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UNIVERSIDADE DO PORTO

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**VACCINE TARGETS IN A MURINE MODEL OF RENAL  
CELL CARCINOMA**

Cátia Isabel Correia dos Reis Fonseca

Dissertação de doutoramento em Ciências Biomédicas

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Dissertação de doutoramento em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Portugal

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*One doesn't discover new lands without consenting to lose sight of the shore for a very long time*

André Gide

*Valeu a pena? Tudo vale a pena  
Se a alma não é pequena.  
Quem quer passar além do Bojador  
Tem que passar além da dor.  
Deus ao mar o perigo e o abismo deu,  
Mas nele é que espelhou o céu*

Fernando Pessoa

*To my Grandmother  
To my Parents, To my Sister  
To Matilde*

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Thanks Dad, for teaching me always to believe.  
To believe that is always possible

*It is never too late to be what you might have been*

-- George Eliot

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## ABSTRACT

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Identification of antigens associated with an effective immune response leading to tumor destruction is a major goal in cancer immunology. GM-CSF proved to be a potent immunostimulatory cytokine following gene transfer into tumor cells. Vaccination with irradiated tumor cells engineered to secrete GM-CSF elicits a potent, specific and long-lasting immunity in multiple murine tumor models, including renal cell carcinoma (RENCA). This vaccination strategy enhances host response through improved tumor antigen presentation by dendritic cells and macrophage. Consistent with the murine findings, clinical testing of this immunization approach also revealed induction of cellular and humoral antitumor responses associated with an extensive necrosis of distant metastasis and targeted destruction of the tumor vasculature.

This study led to the serologic discovery of a large spectrum of broadly expressed self-antigens associated with tumor rejection in RENCA tumor model. Immunoscreening of a tumor-derived cDNA library with sera from mice vaccinated with irradiated wild-type or GM-CSF transduced RENCA cells revealed high-titer IgG antibodies against several proteins involved in carcinogenesis. We demonstrate that antibodies against these antigens are induced upon vaccination, with antibody repertoire increasing with the number of immunizations. In contrast, these proteins are not recognized with serum from naïve mice. Furthermore, enhanced tumor rejection *in vivo* by GM-CSF vaccines proved to be associated with induction of a more diverse antibody repertoire. Our expression studies also showed that some of these RENCA-associated antigens are specifically upregulated in tumor cell lines. Interestingly, database analysis revealed that these serologic-defined proteins are common humoral targets found in other human tumor models as well as autoimmune diseases and viral infections.

In order to assess the role of these proteins as potential tumor-rejection antigens, we next tested different vaccine strategies. These approaches, including naked DNA vaccines, RNA-transfected DCs and gene-modified tumor cells, were not able to induce tumor rejection against live RENCA cells, *in vivo*. Our preliminary results indicate that regulatory T cells, able to inhibit RENCA-specific effector T-cells, can be induced upon vaccination with these serologic-defined antigens, suggesting that immunoregulatory pathways involved with self-tolerance may be responsible for tumor evasion and progression.

This work unveiled new immune targets associated with protective tumor immunity. A better understanding of the molecular mechanisms by which these proteins can trigger

different immunologic responses will be essential to construct better tumor vaccines in the future.

Um dos maiores desafios na área da imunologia tumoral é a identificação de antígenos associados a uma resposta imune eficaz, que culmine na destruição dos tumores. O GM-CSF é uma potente citocina estimuladora do sistema imunitário, após transfecção em células tumorais. A vacinação com células tumorais irradiadas, modificadas para secretarem GM-CSF, induz uma potente, específica e longa imunidade em múltiplos modelos tumorais de ratinho. Esta estratégia de vacinação melhora a resposta imune através do aumento da apresentação de antígenos por células dendríticas e macrófagos. De acordo com os resultados obtidos em ratinhos, incluindo em carcinoma renal (RENCA), os testes clínicos desta estratégia de imunização revelaram também a indução de uma resposta humoral e celular anti-tumoral associada à necrose de metástases e a uma destruição específica da vasculatura do tumor.

Neste estudo, foi possível a descoberta serológica de um largo espectro de auto-antígenos associados a rejeição tumoral no modelo de RENCA. O rastreio de uma biblioteca de cDNA derivada desta células, feito com soro de ratinhos vacinados com células irradiadas não transfectadas ou transfectadas com GM-CSF, revelou a presença de títulos elevados de anticorpos IgG contra muitas proteínas envolvidas em processos carcinogênicos. Demonstrou-se ainda que, após a vacinação, são induzidos anticorpos contra estes antígenos, e que o repertório de anticorpos aumenta com o número de imunizações. Em contraste, estas proteínas não são reconhecidas pelo soro de ratinhos não imunizados. Além disso, o elevado nível de rejeição tumoral observado com vacinas de GM-CSF parece estar associado à indução de um repertório mais diverso de anticorpos. Os estudos de expressão revelaram que estes antígenos associados a RENCA são especificamente mais elevados em linhas celulares tumorais. A análise da base de dados revelou também que proteínas identificadas por serologia são alvo comum de outras respostas imunes, tais como as encontradas em modelos tumorais humanos, ou em doenças auto-imunes e infecções virais.

Para avaliar o papel destas proteínas como potenciais antígenos de rejeição tumoral, foram testadas múltiplas estratégias de vacinação. Estas estratégias, incluindo vacinas de DNA, células dendríticas transfectadas com ARN e células tumorais modificadas, não foram suficientes para induzir a rejeição de células tumorais RENCA, *in vivo*. Os resultados preliminares indicam que células T reguladoras capazes de inibir células T efectoras, específicas para RENCA, podem ser induzidas após vacinação com estes antígenos. Em conjunto, estes resultados sugerem que as vias imuno-reguladoras

envolvidas em auto-tolerância podem ser responsáveis pela evasão e progressão tumoral.

Este trabalho levou à descoberta de novas proteínas associadas à indução de uma resposta imune de rejeição tumoral. O conhecimento mais detalhado dos mecanismos moleculares a partir dos quais esta proteínas podem induzir diferentes respostas imunológicas é essencial para a construção de melhores vacinas anti-tumorais.

L'identification des antigènes générant une réponse immune efficace menant à l'élimination des tumeurs est un objectif majeur de l'immunologie anti-tumorale. Le GM-CSF est un immunostimulateur efficace après transfection du gène dans des cellules tumorales. La vaccination par des cellules tumorales irradiées conditionnées pour produire du GM-CSF génère une immunité efficace, spécifique, et durable dans de multiples modèles de tumeurs chez la souris, incluant le carcinome rénal (RENCA). Cette stratégie vaccinale augmente la réponse de l'hôte via une meilleure présentation de l'antigène tumorale par les cellules dendritiques et les macrophages. Conformément aux travaux menés chez la souris, les études cliniques utilisant cette approche vaccinale ont également révélé l'induction d'une réponse anti-tumorale cellulaire et humorale, associée à une nécrose importante des métastases distantes ainsi qu'à une destruction ciblée de la vascularisation tumorale.

