the generation of cellular and antibody immune responses to autologous tumor associated antigens in patients treated on this protocol. We show that treated patients with a variety of malignancies and low tumor burden had significantly longer survival duration than those with a higher tumor burden when followed over 3+ years.

Cytotoxic immune responses to autologous tumor cell lines, established from primary cancers in three patients with different cancers (renal, gastric, and lung) were shown to develop after therapy. In addition, an increase in antibody activity to a tumor associated antigen, mesothelin, was found in serum samples from treated patients with advanced lung cancer.

Autologous tumor cells are useful tools for *in vitro* studies documenting cellular immune responses as they express tumor associated antigens that are unique to each patient's cancer These tumor cells are targets of HLA-restricted adaptive $CD8^+$ T lymphocyte cytotoxicity responses as well as non-HLA-restricted $CD56^+$ natural killer (NK) cells. As previously reported, an increase in a population of lymphocytes that co-expressed cell surface markers characteristic of both $CD8^+$ T cells and NK cells were found in PBMC obtained after the treatment of patients with different cancers.[17] In the current report, we document that post-treatment PBMC that contained increased numbers of cells with this dual phenotype killed autologous but not allogeneic tumor cell lines. This correlation was found in PBMC from each of the 3 patients with the different cancers, indicating the ability of this protocol to induce cytotoxic T cell responses to resident tumors with different histologic origins.

To characterize the cell (s) mediating the killing, antibodies that react with activating receptors on NK cells and structures expressed on $CD8^+$ cells that interact with ligands on tumor cell lines were added to the cytotoxicity assays. The presence of antibodies that react with NKG2D and DNAM-1 reduced the killing of both autologous and allogeneic cell lines. In contrast, cytotoxicity to autologous cell lines, but not allogeneic lines, was blocked in cultures containing antibodies to T cell receptors (TCR) and cultures containing antibodies reacting with HLA class I molecules, the former expressed on $CD8^+$ cells and the latter expressed on both the T cells and the tumor cell line. Again, this was a consistent observation in studies with cells and autologous tumors from patients with different cancers.

The results of this study do not specifically identify the phenotype of cells mediating autologous target cell cytotoxicity.

The origin of the cells with dual phenotypes is not clearly understood. However, they have been identified in cord blood, in lymphocytes from individuals with autoimmune diseases as well as in those with cancer.[20]

T cells co-expressing $CD3^+$ $CD8^+$ $DC56^+$ have been shown to be effective in adoptive immunotherapy of several cancers.[21] We previously reported that T cells with this phenotype, expanded in vitro from $CD8^+$ cells isolated from post-treatment PBMC, induced responses when injected directly into autologous metastatic lesions in various cancers.[17]

Other investigators reported that overall survival (OS) of patients with NSCLC (non-small cell lung cancer) was significantly increased in patients receiving chemotherapy and infused with cells with this phenotype when compared to the OS of patients receiving chemotherapy only.[22] In a phase II trial, patients with myeloma were infused with a population of cells co- expressing $NKG2D^+$ $CD3^+$ $CD8^+$ and low dose IL-2. Blood from patients receiving these infusions were found to have significant increased numbers of $CD3^+$ $CD8^+$ $CD56^+$, $CD3^+$ $CD8^+$, and $NKG2D^+$ $CD3^+$ cells compared with patients treated with IL-2 alone. Furthermore, blood samples containing the infused cells were found to kill autologous myeloma cells, whereas blood from patients receiving IL-2 alone did not.[23]

Our results, demonstrating the induction of triple-positive cytotoxic T-cell ($CD3^+$ $CD8^+$ $CD56^+$) after treatment of patients with solid tumors, are analogous to those of this study in patients with hematologic malignancies and reaffirms the importance of these cellular immune responses in cancer immunotherapy. Together, these data support the use of these cells in patients with advanced cancer who exhibit progressive disease.

