Vaccination with irradiated, GM-CSF secreting murine renal carcinoma cells elicits a broad antibody response that targets multiple oncogenic pathways

Abstract

Vaccination with irradiated tumor cells engineered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) elicits potent, specific, and long-lasting anti-tumor immunity in multiple murine tumor models. Early stage clinical testing of this vaccination scheme in advanced cancer patients revealed the consistent induction of humoral and cellular anti-tumor responses that accomplished extensive tumor necrosis. GM-CSF secreting tumor cell vaccines increase tumor antigen presentation by dendritic cells and macrophages, but the precise mechanisms underlying immune stimulation remain incompletely understood. To clarify further the contribution of GM-CSF to immune recognition, we undertook a detailed analysis of the antigenic targets of vaccination in the RENCA murine renal cell carcinoma model. In this system, immunization with irradiated wild type RENCA cells elicits moderate levels of tumor protection, whereas GM-CSF secreting cells effectuate increased tumor destruction. Through screening a RENCA cDNA expression library with sera from immunized mice, we found that GM-CSF secretion resulted in a marked diversification of the anti-tumor reaction compared to parental cells. GM-CSF producing tumor vaccines evoked high titer antibody responses to 26 RENCA antigens, while wild type cellular vaccines provoked humoral reactions to only 2 gene products. The targets of vaccine responses were widely expressed, non-mutated proteins, and included antigens that were expressed at higher levels in carcinoma cells compared to normal kidney. Many of the identified gene products participate in well-defined mechanisms that contribute to tumor cell metabolism, death, invasion, and metastasis. Protein disulfide isomerase (PDI), the most frequently
detected clone in the library screening, also elicited high titer antibodies in an advanced myeloid leukemia patient responding to vaccination in the setting of allogeneic bone marrow transplantation. Together, these findings indicate that the enhanced tumor immunity stimulated with GM-CSF involves a diversified antibody repertoire that targets multiple oncogenic pathways.
Introduction

Antitumor innate and adaptive immune responses can be frequently detected in cancer-bearing hosts. The identification and molecular characterization of antigens capable of eliciting an immune-mediated tumor destruction, in patients, has been a major goal in the field of cancer immunology [6]. A large repertoire of proteins evoking a concomitant humoral and cellular immune recognition has now been uncovered [6]. Even though they constitute potential targets for antigen-specific immunotherapy, the biologic role of these responses is still poorly understood.

Live tumor cells inactivated by irradiation have been extensively studied as antitumor vaccines both in murine and human models [56]. Whole tumor cells are a potent vehicle of generating anti-tumor immunity since they provide a large repertoire of unique antigens that can promote the development of a tumor specific immune response. Nonetheless, most tumor cells are considered poorly immunogenic, mainly because they express self-antigens in a non-stimulatory context. Consequently, the use of immunological adjuvants has been addressed in current immunotherapeutic approaches. One strategy used for enhancing the immune-stimulatory capacity of tumor cells, includes cytokine transfection [5, 9, 31, 32]. Previous studies from our group have shown the unique role of GM-CSF as an inflammatory cytokines involved in host defense [8]. The ability of GM-CSF to enhance antitumor immunity was first identified through an in vivo screen of a large number of immunostimulatory molecules. GM-CSF-transduced irradiated tumor vaccines were the most potent stimulators of protective immunity against live tumor challenge [10]. A coordinated humoral and cellular response contributes for the mediated tumor rejection observed in this system. This approach improves the ability of CD11b+ dendritic cells to capture and present cancer antigens to tumor-reactive CD4+ and CD8+ T cells, CD1d-restricted invariant natural killer (NK) T cells, and B cells [19]. Clinical trials
of patients with pancreatic cancer, non-small cell-lung carcinoma, prostate cancer, renal cell carcinoma and metastatic melanoma subjected to this immunization strategy have shown, for the majority of patients biopsied, extensive inflammatory infiltrate within the tumor, that in some cases was associated with tumor regression [24, 42, 43, 49, 50].

Serological identification of antigens by recombinant expression cloning (SEREX) approach has proven to be an efficient method for rapid detection of relevant tumor rejection antigens [41]. This method detects antigens specifically bound to high titers IgG immunoglobulins. Using this technique, our group has identified antigens, recognized by both B and T cells, associated with vaccine-induced tumor destruction in patients with advanced tumors [20, 48]. Moreover this correlation between increased humoral immunity and tumor destruction proved to be conserved between human and murine systems. More recently the same strategy has been used to define autoantigens associated with autoimmune diseases as well as viral-induced antibody targets [29, 30, 46].

Murine models are particularly useful to identify relevant tumor specific antigens (TA) and characterize immunological responses evoked by these antigenic targets that may result in protective anti-tumor immunity. RENCA is an immunogenic tumor cell line with potential interest since vaccination with irradiated, unmodified tumor cells can elicit measurable levels of protective immunity. Nonetheless, vaccination with irradiated, RENCA cells engineered to secrete GM-CSF generates greater levels of protective immunity. This model allows us to explore the contributions of GM-CSF to the enhanced anti-tumor immunity observed; in particular, to understand if this is due to the immune recognition of a broader panel of antigens or due to differences in the proteins targeted by the immune repertoire, evoked by this cytokine. In order to characterize these humoral responses we screened a cDNA library derived from RENCA tumor cells. Using sera from
mice immunized with GM-CSF-transduced vaccines, this work unveiled a broader repertoire of immunologic targets associated with GM-CSF protective tumor immunity, in this model. A humoral response against a larger group of antigens was found present in post immunization sera of mice vaccinated with irradiated GM-CSF-transduced RENCA cells. Moreover, reactivity to these proteins increased as a consequence of vaccination. These antigens represent a diverse group of nuclear, cytoplasmic and membrane proteins, some of which previously identified as immunological targets in different human malignancies. They are involved in key carcinogenic pathways including: Rho/Ras signaling pathway, transcriptional activation, proliferation, metastization and cell death. We further show upregulation of a group of genes, which may account for their immunogenicity observed in RENCA vaccines.