Dans cette étude, nous avons trouvé dans le sérum des titres élevés d'IgG spécifiques des antigènes RENCA après vaccination. Ces titres, déterminés par cytométrie en flux, supportent l'hypothèse d'une réponse humorale contre les antigènes tumoraux induite après vaccination. De manière à étudier plus précisément la spécificité des anticorps, nous avons généré une banque d'expression de cDNA à partir de cellules tumorales RENCA. Cette banque a été criblée pour identifier des antigènes en utilisant des sérums de souris immunisées avec des cellules irradiées, des cellules non modifiées, ou des cellules irradiées sécrétrices de GM-CSF. La comparaison de la réactivité des sérums a également montré l'induction d'un répertoire plus varié associé à l'augmentation du rejet des tumeurs *in vivo* avec les vaccins utilisant le GM-CSF. De plus, nous avons démontré que les anticorps dirigés contre ce panel d'antigènes sont induits après vaccination, avec un répertoire d'anticorps augmentant avec le nombre d'immunisations. A l'opposé, ces protéines ne sont pas reconnus par le sérum de souris naïves.

L'étude des bases de données montre que nos travaux ont amené à la mise en évidence d'un large spectre d'auto-antigènes, largement exprimés, ayant des rôles clé dans les processus de carcinogénèse. De manière remarquable, bien que ces antigènes soient majoritairement des protéines non mutées, intracellulaires, la plupart sont similaires aux auto-antigènes associés au cancer également trouvés dans les maladies auto-immunes ou les infections virales. De plus, nos analyses d'expression de ces protéines montre qu'un groupe particulier d'antigènes associés au RENCA est

spécifiquement surexprimé dans les lignées cellulaires tumorales, ce qui pourrait expliquer leur immunogénicité.

De façon à déterminer le potentiel de ces protéines en tant qu'antigènes associés au rejet des tumeurs, nous avons finalement testé différentes stratégies vaccinales. Ces approches, incluant des vaccins à ADN nu, cellules dendritiques transfectées par de l'ARN ainsi que des cellules tumorales transgéniques, n'ont pas permis d'induire un rejet tumorale des cellules RENCA vivantes *in vivo*. Nos résultats préliminaires indiquent que les cellules T régulatrices, capables d'inhiber les cellules T effectrices spécifiques des RENCA, peuvent être induites après vaccination par ces antigènes isolés par des techniques d'analyses sérologiques. Cette dernière observation suggère un rôle des voies immunorégulatrices impliquées dans la tolérance du soi dans l'échappement de la tumeur au système immunitaire ainsi qu'à sa progression.

## ABBREVIATIONS

---

aa	Amino acid
Ag	Antigen
AP	Alkaline phosphatase
APC	Antigen Presenting Cell
bp	Base pairs
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte antigen 4
(k)Da	(kilo) Dalton
DC	Dendritic cell
DMEM	Dulbecco's modified eagle's medium
DOTAP	N-(2,3-Dioleoyloxy-1-propyl) trimethylammonium methyl sulfate
DTT	Dithio- 1,4- threitol
ddH <sub>2</sub> O	Double Distilled Water
dNTP	Deoxiribonucleoside Triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzymed-linked Immunospot

ER	Endoplasmic reticulum
FACS	Fluorescence-activated Cell Sorting
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte/macrophage Colony Stimulating Factor
Gy	Gray
HBSS	Hank's Balanced Saline Solution
HSPs	Heat shock proteins
IFN	Interferon
IgG	Immunoglobulin isotype G
IFS	Inactivated Fetal Calf Serum
i.m.	Intramuscular
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MOPS	4- Morpholinepropanesulfonic acid
NFDM	Non-fat dry milk
NK cell	Natural killer cell
PBS	Phosphate buffered saline
PCR	Polimerase chain reaction

RPMI 1640	Roswell Park Memorial Institute 1640 medium
SDS	Sodium dodecylsulfate
SEREX	Serologic Analysis of Recombinant cDNA Expression Libraries
SSC buffer	SDS sodium citrate buffer
TAA	Tumor Associated Antigen
TAE	Tris acetate EDTA (buffer)
TIL	Tumor infiltrate lymphocyte
Tregs	Regulatory T cells
TTBS	Tween- Tris- buffered saline
v/v	Volume per volume
VSV	Vesicular Stomatitis Virus
w/v	Weight per volume

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 humoral response 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.

The specific aims of these studies are described below:

- A) Characterize the antigenic targets evoked by immunization with irradiated, unmodified and irradiated, GM-CSF transduced RENCA cells.
- B) Test, *in vivo*, the immunogenic potential of serologic identified RENCA-associated antigens using antigen-based and whole-cell based vaccines
- C) Examine the role of these humoral targets in the balance between tumor immunity *versus* tolerance
- D) Evaluate the conserved immunogenicity between murine and human tumor systems

## INTRODUCTION

### 1.1 Cancer Immunity: Concepts

One of the main goals in the field of cancer immunology has been to develop approaches that specifically stimulate the immune system to control tumor growth *in vivo*. Cancer vaccination – or active immunotherapy - is based on William Coley's first observations that patients with advanced cancer injected with bacterial extracts experienced durable tumor regressions (Coley 1991). Coley's observation led later on to the Cancer Immunosurveillance hypothesis, postulated by Burnet and Thomas, in 1957, that the immune system constantly surveys the body for transformed cells and is able not only to recognize, but also eliminate tumors based on their expression of tumor-associated antigens (TAA). More than a hundred years after, tumor immunologists' work still focus on the idea that the immune system can be manipulated to recognize cancer cells and eliminate them in a selective way.

The molecular identification of TAA was originally demonstrated in rodents and was based on the findings that tumors induced in animal models were frequently rejected when transplanted to syngeneic hosts, whereas transplants of normal tissue between syngeneic hosts were accepted (Gross 1943; Foley 1953; Prehn and Main 1957). Even though this concept of Immunosurveillance has been under criticism, there is now accumulating data suggesting the importance of the immune system in controlling tumor malignancy and the contributions of both innate and adaptive immunity to this response. In the past years, studies using mice with defined immunological defects have shown greater susceptibility to spontaneous and induced tumors, with many of these tumors rejected if transplanted into normal hosts (Girardi *et al.* 2001; Shankaran *et al.* 2001; Smyth *et al.* 2001).