Although patients receiving DC-based cancer vaccines generated in vitro have been shown to develop immune responses to the intended target in in vitro assays, few studies have shown that these vaccines induce clinical responses to resident tumors.[24] In contrast, anti-tumor immune response and tumor resolution have been reported in mouse models and in human malignancies wherein

autologous DC at various stages of maturity were injected directly into tumors.[25-33] It is important to note that in these studies, the tumor-bearing host also received chemotherapy or radiation.

Tumor resolution was reported in patients with several different solid cancers in their advanced stages when mature DC were injected directly into the tumors.[34] In that trial, increased amounts of selected cytokines produced by injected DC correlated with stable disease and prolonged survival. Concordant development of an immune response to resident tumors was not reported.

We are not aware of any reports documenting the induction of dual-positive anti-cancer cytotoxic T-cells in recipients receiving cancer vaccines armed in vitro. Our results provide evidence that anti-cancer cytotoxic T-cells are generated or that the existing responses expand when autologous DC are antigen-armed in-situ

Vaccines are intended to establish immunologic memory to the targeted antigens We conducted studies to determine if immunologic memory was generated in patients treated on this protocol. PBMC obtained from patients before and after treatment were exposed in vitro to autologous or allogeneic tumor cell line lysates and tested for cytotoxic activity against autologous or allogeneic tumor cell lines. The cytotoxic activity to autologous but not allogeneic tumor cell lines increased post-treatment in PBMC that were exposed to autologous lysates. Co-culturing with allogeneic tumor cell lysates did not increase the cytolysis of either target. These results were replicated in cells obtained from patients with diverse types of cancer. Another measure of memory is the induction of cytokines by lymphoid cells upon exposure to the antigen(s) which induced a primary immune response. Supernatants from PBMCs co-cultured with lysates of autologous and allogeneic tumor cell lines were harvested and tested for cytokines. Increased levels of selected cytokines were found in the supernatants from cells exposed to autologous tumor cell lysates, but not in those exposed to allogeneic tumor cell line lysates.

Cytokines are produced by CD8+ and CD4 $^{+}$T cells upon the recognition of peptides presented by HLA class I and II molecules on DC. These cytokines play essential roles in the generation of cellular and humoral immunity.[35] Antigen exposure also induces regulatory immune responses. Fox-P3 expression, indicative of the induction of regulatory T-cell activity, was not detected in lysate-exposed PBMC from patients with lung cancers.

It has been established that a cellular immune response to a resident solid tumor develops before clinical recognition. Interestingly, antibodies to tumor-associated antigens have also been found in patients prior to overt clinical manifestation. However antibodies to tumor antigens have not been exploited in cancer immunotherapy to the extent of the cellular arm of the adaptive immune response .[36,37]

We have previously reported an increase in antibodies against mesothelin (a tumor-associated antigen) in serum samples obtained from patients with lung cancer.[18] Mesothelin was originally described in mesothelioma and ovarian cancer.[38,39] Antibodies against mesothelin have been detected in sera from patients with mesothelioma and have been used to monitor mesothelioma progression.[40] This membrane-bound protein is undetectable in most normal tissues but is expressed at high levels in several solid tumors, including lung, ovarian, and pancreas, thus a target for immunotherapy.[41-45] Monoclonal antibody therapies for mesothelin-expressing cancers have shown limited efficacy. CAR-T cells have been developed and employed in the treatment of mesothelin expressing tumors.[42]

Conclusions The goal in the treatment of patients with advanced and recurrent cancers is to enhance their quality of life and prolong their survival. The results of our studies clearly document that direct injection of autologous dendritic cells into recurrent and advanced solid tumors combined with radiation to the injection site results in the generation of immune responses to tumor-associated antigens. Moreover, patients with different advanced cancers and low tumor burden treated with this protocol had significantly extended disease-free survival compared to patients with a high tumor burden. Notably, local, and systemic toxicity due to cytokine release that often accompanies treatment with CAR-T cells has not been observed. This is an important consideration, given the general physical condition of patients with advanced solid tumors. These factors support the conclusion that this treatment approach significantly benefits patients with advanced solid tumor regardless of histological origin.

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