Finally, the current work allowed us to unveil a new immunologic target conserved between murine and human tumor models. High-titer IgG antibodies specific for PDI, were detected in patients immunized with GM-CSF-secreting tumor cell vaccines (GVAX). Furthermore, we show that increased reactivity to PDI developed as a result of vaccination in a patient that responded well to this immunotherapy.
Material and Methods

Murine studies Tumor Models

Adult female BALB/c mice, 8-12 weeks of age were purchased from Taconic Farms. All animal procedures were performed according to Dana-Farber Cancer Institute approved protocols and conducted under Institutional Animal Care and Use Committee guidelines. 8 Mice (8 per group) were immunized s.c. on the abdominal wall with $5 \times 10^5$ irradiated (35Gy) GM-CSF transduced or wild-type RENCA cells at weekly intervals up to 10 times. Sera used in the experiments was collected by eye bleeding and pooled from the 8 mice from each immunized group.

RENCA (Renal Cell Carcinoma), CMS5 (Fibrosarcoma) and CT-26 (colon tumor) murine cell lines (syngeneic to BALB/c mice) were cultured in vitro in DMEM containing 10% (v/v) inactivated fetal calf serum (IFS), 100 units/ml penicillin/ streptomycin, 1 mM non-essential aminoacids and 10 mM HEPES buffer (pH 7.4).

Generation of GM-CSF-secreting Tumor cells

To study the effects of GM-CSF secreting RENCA cells we used retroviral mediated gene transfer to engineer RENCA cells to secrete high levels of this cytokine. The cDNA for the murine GM-CSF was amplified by reverse transcription PCR and subcloned into pMFG.S, a replication-deficient retroviral vector (pUC19/MMLV-based) as described previously [10]. Protein coding sequences were inserted between the Nco/Xba and Bam HI sites in order to keep the position of the initiator ATG, and a minimal 3' nontranslated sequence is included in the insert. The pMFG vector uses the MMLV long terminal repeat sequences to generate both a full-length viral RNA (for encapsidation into viral particles) and a subgenomic RNA that is responsible for expression of inserted sequences. Resulting construct (pMFG-GM-CSF) was transfected into 293GPG cells to generate high-titer
stocks of concentrated recombinant virus with amphotropic range [39]. Wild-type RENCA cells were infected with these viral stocks.

**Bioactivity of GM-CSF-secreting RENCA cells**

GM-CSF-secreting RENCA cells generated approximately 300ng/10^6 cells/48h of bioactive protein, as determined by ELISA (mouse GM-CSF BD OptEIA ELISA Set) according to the manufacturer’s instructions. Briefly, ELISA plates (Corning) were overnight coated with GM-CSF specific coating antibody, at 4°C. Next day, after several washings, the wells were blocked for at least 1 h at room temperature. Standard dilutions and equal amounts of supernatant from transduced cell lines were incubated for 2 h at RT, washed, and incubated with 100 µl of detection antibody for 1 h. Substrate solution is added after final washings in the dark. Absorbance is read at 450 nm within 30 min of stop solution.

**cDNA Library Construction and Screening**

A cDNA expression library was generated and screened as described [20]. Briefly, total RNA was isolated from RENCA cell line by using guanidine isothiacyanate, and mRNA was selected with two rounds of oligo(dT) cellulose. The cDNA expression library was constructed in the Lambda Zap vector by using a commercial cDNA library kit (ZAP-cDNA Gigapack III Gold cloning kit, Stratagene) according to the manufacturer’s procedures. Immunologic screening of our RENCA cDNA expression library was done according to the manufacturer's instruction (picoBlue Immunoscreening Kit, Stratagene). To remove antibodies reactive against antigens related to the vector system or bacteria, pooled serum collected from vaccinated mice was preabsorbed four times against bacteria lysed by nonrecombinant ZAP Express phages. Plaques (1X10^6) were screened with pre-cleared postvaccination sera at a 1:300 dilution in TBS/0.1% Tween-20/2% nonfat dried milk (NFDM) and 0.01% (w/v) sodium azide. Antigen-antibody complexes were detected with
goat anti-mouse pan IgG antibody conjugated to alkaline phosphatase (Jackson ImmunoResearch laboratories).

**Phage-plate Assay**

Phages from positive clones were mixed with nonreactive phages of the cDNA library as internal negative control, at a ratio 1:10. This mix was used to transfect 200µl of XL1-Blue MRF’ bacteria. The phage and bacteria were plated onto NZY agar plates. Immunoscreening assay described above was used to detect specific binding of IgG antibody present in the pre-cleared sera to recombinant proteins expressed on the positive lytic plaques. Semi-quantitative analysis of antibody titers was assessed by antibody reactivity determined by phage-plate assay, in serial samples.