Work from Shankaran *et al.* has shown that the immune system can also promote the emergency of primary tumors with reduced immunogenicity that are capable of escaping immune recognition and destruction (Shankaran *et al.* 2001). These findings led to a new, broader and more dynamic concept that emphasizes the role of immunity not only in preventing but also shaping tumor immunogenicity. The Cancer Immunoediting model describes this dual host-protective versus tumor-sculpting action of the immune system in cancer, in three phases: i) *elimination* (or immunosurveillance), when innate and tumor-specific adaptive immunity provide the host with a capacity to completely eradicate the

developing tumor; ii) *equilibrium*, as the period of latency in which tumor's immunogenic phenotype is being shaped by immunological pressure; iii) and *escape* (Dunn *et al.* 2002; Dunn *et al.* 2004). This last phase refers to tumor outgrowth without immunological restraints.

In humans, several lines of evidence contributed to this idea of tumor immunity: i) occasional spontaneous regressions of cancers in immunocompetent hosts and increased cancer incidence in immunocompromised individuals; ii) spontaneous antitumor immune response detected in cancer patients; iii) accumulation of immune cells at the tumor sites as a possible positive prognostic indicator of patient survival (Starzl *et al.* 1970; Zhang *et al.* 2003).

An immune response is a multistep process, requiring antigen presentation, activation and expansion of specific immune effector cells, and their localization at the site of challenge. A series of events have to take place to initiate an effective immune response, including danger signals, secretion of cytokines and other inflammatory mediators, as well as presence of professional antigen-presenting cells (APC) that are responsible to take up antigen, mature and migrate to lymph nodes, where they present the antigen to T cells.

Cancer cells can induce immuno recognition through activation of both arms of the immune system. In one pathway, tumor cells can be directly detected by components of the innate immunity (NK, phagocytes, DC) that use pattern recognition receptors, as well as other cell-surface markers. The adaptive arm of the immune system uses a direct and indirect pathway - also called *cross-priming* - to recognize tumor cells. In contrast to tumors that lack the expression of important stimulatory molecules (e.g. B7-1, B7-2), activated DC can, after phagocytose tumor cell debris and process it for MHC presentation, up-regulate the expression of co-stimulatory molecules. After migrating to regional lymph nodes, they are able to stimulate in a tumor-specific fashion CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes (Banchereau and Steinman 1998). Subsequently, CD4<sup>+</sup> T cells can provide help for B-cell antibody production.

Several factors can contribute to the failure of tumor immunity. Inefficient tumor antigen presentation is one mechanism that may underlie tumor escape and is associated with the maturation state of DCs. A critical step for stimulation and maturation of these innate immune cells is the presence of cytokines in the tumor milieu.

An improved understanding of the cellular and molecular mechanisms that lead to immunologic tumor rejection, as well as elucidating how tumors escape immune detection and elimination, will have important implications for cancer therapy in humans.

## **1.2 Whole Tumor Cell Vaccines**

Live whole tumor cells inactivated by irradiation were the first type of antitumor vaccines and have been extensively used both in murine and humans (Ward *et al.* 2002). Whole tumor cells are a potent vehicle of generating anti-tumor immunity since they provide a large repertoire of potential antigens that can promote the development of a broadly active immune response. In most cases, although humoral and cellular responses are induced in the host, this antitumor immunity is not sufficiently potent to prevent the progression of the disease. Though whole tumor cells are a good source of antigens, additional stimuli, as those provided by immunological adjuvants, is necessary to overcome the induction of tumor-specific T cell anergy (Matzinger 1994; Staveley-O'Carroll *et al.* 1998). 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 to be considered when designing rational immunotherapeutic approaches.

## **1.3 Cytokine-based vaccines**

### **Cytokines in the Tumor Microenvironment / Tumor Milieu**

Accumulating evidence from both human and mouse studies, support the key involvement of cytokines in promoting tumor immunity, inflammation and carcinogenesis (Dranoff 2004). Cytokines in the tumor microenvironment are also known to be a key variable in limiting the immunogenicity of nascent cancers. Cytokines can be produced by the host stromal and immune cells, in response to molecules secreted by the cancer cells, or as part of the inflammation process that is associated with tumor growth. Cytokines opposing roles at the tumor site can influence the immune response toward tolerance or immunity. Numerous studies established the ability of cytokine-secreting tumors to function as cellular vaccines able to augment systemic immunity against wild-type tumors. The pioneer work of Forni and colleagues was essential to demonstrate the potential of manipulating the cytokine milieu in order to induce dramatic changes in the host immune response (Forni *et al.* 1988). They showed that peritumoral injection of particular cytokines, particularly interleukin-2, could induce tumor destruction, involving the coordinated activity of neutrophils, eosinophils, macrophages, natural killer cells, and lymphocytes. Moreover, this immune response could, in some cases, generate protective immunity against tumor challenge. These provocative findings, that host response to tumor challenge can be dramatically influenced by inoculation of tumor cells genetically engineered to express particular cytokines, were the base of additional studies by Dranoff

*et al.* to compare the ability of different cytokines and other molecules to enhance the immunogenicity of tumor cells (Dranoff *et al.* 1993).

#### **1.4 GM-CSF-secreting Whole Tumor Cell Vaccines**

Inflammation constitutes an essential “danger” signal to induce recruitment of leukocytes and initiate an efficient antigen presentation. Cytokine gene transfer into tumors has been used to address this issue.