**Sequence Analysis of Positive Clones**

DNA plasmids from positive clones were isolated using commercially available kits (QIAGEN). The length of DNA inserts was determined after double Eco RI and Xho I restriction endonuclease digestion (Biolabs) and run in standard TAE agarose gel electrophoresis. After sequencing the cDNA inserts (Molecular Biology Core Facility, Dana-Farber Cancer Institute), alignments with GenBank database were performed using the National Center for Biotechnology Information (NCBI) BLASTN and BLASTX algorithms, to identify identities and homologies of genes. The Cancer Immunome Database (www2.licr.org/cancerimmunomeDB) was also analyzed for representation of human orthologs of our cloned mouse antigens.
Total RNA Isolation

Total RNA was isolated from tumor cells or normal tissues with TRizol (Gibco/BRL) according to manufacturer's recommendations. In brief, after adding Trizol Reagent for sample homogenization or lysis, an appropriate amount of chloroform was mixed. Following centrifugation, the upper aqueous phase was recovered and total RNA precipitated with isopropyl alcohol. After washing with 75% ethanol, the RNA pellet was briefly dried and subsequently dissolved in RNase free ddH₂O and stored at -80°C.

Reverse Transcriptase Reaction

Superscript II Reverse Transcriptase (RT, Invitrogen) was used for the first strand cDNA synthesis according to the manufacturer’s instructions. 1-5µg of total RNA and oligo(dT) (Roche Molecular Diagnostics) were heated up to 80°C. The contents were chilled on ice and a mix of dithiothreitol (DTT, Invitrogen), RT reaction buffer (250 mM Tris·Cl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, Invitrogen) and 10 mM deoxy nucleotide triphosphate mix (dNTP, Roche Molecular Diagnostics) were added. The tube was warmed to 42°C and the RT was added. After an incubation of 1 hour, the enzyme was deactivated by heating to 95°C. Rnase H was added for 20 minutes at 70°C to remove the RNA complementary to the cDNA.

Northern Blotting

10 µg total RNA was mixed with the appropriate volume of RNA sample loading buffer containing ethidium bromide (R4268, Sigma) and incubated at 65°C for 10 minutes. Samples and a size marker (Millenium Marker, Ambion) were loaded into an agarose formaldehyde gel and electrophoresed in 1x MOPS running buffer. After overnight transfer, positively charged nylon membrane (Hybond-XL, Amersham Biosciences) RNA was covalently bound to the membrane by UV-crosslinking (UV Stratalinker 2400,
Stratagene). For the probes preparation, 25 ng of template DNA ranging from 500 to 1500 nucleotides was labeled with (NEN/Perkin Elmer Life Sciences) according to the manufacturer's instructions (Prime-It II Random Primer Labeling Kit, Stratagene). Hybridizations were conducted with 0.5-1.5 kb $^{32}$P-labeled probes, at 68°C, for 1 h in the appropriate amount of hybridization solution (ExpressHyb, Clontech). This probe solution was added to the pre-hybridized membrane and incubated for one hour to overnight. Autoradiography was performed by exposing the membrane to film (Kodak X-OMAT-AR) and an intensifying screen at -80°C for 1-5 days. Thereafter, the filters were stripped and rehybridized with 18S ribosomal RNA or GAPDH (Glycerol 3-phosphate dehydrogenase) as a loading control.

**Immunoblotting (Western)**

Whole cell lysates from normal tumor cell lines and normal tissue were prepared in a lysis buffer containing the detergent NP-40 and protease inhibitors. Samples were then centrifuged and the supernatant stored at -80°C, after protein concentration was determined with a BioRad protein assay. Lysate were subjected to protein gel electrophoresis using 10-12% SDS-PAGE with Tris-glycine buffer and transferred onto a polyvinylidene fluoride membrane (PVDF) membrane (Millipore) with a wet transfer system (BioRad) according to the manufacturer’s instructions. Membrane was blocked with 5% (w/v) NFDM/ PBS overnight at 4°C, or 2 hours at room temperature. A goat anti-SSRP1 antibody (Santa Cruz, Inc.) was diluted in 5% (w/v) NFDM/TTBS and incubated at room temperature for 1 hour. Membrane was incubated at room temperature for 1 hour with the secondary HRP-labeled antibody, diluted in 5% (w/v) NFDM/ TTBS. After several washes with TTBS the substrate (Westen Lightening kit NEN/Perkin Elmer) was
added and the membrane was exposed (X-Omat Blue, Kodak). Antibody to β-actin (Sigma, St Louis, MO) was used as a control to ensure equal loading of lanes.

**FACS Analysis**

RENCA cells ($5 \times 10^5$) were incubated with mouse serum (1:100 dilution), obtained from a pool of 8 mice immunized with R-GM vaccines or mouse IgG isotype control, for 3 hours at room temperature. Cell were washed twice and resuspended in 2% IFS (inactivated fetal calf serum)/PBS. Fluorescent staining of RENCA cells with sera was performed by using a secondary (phycoerythrin) PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). Stained cells were analyzed on a FACScan cytometer (Becton Dickinson).

**ELISA**

Elisa plates (nunc) were coated overnight at 4°C with 1-5µg/ml of 6x Histidine-tagged full-length recombinant human PDI protein (hist-PDI) (ProSpec-TechnoGene) in a carbonate buffer, pH 9.6. The same molarity of a 6x histidine peptide (New England peptide) was also used as a control. All wells were blocked overnight with 2%NFDM/PBS at 4°C, washed and incubated in triplicate with 100µl of patient sera or a mouse protein disulfide isomerase (PDI) antibody (BD Biosciences, San Jose, CA) as a positive control, diluted 1:100 in 2% NFDM/PBS, overnight at 4°C. A goat anti-human IgG conjugated alkaline-phospatase (Jackson) was added at room temperature and the plate was developed with pNPP substrate (Sigma). In this assay, antibody reactivity with purified hist-PDI was subtracted from antibody reactivity to histidine peptide. Another human recombinant histidine-tagged protein, Visfatin (hist-Visfatin) (Alexis Biochemicals) was also tested as a control.
Clinical Samples

Sera were obtained from vaccinated melanoma, non-small-cell lung carcinoma, and leukemia patients and healthy donors, enrolled on IRB approved Dana-Farber Cancer Institute clinical protocols. The details of these studies design and methods of vaccine production have been presented previously [11, 42].

Patient B was enrolled in a GVAX leukemia cell vaccination trial after non-myeloablative transplantation (NST), for advanced myeloid malignancies. Briefly, leukemia cells are harvested prior to transplant admission. Vaccines are generated by adenoviral GM-CSF transductin (Ad-GM-CSF), irradiated and cryopreserved. Patients are administered 6 vaccines each ($10^6$ to $10^7$ cells per vaccine). The subject was in remission at 5 months post transplant but was not in remission at 1 month post transplant, when vaccinations started. The subject remains in remission 8 months post transplant.
Results

Humoral response induced by vaccination with GM-CSF-secreting RENCA cells

We previously showed that irradiated RENCA cells engineered to secret GM-CSF (R-GM) are more efficient than wild-type (R-WT) cells alone in inducing tumor protection against live tumor cells [10]. To assess if this immunogenicity was associated with the induction of a humoral response, sera were collected from non-immunized mice (Pre) or mice vaccinated, at weekly intervals, with irradiated R-GM (Post) cells and their recognition of cell surface markers analysed and compared by flow cytometry. A secondary anti-mouse IgG antibody was used to determine antibody titers recognizing surface proteins on RENCA cells. Sera from naïve mice and control isotype antibody showed no reactivity (Fig. 1A and 1B, respectively), while minimal staining was observed with sera collected after one or two vaccinations (data not shown). In contrast, sera obtained after ten vaccinations strongly recognized RENCA cells (Fig.1C).

RENCA cDNA library construction and immunoscreening

In order to identify the targets of antibody responses stimulated by GM-CSF secreting RENCA cells we used a serology-based expression cloning strategy (SEREX). A cDNA expression library derived from RENCA cells was established and used for the immunologic screening. Pooled sera from mice vaccinated 10 times with irradiated, GM-CSF secreting cells or irradiated wild type RENCA cells was used at 1:300 dilutions to screen the library. Positive plaques were isolated by the reactivity of the recombinant proteins with high-titer IgG antibodies present in the sera of immunized mice. Positive plaques were re-plated for a secondary and tertiary screening until clonality was reached. As shown in Table I, library screening with sera from from GM-CSF secreting cells
resulted in the identification of 177 clones versus 3 clones with sera from wild-type RENCA vaccines, suggesting that GM-CSF stimulates an intensified antibody response.

**Serologic differences induced by GM-CSF-transduced RENCA vaccines**

To identify the humoral targets detected with sera from vaccinated mice, cDNA inserts from positive clones were isolated and their DNA sequence aligned against the GeneBank database (Table I). Out of 180 immunoreactive clones, sequence analysis and homology search revealed that they represent a total of 28 unique antigens. 21 of the gene products identified during our serologic analysis corresponding to proteins with known function (Table II). Database searches show that these gene products are involved in a wide range of cellular functions including transcription, translation, proliferation, migration, and stress response. We grouped these serologically defined proteins according to their role in the cell, and to the major signaling pathways they are associated with. These classifications include DNA/RNA binding proteins, proteins involved in cell metabolism, cytokines, proteins associated with the Ras/Rho signaling pathway, stress-inducible gene products, and cell-death associated proteins. Database search indicates that the majority of these proteins were intracellular. Nevertheless, membrane and secreted proteins were also detected.

**Antibody response against RENCA-associated antigens is a result of vaccination and increases with the number of immunizations**

A comparison of serum reactivity confirmed that all isolated clones from the library are recognized by GM-CSF secreting vaccines (including the clones initially isolated by R-WT sera). In contrast, only 2 out of these 28 clones are positive when detected by R-WT
sera (Table I). These results show that GM-CSF-transduced RENCA cells induce a quantitatively different serological response when compared with wild-type tumor cells.

Once positive clones were plaque purified, a phage plate assay was undertaken to determine whether these antigenic targets were specifically induced by vaccination. Even though this is not a quantitative method, the phage plate assay allows a simple and rapid-quantification of antibody responses and differences in the intensity can be clearly observed (Fig.2). Comparison of reactivity against a panel of isolated clones was performed using sera collected from naïve mice (Pre) and sera from vaccinated mice used for the initial library screening (Post).