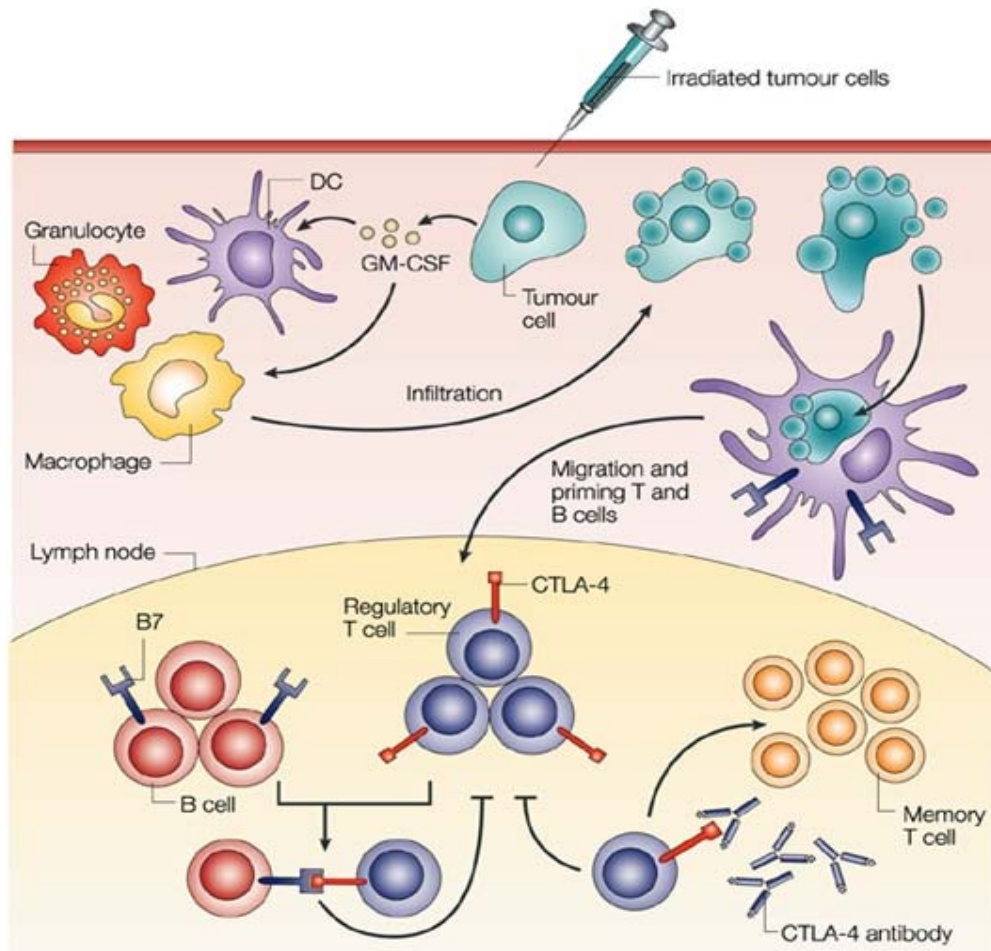
GM-CSF is one of the most important inflammatory cytokines involved in host defense (Hamilton 2002). Different GM-CSF-activated signaling pathways are critical in regulating the proliferation, differentiation, and maturation of myeloid cells and stimulating macrophage proliferation. Additionally, GM-CSF primes the respiratory burst and enhances the effector function of mature granulocytes and mononuclear phagocytes. GM-CSF stimulates phagocytosis by up-regulating the expression of surface molecules, as Fc $\gamma$ RI, Fc $\gamma$ RII and complement receptors, in most phagocytes (including neutrophils, macrophages, eosinophils and dendritic cells). This cytokine not only facilitates antigen uptake, but also improves antigen presentation by APC through increased expression of major histocompatibility (MHC) class II and co-stimulatory molecules. In monocytes / macrophages, GM-CSF can stimulate the production of multiple pro-inflammatory cytokines, and is able to induce the expression of critical adhesion molecules, promoting their migration to the inflammatory foci. GM-CSF, alone or with IL-4 or TNF- $\alpha$ , promotes the development of dendritic cells from murine and human hematopoietic precursors (Xu *et al.* 1995).

The ability of GM-CSF to enhance antitumor immunity was first identified through an *in vivo* screen of a large number of immunostimulatory molecules (Dranoff *et al.* 1993). High-efficiency gene transfer system was used in order to compare the immunostimulatory activity of a gene product or mixture of gene products best able to stimulate anti-tumor immunity in a wide variety of tumor models. A large panel of high titer retroviral vectors expressing a variety of cytokines, adhesion molecules and co-stimulatory molecules was generated. The vaccination properties of both live and irradiated tumor cells transduced with the viruses was compared in several different murine tumor models. Even though several gene products increased protective immunity to several degrees, GM-CSF gene-transduced, irradiated tumor vaccines were the most potent inducers of long-lasting, specific tumor immunity, even in poorly immunogenic tumor models (e.g. B16). In spite of the significant vaccination activity of some of the non-transduced cells lines (e.g. RENCA and CMS5 cell lines) in eliciting systemic immunity, irradiated GM-CSF-expressing cells were more effective than irradiated cells alone. The

mechanism underlying the potent ability of GM-CSF to improve antitumor immunity involves the enhancement of tumor antigen presentation by recruitment of host APC (Dranoff *et al.* 1993; Huang *et al.* 1994). Vaccination with irradiated tumor cells engineered to secrete GM-CSF stimulates infiltration of DC, macrophages and granulocytes at the immunization site (Figure 1.1). This coordinated cellular reaction promotes the efficient phagocytosis of tumor debris by DC. This vaccination further induces DC to mature and migrate to regional lymph nodes to prime tumor-specific T and B cells.

A coordinated humoral and cellular response involving antibodies, CD4+ and CD8+ tumor-specific T cells, and CD1d-restricted invariant NKT cells contributes for the mediated tumor rejection seen in this system. The broad cytokine production elicited by vaccination with GM-CSF-secreting tumor cells is consistent with a requirement of CD4+ T cells for priming and is also consistent with a pivot role of antibodies in GM-CSF-stimulated immunity. IgG antibodies recognizing tumor cells were also induced by this immunization.

DCs are potent APC with a crucial role in priming antigen-specific immune responses (Banchereau *et al.* 2000). DC specialized ability to capture antigens in peripheral tissues, to process this material efficiently into MHC class I and II pathways, to up-regulate costimulatory molecules upon maturation, and to migrate to secondary lymphoid tissues, renders them unique in stimulating immunity. In order to identify specific properties of these DC in tumor protection, the biological activities of B16 melanoma cells engineered to secrete GM-CSF or Flt3-ligand were compared (Mach *et al.* 2000). Although GM-CSF and Flt3 cytokines can promote a marked expansion of CD11c+ DC locally and systemically, GM-CSF-expressing cells induced higher levels of protective immunity. Several differences between DCs elicited by GM-CSF and Flt3 may be responsible for the distinct vaccinations outcomes: GM-CSF generates a population of mature CD11b+, CD8+ DC, with higher ability to capture and process dying tumor cells and may contribute to enhanced priming. GM-CSF also evoked higher levels of co-stimulatory molecules associated with a greater functional maturation status in these cells. Because dying tumor cells provide the antigens for the immunization, the presence of these specialized DC at the site of vaccination, may contribute to enhanced priming by reducing the amount of antigen necessary to trigger T cell proliferation. Differences in the ability of GM-CSF and Flt3 to stimulate CD1d-restricted invariant NKT-cells also contributed for the differences observed in tumor protection (Mach *et al.* 2000).



Dranoff, G. ; 2004 (Dranoff 2004)

**Figure 1.1: GM-CSF secreting tumor-cell vaccines and CTLA-4 antibody blockade show synergistic antitumor effects.**

## 1.5 GM-CSF Tumor Vaccines: from Mice to Men

### GM-CSF transduced autologous tumor vaccines: Clinical trials

Cancer vaccination strategies have focused on the use of autologous and allogeneic tumor cells genetically modified to express a range of different immunomodulatory genes, including cytokines, co-stimulatory molecules, and tumor antigens.