Seroreactivity of the purified clones was assessed semi-quantitatively by comparing the signal obtained with Pre and Post vaccination sera from GM-CSF secreting cells. As summarized in Table III, antibody reactivity to each of the isolated gene products was present after immunization. In contrast, no reactivity was observed using sera from non-vaccinated mice (Pre).

The phage plate assay allows a simple and rapid-quantification of antibody responses. Using this approach, we determined if the number of immunizations could induce differences in the antibody repertoire. Using this approach, we determined if the number of immunizations could induce differences in the antibody repertoire against a panel of isolated clones. To assess this, sera pooled from naïve or vaccinated mice were collected after 1, 2, 3 or 10 inoculations (W1, W2, W3 and W10, respectively). The intensity of antibody response, against the same target antigens, was compared in a phage plate assay at 1:300 dilution. Upon incubation with replica-plated phages, no reactivity was observed with sera from naïve mice or sera from early time points, W1 and W2 (Table III). Evidence of antibody reactivity could only be detected after the third immunization. In addition, the strongest antibody response to this panel of antigens was observed with the
latest time point, collected after 10 vaccinations (W10). These observations show that increasing the number of immunizations stimulates a more potent antibody response.

Upregulation of RENCA antigens in tumor cells as a potential mechanism of immunogenicity

In tumor cells, one mechanism that can result in the generation of antigenic epitopes recognized by the immune system relates to mutations. Several examples, including mutated ras oncprotein and the p53 tumor suppressor protein have been shown in the literature [1, 13]. However, we did not find any mutations in any of the genes isolated by library screening when their nucleotide sequence was compared against NCBI database. Nevertheless, previous studies have shown that immunogenicity of non-mutated cancer antigens might be related to increased expression in tumor cells (gp100 and Mart1 in melanoma or PSA in prostate cancer). Thus, we hypothesized that the same mechanism could apply to the identified RENCA antigens. In order to address this question, we examined mRNA levels of a panel of identified genes, to evaluate whether their overexpression in RENCA tumor cells when compared to normal kidney could be responsible for the observed immunogenicity. A series of Northern blots were performed, and cDNAs from the corresponding clones were used as a probe in hybridization experiments against total RNA obtained from a variety of tumor cell lines and normal tissues.

As shown in Figure 3, proteins involved in the the Ras/Rho signaling pathway, including ROCK2, FDS, GNB2, IQGAP1 and CD44 show increased transcript levels in RENCA cells. In contrast, absent or low mRNA transcript levels were found in the kidney as well as in other normal tissues tested, including spleen and liver; the only exception being high levels of FDS in the liver, which can be explained by the essential role of this
enzyme in cholesterol synthesis in this organ. Interestingly, we also found overexpression of these genes in the B16 murine melanoma cell line. A similar pattern of upregulation in tumor cell lines was observed for two transcription activators: SSRP1 and TCEA1, when compared with normal tissues (Fig.3). Furthermore, we were able to confirm overexpression of SSRP1 protein by Western blot analysis. Figure 4 shows high expression levels in RENCA cells but, on the contrary, it is low or undetected in kidney. Increased protein levels of SSRP1 were also detected in two other tumor cell lines B16 and CT-26 (colon carcinoma), but not CMS5 (fibrosarcoma).

A third mechanism that may be associated with tumor protein immunogenicity is alternative splicing. CD44 is encoded by a single gene, but multiple forms can be generated by alternative RNA splicing [53]. Some of these isoforms have been associated with tumor progression [57]. Accordingly, Northern blot analysis of CD44 reveals multiple bands in B16 and RENCA tumor cell lines with different molecular weights, potentially corresponding to multiple isoforms that are weakly expressed or not present in normal tissue.

Overall, these data show that overexpression of genes involved in two key carcinogenic pathways-Rho/Ras signaling pathway and transcriptional activation - may account for their immunogenicity observed in RENCA vaccines. Moreover, these findings raise the possibility that alternative spliced variants, detected in these tumor cells, might be another possible mechanism of immunogenicity associated with some of these gene products.

**High-titer antibody response against PDI is present in advanced tumor patients**

Several of the murine tumor antigens that we pulled out from our library have been previously identified in patients with other tumors and have their human orthologs
represented in the SEREX database (www.licr.org/serex). Moreover our own group has shown that the correlation between increased humoral immunity to ATP6S1 and tumor destruction identified in a long-term responding patient was conserved between human and murine systems [20]. Thus, we hypothesized that a similar relationship could be observed to vaccine targets found in our murine model.

Even though a broad repertoire of RENCA antigens have been recognized in several tumor models we decided to focus our attention on PDI given that this gene product was frequently detected in our immunoscreening. To test this idea, we established an ELISA with bacterially produced recombinant human histidine-tagged PDI (Hist-PDI), and analyzed sera from a variety of advanced cancer patients including 19 metastatic melanoma, 7 metastatic NSCLC patients, 6 myeloid leukemia patients, and 30 healthy blood-bank donors as controls. Two patients analysed, M18 (melanoma) and B (AML), harbored strong IgG antibody response against PDI in contrast to none of the controls (Fig. 5). Western blot analysis confirmed patient B reactivity (Fig. 6). Subject M18 developed anti-PDI antibodies in the absence of immunotherapy, indicating that this antigen can be a target of endogenous anti-tumor immunity. However, patient B was enrolled in a trial of vaccination with irradiated, autologous myeloid leukemia cells engineered to secret GM-CSF following non-myeloablative allogeneic bone marrow transplantation. To define the impact of immunotherapy on antibodies to PDI, we performed a longitudinal analysis (Fig. 7). While antibodies were not detected prior to transplantation or early during vaccination, potent humoral reactions were detected in association with the delayed induction of a complete clinical and hematologic response. Interestingly, this was temporarily related to a reduction in immune suppression (administered for prevention of graft-versus-host disease), raising the possibility that vaccination early post-transplantation might enhance anti-leukemia immunity.
Discussion