Based on the results of murine preclinical studies, the role of GM-CSF-transduced vaccines in stimulating tumor immunity was tested in humans (Soiffer *et al.* 2003). A Phase I clinical trial in patients with metastatic melanoma was conducted (Dranoff *et al.* 1997). Briefly surgically excised tumors were processed to a single-cell suspension, transduced with replication defective retroviruses expressing GM-CSF, irradiated and used to immunize patients with metastatic melanoma. Initial evaluation of GM-CSF-based

vaccines demonstrated a consistent induction of immunity in patients with no significant toxicity associated. Pathological examination at the site of injection of irradiated GM-CSF-secreting tumor cells revealed an intense local reaction associated with a dense infiltrate of mature DCs, macrophages, eosinophils, CD4+ and CD8+ T lymphocytes, as well as plasma cells that could contribute to substantial destruction of metastases. Vaccination stimulated a strong antibody reaction directed against melanoma cell-surface and intracellular antigens (Hodi *et al.* 2002). The evaluation of this vaccination strategy in patients with advanced melanoma revealed the consistent and coordinate induction of cellular and humoral responses capable of inducing a substantial necrosis of distant metastases. As a result, an extensive tumor destruction, fibrosis and edema were seen in most of the patients. Lymphocytes harvested from infiltrated metastases displayed potent specific cytotoxicity and secreted a broad profile of cytokines in response to the autologous tumor cells. High-titer anti-tumor antibodies were present in post-vaccination sera. Another feature of the anti-melanoma response was the targeted destruction of the tumor vasculature, where lymphocytes, eosinophils and neutrophils were closely associated with the dying tumor blood vessels.

A number of genetically modified autologous or allogeneic tumor cell vaccines have now been tested in clinical trials. This immunization strategy has been tested in patients with renal-cell carcinoma, prostate carcinoma, metastatic melanoma, and pancreatic cancer and confirmed the biological activity and safety of GM-CSF-based tumor cell vaccines (Simons *et al.* 1997; Simons *et al.* 1999; Jaffee *et al.* 2001). The majority of patients' biopsies demonstrated extensive inflammatory infiltrate within the tumors, sometimes associated with increased tumor-specific lymphocyte activity and tumor regression. In order to avoid the need of establishing primary tumor cell-cultures from each patient, a new approach involving the use of adenoviral vectors, which can readily infect resting target cells without the need of target cells replication for infection, was employed.

Clinical testing of GM-CSF-secreting tumor cell vaccines in tumor patients with metastatic melanoma has demonstrated that the principles revealed in the murine systems can be directly relevant to cancer in humans (Soiffer *et al.* 2003).

## **1.6 Combinatorial Immunotherapeutic Strategies**

### **Synergistic antitumor effect of GM-CSF based Vaccines and CTLA-4 antibody blockade.**

New insights into the mechanisms by which T and B cells are successfully activated and by which tumors can evade immune recognition has led to the development of

combinatorial immunotherapeutic approaches that enhance vaccine-induced anti-tumor responses.

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is a fundamental T-cell checkpoint that limits the magnitude of immune responses (Peggs *et al.* 2006). CTLA-4 is a tightly regulated surface molecule, present on CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes that plays an important role in downregulating T cells response. Upon engagement by B7-1 or B7-2 present on DCs, CTLA-4 signaling in activated T cells induces cell-cycle arrest and diminish cytokine production (Doyle *et al.* 2001; Salomon and Bluestone 2001). Blockade of CTLA-4 using anti-CTLA-4 antibodies can induce rejection of several types of established transplantable tumors in mice (e.g. colon carcinoma, fibrosarcoma, lymphoma and renal carcinoma) (Leach *et al.* 1996; Yang *et al.* 1997; Sotomayor *et al.* 1999). Allison and colleagues have shown that transient antibody-mediated blockade of CTLA-4 function could increase the anti-tumor effects of GM-CSF-secreting tumor vaccines in several poorly immunogenic mouse models (van Elsas *et al.* 1999; Suttmüller *et al.* 2001). This synergistic effect using CTLA-4 antibody blockade in combination with GM-CSF vaccines has also been shown to increase tumor immunity in patients, albeit with a risk of breaking tolerance against self-antigens (Hodi *et al.* 2003). Anti-CTLA-4 antibody administration induced tumor regression and immune infiltrates in melanoma and ovarian patients, who had been previously vaccinated with irradiated, autologous GM-CSF-secreting tumor cells (Hodi *et al.* 2003).

### **1.7 Tumor-Associated Antigens**

Over the last century, tumor immunologists have been trying to address two fundamental questions: can the immune system discriminate between normal and tumor cells? And can one use this as a tool to selectively eliminate cancer?

The development of successful vaccines for tumor immunotherapy requires the identification of cellular antigens that are primarily associated with tumor cells. These antigens have to be delivered in a way that produces the appropriate immune response to control tumor growth. A variety of genetic and biochemical techniques have been developed to identify tumor-associated antigens that can be used to discriminate between cancer and normal cells. Tumor antigens can be classified according to the type of immune response they elicit: humoral or cellular (CD4<sup>+</sup> or CD8<sup>+</sup> cytotoxic T lymphocytes). Antigens specifically recognized by tumor-specific CTLs in the context of MHC class I molecules were the first group of tumor antigens to be identified (Lurquin *et al.* 1989). The initial focus on CD8<sup>+</sup> T antitumor response cells derived from two major facts: i) was that most tumors are positive for MHC class I but negative to MHC class II; ii) CD8<sup>+</sup> cytotoxic T lymphocytes are able to induce direct tumor killing by recognition of

peptide antigens, presented by the tumor's MHC class I molecules (Boon and van der Bruggen 1996).

CD4<sup>+</sup> T or T helper (Th) cells are also essential components of the immune system and can mediate a number of antitumor effector pathways inducing a potent and long-lasting immunity (Sahin *et al.* 1995; Overwijk *et al.* 1999). CD4<sup>+</sup> T cells critical role in induced anti-tumor immunity was first demonstrated by abrogation of antitumor immunity in experiments using CD4-knockout or antibody-depleted mice (Toes *et al.* 1999). Other murine studies have shown that CD4<sup>+</sup> T cells can eradicate tumor in the absence of CD8<sup>+</sup> T cells. There is now accumulating evidence that CD4<sup>+</sup> T cells key role in tumor immunity is due, not only to the ability to provide help in priming CD8<sup>+</sup> CTL, but also to the ability to stimulate the innate arm of the immune system (macrophage and eosinophils activation) at tumor site (Hung *et al.* 1998). In addition, they can also sensitize tumor cells to CTL lysis through secretion of effector cytokines, such as IFN- $\gamma$ . Two predominant Th cell subtypes exist, Th1 and Th2. Th1 cells, characterized by secretion of IFN- $\gamma$  and TNF- $\alpha$ , are primarily responsible for activating and regulating the development and persistence of CTL. In addition, Th1 cells activate antigen-presenting cells (APC) and induce production of the type of antibodies that can enhance the uptake of infected cells or tumor cells into APC. Th2 cells favor a predominantly humoral response. Specifically, modulating the Th1 cell response against a tumor antigen may lead to effective immune-based therapies. Th1 cells can also directly kill tumor cells via release of cytokines that activate death receptors on the tumor cell surface.

T cell defined antigens were initially isolated using a technique developed by Boon and colleagues (Brichard *et al.* 1993; Coulie *et al.* 1994). This technique utilizes tumor-reactive CTL clones, isolated from patients, to screen target cells that have been transfected with a cDNA library derived from the autologous tumor cell. In addition, two other approaches have been used, one involving the purification of peptides eluted from MHC complexes derived from tumor cell membranes, and another, called reverse immunology, that uses candidate tumor antigens to stimulate lymphocytes *in vitro* and then test their ability to specifically kill tumor cells that are known to express the antigen (Cox *et al.* 1994; Mandelboim *et al.* 1994).

Another technique to identify immunogenic tumor antigens was introduced by Pfreundschuh and colleagues, and was based on the detection of a humoral response against autologous tumor cells, by screening a phage expression library with serum from cancer patients. This method to detect tumor antigens specifically bound to high titers of IgG was called SEREX (for serological identification of antigens by recombinant expression cloning) (Sahin *et al.* 1995). Since isotype switching from IgM to IgG implies the presence of specific help from CD4<sup>+</sup> T cells, the rationale was that a T-cell response

against these serologically defined antigens should be present. Detection of antibody responses against known CTL-defined antigens (e.g. MAGE-1 and tyrosinase) raised the question whether specific humoral and cellular responses against tumor antigens can occur simultaneously in a given patient. Characterization of B cell response in patients with different tumors demonstrated the presence of high titer IgG antibodies, to a diversity of tumor-associated antigens (Sahin *et al.* 1995; Sahin *et al.* 1997; Old and Chen 1998; Scanlan *et al.* 1999; Scanlan *et al.* 2002). Subsequently, several of these antigens (e.g. NY-ESO) have been shown to be also targets of specific T-cell responses *in vivo* (Chen *et al.* 1998; Jager *et al.* 2000; Jager *et al.* 2000). Additionally, histological examination of the vaccination site and regressing tumors in patients who respond to tumor vaccines, have shown the presence of a diverse inflammatory response including B and T cells (Hodi *et al.* 2002; Schmollinger *et al.* 2003). In animal models, it also became clear that tumor rejection *in vivo* was associated with an immune response involving the interaction of antibodies, as well as B and T cells (Nishikawa *et al.* 2001). These observations contributed to the hypothesis that effective tumor rejection *in vivo* results from a coordinated immune response involving different classes of effector cells, targeting a number of TAA.

A broad repertoire of tumor antigens recognized by antibodies, as well as CD4+ and CD8+ T lymphocytes in cancer-bearing hosts, is now uncovered. Based on their expression pattern, TAA can be classified in four major groups: i) *shared tumor antigens*, representing antigens encoded by genes that are silent in most normal tissues, but are activated in various types of tumors; ii) *tissue-differentiation antigens*, that show a lineage specific expression in tumors and also in normal cells of the same origin (e.g. Tyrosinase is expressed in melanoma and melanocytes); iii) *tumor-specific antigens*, that are expressed in cancer cells but not in normal cells, and can arise as a result of mutations or alternative splicing; and iv) *overexpressed tumor antigens*, which are expressed both in normal and cancer cells, but at different levels. Cancer-testis antigens are a specific group of shared TAA that are normally expressed in spermatozoa and silenced in somatic cells, but during cancer development, their expression re-emerges (Scanlan *et al.* 2004). Because members of this group are frequently expressed in tumors of different histological type, they have been extensively study as targets for antigen-specific immunotherapy in cancer.

## **1.8 Renal Cell Carcinoma (RENCA) as a Tumor Model**

Animal models are an excellent tool to understand basic paradigms of tumor immunology, particularly the mechanisms underlying anti-tumor immune responses. GM-

CSF-based tumor vaccines are a good example where clinical testing of this immunization strategy in patients with advanced melanoma could validate some of the principles seen in the poorly immunogenic B16 tumor mouse model.

Murine models are particularly useful to identify relevant tumor specific antigens and characterize immunological responses evoked by these antigenic targets that may result in protective anti-tumor immunity. The ultimate therapeutic goal of tumor antigen identification is their use as tumor rejection antigens in recombinant vaccine strategies, and evaluate whether they can elicit a significant clinical response in patients. Since murine models provide an *in vivo* milieu that mimics, as closely as possible human cancers, they play a critical role in pre-clinical testing of novel immunotherapies.

RENCA is an immunogenic tumor cell line with potential interest since vaccination with irradiated, unmodified tumor cells can elicit measurable levels of protective immunity (Dranoff *et al.* 1993). Nonetheless, vaccination with irradiated, RENCA cells engineered to secrete GM-CSF generates greater levels of protective immunity (Dranoff *et al.* 1993). This model provides the basis to understand the contribution of GM-CSF cytokine to enhanced anti-tumor immunity, in particular, to understand if augmented anti-tumor immunity is due to recognition of additional antigens or due to differences in the antigen targets recognized by the immune response. Furthermore, the potential role of these candidate tumor rejection antigens can easily be assessed in different antigen-specific vaccine strategies that have demonstrated efficacy in other murine models.

GM-CSF secreting RENCA cells constitute an experimental system with important implications for the clinical application of GM-CSF transduced tumor cells as therapeutic vaccines. Additionally, this model can also help to understand the basic immunological principles associated with the use of this adjuvant cytokine.