The current studies were undertaken to better understand the role of GM-CSF in enhanced tumor protection. Through an immunoscreening of a RENCA cDNA expression library we identified high titer antibodies against 26 tumor antigens in GM-CSF producing tumor vaccines whereas wild-type cellular vaccines induced a humoral response to only 2 gene products. In addition, we demonstrate that immune response against this panel of immunogenic antigens is induced upon vaccination, with the number of detectable antibodies increasing with the number of immunizations. In contrast none of these proteins is recognized with serum from naïve mice. Overall, these findings suggest that GM-CSF transduced tumor cells evoke a broader immune recognition contributing for the enhanced antitumor effect previously observed in vivo, in this model [10].

The SEREX analysis of human and murine tumors has identified a large repertoire of tumor antigens that elicit humoral immune responses in tumor-bearing hosts [35, 41]. Even though this technique was introduced to identify tumor specific products, so far, most of the serologically defined antigens identified are not restricted to tumor, but are broadly expressed, non-mutated self-antigens [45]. In our model, characterization of the antibody repertoire evoked by GM-CSF producing vaccines led to the identification of a large panel of self, nonmutated RENCA associated tumor antigens. With few exceptions intracellular proteins with ubiquitous expression and wide tissue distribution were clearly the dominant autoantigens. Interestingly, this tumor antigenic repertoire shares common elements with autoantigens found in patients with autoimmune diseases (SSRP1, Histone 1, HnRNP), as well as antigenic targets of virus-induced autoantibody responses (ROCK2) [13, 14, 18, 30, 34]. Even though the basis for this self-protein immunogenicity is not completely understood, these data support the notion that different kind of events (e.g.
viral infection, transformation) can trigger the immune system to respond to previously ignored antigens.

Immunogenicity of tumor specific antigens can be associated with genetic mutations or polymorphisms. These can, by affecting antigen processing (immunogenic neoepitopes), or improving peptide binding to MHC, induce an immune response associated with T cell recognition and antibody secretion [22, 28]. To investigate whether this was the case for any of the antigens found in our library screening, DNA sequence of the immunoreactive clones was compared with the GeneBank database. No mutations were found, suggesting that genetic alterations do not contribute to the high titer antibody response elicited by these proteins.

Another mechanism underlying the immunogenicity of most non-mutated, self antigens is their overexpression in tumors. One group of antigens isolated in our immunoscreening, with particular immunologic relevance, includes ROCK2, FDS, GNB2, IQGAP1, CD44 and ARF4. These proteins, involved in the Ras/Rho signaling pathway, are key regulators associated with actin reorganization, cell-motility, cell-cell and cell-extra-cellular matrix adhesion, as well as proliferation, apoptosis and metastization [4, 23, 26, 36]. Such pathway plays a pivotal role in the regulation of numerous cellular functions associated with malignant transformation. In addition to their function, aberrant expression as well as mutations of some of these gene products in tumor cells has also been associated with cancer progression [37, 52].

To further explore the mechanism of immunogenicity of these tumor associated antigens, we performed a series of studies to define tissue expression. Using Northern blot analysis we found that several of the identified antigens including ROCK2, TFIIS, FDS, SSRP1, CD44, IQGAP1 and GNB2 were upregulated in RENCA tumor cells (as well as in B16, a tumorigenic murine melanoma cell line) when compared to kidney tissue. For
SSRP1, we were also able to confirm, by western blot analysis, protein overexpression in these tumor cell lines.

DNA amplification is frequent in tumors and may result in immunogenic antigens by increasing protein levels without any additional DNA mutations. This has been suggested as a mechanism by which self-proteins are recognized by the immune system. Even though we can not rule out that additional posttranslational modifications may take place when these proteins are expressed in tumor cells, these results imply that overexpression may be associated with their immunogenic potential.

Farnesyl diphosphate synthase (FDS) catalyzes the formation of farnesyl diphosphate, a key intermediate in the mevalonate pathway responsible for the synthesis of cholesterol and isoprenoids. These metabolites are involved in the posttranslational modifications essential for the proper function of many regulatory proteins including Ras and Rho GTPases [40]. Alterations in the mevalonate pathway are known to be associated with malignant cell growth [15].

ROCK II or protein serine/threonine kinase is a downstream effector of Rho, a GTPase of the Ras superfamily [21, 55]. Elevated expression of RhoA and RhoC, as well as that of the Rho effector proteins ROCK I and ROCK II, is also commonly observed in human cancers and are often associated with a more invasive and metastatic phenotype [2, 7, 27, 45].

GNB2 is a subunit of heterotrimeric G-proteins that function as downstream effectors of G-protein coupled receptors (GPCR) on the surface. Recent studies have indicated that activation of these proteins can lead to the oncogenic transformation of different cell types. In addition, their aberrant expression has been associated with tumor proliferation. IQGAP1 and CD44 members of the Rho-signaling pathway are key players in mediating cell-cell adhesion and tumor cell migration. IQGAP1, a downstream effector
of two Rho GTPases, Rac1 and Cdc42, function as an inhibitor of cadherin-mediated cell
adhension. IQGAP1 has a fundamental role in cell motility and invasion [33].