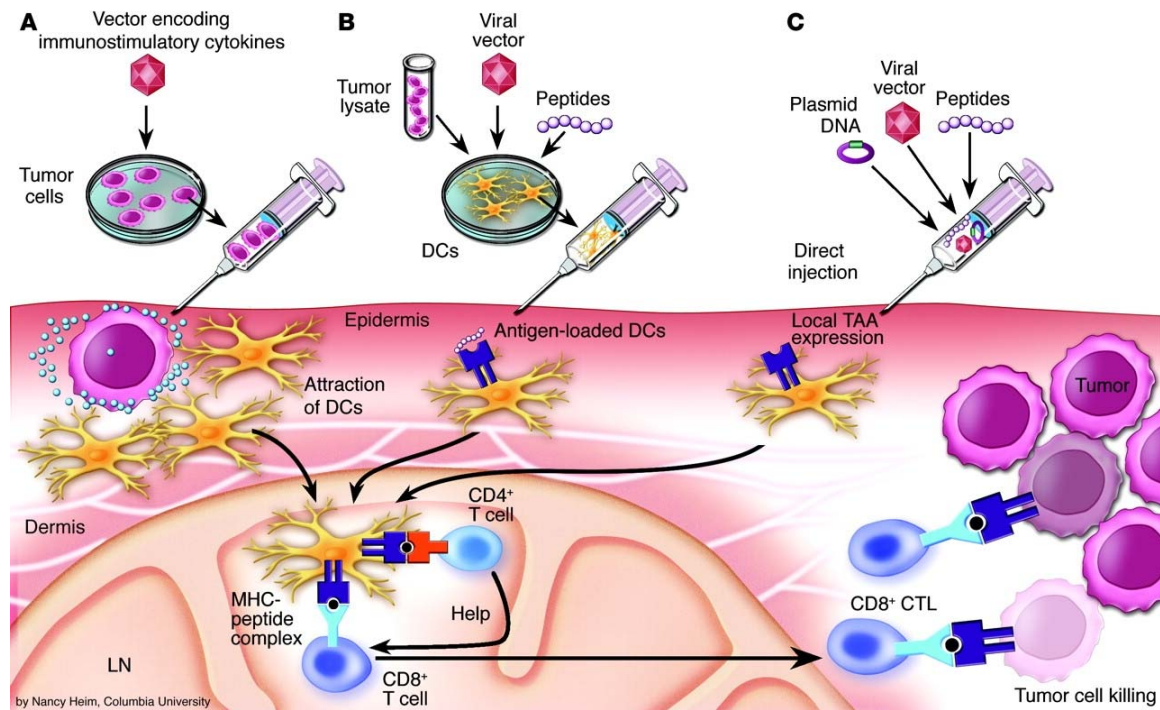
## **1.9 Tumor Vaccines**

Tumor vaccines can be based on cancer cells or on the genetic identification of tumor associated antigens (Figure 1.2). Various cancer cell derived strategies have been developed to induce tumor-specific immune response against autologous malignant cells (Boon *et al.* 1997; Rosenberg 1997). These include whole tumor cell vaccines (both autologous and allogeneic preparations), genetically modified tumor vaccines (genes encoding cytokines, chemokines or co-stimulatory molecules), cancer cell extracts (lysates, membranes and heat-shock proteins) and cancer cells fused to APC.

Tumor associated antigens (TAA) recognized by cellular and humoral effectors of the immune system are potential targets for antigen specific cancer immunotherapy. Vaccines based on the genetic identification of tumor antigens include purified cancer

antigens (natural or recombinant), synthetic peptides, naked DNA (e.g. plasmids, recombinant viruses and bacteria, and antigen-modified DCs vaccines.

Some cancer vaccine modalities and the rationale behind their application to induce an antitumor response will be discussed.



by Nancy Heim, Columbia University  
**Berzofsky JA, et al.; (Berzofsky et al. 2004)**

**Figure 1.2: Approaches to Anti-Tumor Vaccines.**

### 1.10 Antigen-based Vaccines

The discovery of TAA and the identification of their immunodominant epitopes led to the development of immunotherapies. These rely on the specific stimulation of the immune system against these defined TA to mediate tumor destruction. One of the advantages of the molecular characterization of TAA and their utilization as anti-cancer vaccines is also to be able to follow the dynamics of the developing immune response in cancer-bearing hosts.

There are two main issues to consider when designing effective antigen-specific cancer vaccines: i) the identification of potent tumor rejection antigens; ii) how to stimulate them to induce an effective, specific and long-lasting anti-tumor immune response, by preventing immune evasion and avoiding autoimmunity.

One of the challenges in using antigen-based immunotherapies is to define which tumor antigens are the best targets for the development of effective immunotherapy.

Tumor antigens can be poor, intermediate or strong tumor rejection antigens depending on how an immune response elicited against a tumor antigen will cause rejection of the tumor growth, *in vivo*. In addition, development of strategies to improve *in vivo* delivery of these antigens is another challenging step. Multiple approaches for the active immunization of patients, using the products of these tumor antigens, are currently being explored in clinic.

### **1.10.1 DNA Vaccines**

One of the hallmarks of DNA vaccination is the development of a robust, long-lasting, antigen-specific cellular and humoral immune response which makes it a suitable approach for cancer immunotherapy.

Plasmid (naked) DNA vaccines are simple vehicles to deliver tumor antigens that can result in protein expression and immunity (Wolff *et al.* 1990). DNA vaccines induce, upon *de novo* synthesis of antigen in transfected cells and can stimulate antigen-specific cellular and humoral-mediated immunity (Ulmer *et al.* 1993). DCs are the principal cells initiating the immune response after DNA vaccination, as they are key mediators of immune responses between resident somatic cells and T cells in the lymph nodes.

Antigens encoded by plasmid DNA delivered to the skin (gene gun) or injected in the muscle, can be processed and presented to induce an immune response by several mechanisms (Tang *et al.* 1992). Bombardment of the epidermis with plasmid coated onto gold particles can directly transfect epidermal keratinocytes and also Langerhan cells, which were shown to rapidly migrate to lymph nodes (Porgador *et al.* 1998). On the other hand, intramuscular injection (i.m.) of plasmid leads predominantly to transfection of myocytes and cross-priming by DC. Cross-priming occurs when professional APCs process secreted peptides or proteins from somatic cells and / or other APCs by phagocytosis of either apoptotic or necrotic bodies (Albert *et al.* 1998; Albert *et al.* 1998).

The type and magnitude of immune responses to DNA vaccines can be modulated by the use of adjuvants encoding cytokines, co-stimulatory molecules or a ligand. A variety of these molecules delivered as DNA can improve APC activation, expansion, or maturation following antigen uptake and processing *in vivo*. Additionally, DNA vaccines provide their own adjuvant in the form of unmethylated bacterial CpG sequences. These can induce an innate immune response able to boost the efficacy of these vaccines.