Another gene product identified in our screen shown to be overexpressed in
tumor cells was SSRP1, a protein belonging to the HMG family. SSRP1 functions as a co-
regulator for transcription, and this regulation is executed by interacting with other
transcriptional activators such as SRF Drosophila GATA factor, and p63 [51]. It can also
heterodimerize with Spt16 to form FACT, a complex initially shown to facilitate
chromatin transcription [38]. Serum response factor (SRF) is a transcription factor that
controls a wide range of genes involved in cell proliferation and differentiation. Interaction
of SSRP1 with SRF dramatically increases the DNA binding activity of SRF, resulting in
synergistic transcriptional activation of native and artificial SRF-dependent promoters.
Antibodies against this protein have been reported in a small series of systemic lupus
erthematosus (SLE) patients [14, 44].

Interestingly, one antigen that we pooled out from the library was detected
repeatedly among the isolated clones. Nevertheless, no upregulation was found for this
protein (data not shown), which might suggest a high level of representation in the cDNA
expression library. About 86% percent of the immunoreactive antigens, initially isolated
from the cDNA library using R-GM sera, corresponded to Protein Disulfide Isomerase
(PDI). PDI is a member of a large family of enzymes involved in the quality control
system for the correct folding and disulfide bonding of nascent proteins in the ER [12].
PDI has also been found present both in the cytosol and on the cell surface where is
postulated that works as a reductase, cleaving the disulfide bonds of other surface proteins
and may also control their function by regulating their redox state [3, 25]. Additionally,
this protein is expressed on the surface of cancer cells; particularly was shown to be
present in the cell membrane of B cells from B-CLL patients and involved in the
Huang et al.'s report reveals for the first time the amplification of ErbB2/PI3K signaling by a disulfide isomerase leading, subsequently, to the activation of RhoA and β-catenin, in a model of tumor cell migration and invasion. Interestingly, in our own system we uncovered a panel of gene products, specifically CD44, IQGAP1, GNB2, FDS and ROCK, which are directly and/or indirectly involved in these oncogenic pathways. Overall, these data suggest that GM-CSF vaccines may target specific gene products participating in well-defined carcinogenic and cancer cell death pathways.

Previously our group has uncovered conserved tumor immunogenicity from humans to mice [20, 47]. Analysis of the immune response of long-term responding melanoma patients vaccinated with autologous, GM-CSF secreting tumor cells, led to the identification of two tumor antigens: ATP6S1 and MLIAP. Furthermore, the same correlation, between increased humoral immunity to ATPS1 and immune-mediated tumor destruction, proved to be conserved in the B16 melanoma murine model. Thus, we hypothesized that the same correlation could apply from our murine model to cancer patients.

Here we analyzed immune responses specific against PDI protein in a panel of advanced tumor patients. Our ELISA data shows strong reactivity from 2 of the patients tested, In contrast no or minimal humoral response response was found in control healthy donors tested. These observations were confirmed by western blot, where sera from both patients recognized the full length recombinant hist-PDI protein indicating that antibody titers can be elicited in some of these patients.

One of the patients eliciting a specific antibody response against PDI was enrolled in an AML-GVAX study and showed complete remission after treatment. Remarkably,
longitudinal analyses of this patient, suggests that increased antibody titers developed as a result of immunizations and coincide with the end of the immunosuppressive treatment and the beginning of a positive clinical response. These results suggest PDI might be a common immunogenic target of GM-CSF-secreting vaccines both in murine and human tumor models.

In summary, we uncovered a potent humoral response to a multitude of tumor antigens targeting multiple oncogenic pathways, associated with GM-CSF-induced tumor protection in RENCA. We found a panel of these proteins to be overexpressed in tumors suggesting a possible mechanism of immunogenicity. Moreover, one of these humoral targets- PDI- revealed conserved immunogenicity with sera from patients who showed a positive clinical response after GM-CSF immunotherapy. Our study thus, raises the question that the same antigenic target associated with an effective antitumor immunity can be recognized by humoral responses elicited by GM-CSF-secreting vaccines, both in mice and humans. Nevertheless, the role of these antibodies in tumor destruction remains to be clarified. This conserved immunogenicity between human and murine tumor models underlie the potential use of these antigens in the clinic for immunotherapy of different tumor malignancies.
Bibliography

42. Salgia, R., et al., *Vaccination with irradiated autologous tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor augments antitumor immunity*
Acknowledgments

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Table I: Clones identified by serologic screening of a RENCA cDNA library

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<tr>
<th></th>
<th>Sera*</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>RENCA-WT&lt;sup&gt;i&lt;/sup&gt;</td>
<td>RENCA-GM&lt;sup&gt;ii&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Positive clones</td>
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<td></td>
</tr>
<tr>
<td>Unique Antigens</td>
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<td>20</td>
<td></td>
</tr>
<tr>
<td>known function</td>
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<td>Gene Products with</td>
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</tr>
<tr>
<td>unknown function</td>
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</table>

Note: Four clones with homology with mitochondrial DNA were not included.

* Precleared sera diluted 1:300 was obtained as a pool from mice vaccinated 10 times, one week apart.

i) serum from mice vaccinated with 5X10<sup>5</sup> irradiated wild-type RENCA cells.

ii) serum from mice vaccinated with 5X10<sup>5</sup> irradiated, GM-CSF-secreting RENCA cells.
**Table II: Functional characterization of RENCA gene products identified by serologic screening**

<table>
<thead>
<tr>
<th>Function</th>
<th>Abbreviation</th>
<th>Gene products Identity/Homology</th>
<th>Serum*</th>
<th>Localization</th>
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<td>Translation initiation factor 4</td>
<td>GM</td>
<td>Intracellular</td>
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<tr>
<td></td>
<td>RPL15</td>
<td>Ribosomal protein L15</td>
<td>GM</td>
<td>Intracellular</td>
</tr>
<tr>
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<td>PDI/Erp59/Ph4b</td>
<td>Protein disulfide isomerase</td>
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</tr>
<tr>
<td></td>
<td>PSMB5</td>
<td>Proteosome subunit, beta 5</td>
<td>GM</td>
<td>Membrane Secreted</td>
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<td><strong>DNA/RNA binding</strong></td>
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<tr>
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<td></td>
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<td></td>
<td>SSRP1</td>
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<tr>
<td></td>
<td>FDS</td>
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<td>GM</td>
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<tr>
<td></td>
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<td>GM</td>
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<tr>
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<tr>
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<td>PBCEF/Visfatin</td>
<td>Pre-B colony enhancing factor</td>
<td>GM</td>
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</table>

* Serum obtained from mice vaccinated with irradiated wild type RENCA cells (WT) or GM-CSF secreting cells (GM), diluted 1:300.
Table III: Antibody repertoire increases with the number of vaccinations.

<table>
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<tr>
<th>Clone Name</th>
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</table>

Time course of antibody reactivity was determined by phage-plate assay. Clones isolated from RENCA cDNA library with sera from 10 weeks vaccinated mice (Post) show no reactivity with preimmune (Pre) sera from syngeneic naïve mice. Quantification was based on the intensity of reactivity of positive plaques. Reactivity: (-) negative, (+) weak, (++) moderate, (+++) strong. Sera were collected after different number of immunizations and quantification was based on the intensity of reactivity of the positive plaques. W1, W2, W3, W10 - pooled sera from each group collected after one, two, three or 10 vaccinations with irradiated, 5X10^5 GM secreting RENCA cells, respectively.
Figure 1: Humoral response to cell surface antigens induced by vaccination with irradiated GM-CSF secreting RENCA cells. Flow cytometry analysis of RENCA cells after treatment with A) isotype control antibody or pooled sera diluted 1/100 from B) naïve (Pre) or C) mice vaccinated 10 times with R-GM (Post), were tested against cell surface antigens using a secondary PE-labelled goat anti-mouse IgG antibody.
Figure 2: Semi-quantitative analysis of seroreactivity using phage plate assay. Assessment of antibody reactivity determined by phage-plate assay in serial samples ranging from negative (-) to weak (+), moderate (++) or strong intensity (+++). A mix of isolated positive and control negative clones was plated and used to compare IgG antibody titers.
Figure 3: Northern blot analysis of ROCK2, FPPS, GNB2, CD44, IQGAP1, SSRP1 and TFIIIS. mRNA expression of RENCA antigens was analyzed using different murine tumor cell lines (RENCA, B16, CT-26 and CMS5) and normal tissue (mouse kidney, liver, spleen). Membranes were hybridized with cDNA probes (indicated on the left). Multiple splice variants are observed when CD44 cDNA is used as a probe. Loading controls for each lane on the same blot were revealed by hybridization with 18S ribosomal probe.
Figure 4: Western blot analysis of SSRP1 shows increased expression in tumor cell lines. Expression of SSRP1 mouse protein was assessed in whole cell lysates from different mouse tumor cell lines (B16, CT-26, CMS5 and RENCA) and kidney. SSRP1 protein was detected by Western blotting with anti-SSRP1 goat polyclonal antibody.
Figure 5: PDI is immunorecognized by tumor patients. Sera samples from healthy donors (n=30) and advanced tumor patients with metastatic melanoma (n=19), non-small cell lung carcinoma (NSCLC) (n=7), and advanced myeloid leukemia (n=6), were tested for reactivity to recombinant PDI (1 μg) by ELISA, at a dilution of 1:100.
Figure 6: Analysis of PDI immunoreactivity in a GVAX treated tumor patient. Immunoblotting against hist-PDI (P) or hist-Visfatin (V) with postvaccination sera (1:500 dilution) and an anti-human IgG secondary antibody. Hist-PDI detection was confirmed with patient B sera. No reactivity was observed with a normal donor sera tested.
Figure 7: Vaccination induces PDI-specific humoral immune response. IgG antibody titers against recombinant hist-PDI (5µg) were determined in serial serum samples (1:100 dilution) from patient B, who was in complete remission after GVAX treatment (♦). Downward arrows indicate AML-GVAX immunizations after non-myeloablative stem cell transplantation (NSCT) (upward arrow). This patient was in remission at 5 months post transplant (dashed double arrow) but was not in remission at 1 month post transplant. She remains in remission as of the last marrow biopsy which was done 8 months post transplant. Reactivity against recombinant human Visfatin, an histidine-tagged protein, was run as control (▪).