### **1.10.2 Dendritic Cell (DC) Vaccines**

DCs are the most efficient antigen-presenting cells (APC) capable of inducing immunity to newly introduced Ag (Banchereau and Steinman 1998; Banchereau *et al.*

2000). These professional APC are the most powerful stimulators of naïve T cells. They have been successfully used as cellular adjuvants in mice to elicit protective T cell-mediated immunity against pathogens and tumors (Banchereau and Steinman 1998; Pulendran *et al.* 2001; Schuler *et al.* 2003).

Immature DCs have a high capability for antigen capture and processing. When DCs encounter inflammatory mediators (e.g. bacterial LPS or TNF- $\alpha$ ) or interact with CD40 ligand on T helper cells, they become mature. Upon maturation DCs lose the ability to capture antigen. They also upregulate MHC, co-stimulatory molecules (CD80 and CD86), and the chemokine receptor CCR7, and they acquire an increase capability to migrate to T cell areas, where they can initiate or “prime” an immune response (Trombetta and Mellman 2005). Based on the central role of these professional APC in initiating immune responses, a variety of strategies have been developed to use DC to stimulate immunity against tumor antigens. Most of these strategies rely on the activation and maturation of DCs *ex vivo* to elicit tumor-specific immunity. *Ex vivo* modification of both human and mouse DCs with genes encoding tumor-antigens, including self-antigens, have been shown to effectively stimulate T cell response *in vitro*. Moreover, in various murine models induction of long-term immunity could be elicited against tumors expressing the corresponding antigens (Gabrilovich *et al.* 1996; Ashley *et al.* 1997). Most of these experiments involve *in vitro* isolation of DCs followed by pulsing with TAs expressed as peptides (Gabrilovich *et al.* 1996), proteins (Paglia *et al.* 1996; Ashley *et al.* 1997) or nucleic acids (Ashley *et al.* 1997; Chen *et al.* 2003). DCs “pulsed” with antigens can efficiently process and present them as MHC-peptide complexes. *Ex vivo* loaded DCs reinfused to tumor-bearing recipients can then elicit T-cell-mediated tumor destruction (Fong and Engleman 2000). Several clinical trials have tested *ex vivo* expanded and primed DCs as vaccines. Two main approaches are currently used to obtain large number of these DCs: i) purification of immature DC precursors from peripheral blood (Fong and Engleman 2000); ii) *ex vivo* differentiation of DC from CD34+ hematopoietic progenitor cells (by culture them with GM-CSF and IL-4) (Mackensen *et al.* 2000; Banchereau *et al.* 2001). DC maturation can be induced with CD40 ligand, LPS, or TNF- $\alpha$ .

DCs modified to express both tumor antigens and co-stimulatory molecules can lead to immunologic memory able to induce protection against subsequent tumor challenges (Wiethe *et al.* 2003).

### **1.10.3 Recombinant-viral Vectors**

The use of recombinant viruses, both as vaccines, or as cytokine gene transfer studies, have been under intensive focus in the field of cancer immunotherapy. Viral-

based systems use recombinant viruses, where genes encoding viral proteins are replaced by the gene of interest. Retroviral and adenoviral vectors permit stable integration of therapeutic genes into the chromosomal DNA of the target cell. These vectors have been used mostly for *ex vivo* gene therapy, involving transduction of the target cells *in vitro* and subsequent reintroduction of the modified cells into the tumor-bearing host. Our group has previously shown that vaccination with irradiated autologous melanoma cells, retroviral or adenoviral-transduced with GM-CSF can generate potent antitumor immunity in melanoma patients (Soiffer *et al.* 1998; Soiffer *et al.* 2003). Adenoviral vectors are able to transduce resting target cells and show only minimal toxicities with *ex vivo* applications, which makes these vectors an attractive alternative for vaccine production (Soiffer *et al.* 2003).

The first studies showing the capacity of recombinant adenoviruses to induce antitumor immunity used  $\beta$ -galactosidase as a model tumor antigen (Chen *et al.* 1996). A number of trials utilizing recombinant viruses expressing tumor antigens, such as CEA or PSA, with or without immunostimulatory cytokines, have now been reported (Marshall *et al.* 2000; Zhu *et al.* 2000). Restifo *et al.* have also demonstrated the generation of antigen-specific immunity using vaccinia and fowlpox constructs, resulting in the protection against tumor challenges (McCabe *et al.* 1995; Wang *et al.* 1995).

### **1.11 Tumor Immunity versus Tumor Escape and Progression**

The immune system can, under different stimuli, induce an immune response leading to immunity or preventing it leading to tolerance. On the other hand, tumors have developed strategies of actively evade or silence / suppress an immune response. It's now clear that both, the characteristics of the tumor, as well as of the tumor microenvironment and systemic factors, can contribute for immune evasion and progression (Restifo *et al.* 1993; Ganss and Hanahan 1998).

Tumor escape, resulting from changes within the tumor itself, is associated with alteration in the antigen processing and presentation pathway. These can lead to tumors poor immunogenicity and affect tumor immune recognition. They include loss of antigen expression, loss / very low expression of MHC class I and II molecules, as well as deficiencies in other components of this pathway (including TAP1 and the immunoproteasome subunits LMP2 and LMP7), shedding of NKG2D ligands (Groh *et al.* 2002) and unresponsiveness to IFN- $\gamma$  (Kaplan *et al.* 1998). Tumors are also poor APCs. Their lack of co-stimulatory molecules on the surface and failure to produce stimulatory cytokines makes them poorly immunogenic or even tolerogenic. Tumors can also present defects in the death-receptor signaling pathway, as well as express anti-apoptotic signals

as mechanisms of escape immune destruction (Catlett-Falcone *et al.* 1999; Takeda *et al.* 2002).

Inhibition of the protective functions of the immune system may also facilitate tumor escape. Indirect presentation of tumor antigens by DC is thought to play a more critical role in determining antitumor immunity, rather than the role of direct immune recognition. The interaction between T cells and DC is critically influenced by the maturation stage of the DC. Mature DC, have a potent ability to activate T cells but in contrast, immature DC can be tolerogenic. Lack of proinflammatory mediators, that induce maturation of DC, as well as persistence of antigen presentation by non-co-stimulatory tumor cells, favors tumor-specific T cell tolerance. Lack of functional mature DCs and abundance of suppressive DCs can reduce the TAA-specific T-cell priming in draining lymph nodes, as well as the TAA-specific effectors immunity in the tumor microenvironment.

Cross-presentation refers to the unique ability of APC, such DC and macrophages, to acquire antigen from donor cells (e.g. tumor cells) and present the captured antigens via their own MHC class I molecules to CD8 T cells. Cross-presentation is involved in the maintenance of tolerance to self-antigens (cross-tolerance), as well as in the induction of immune responses (cross-priming). The different outcomes (tolerance vs. immunity) will depend on the presence or absence of inflammatory, as well as co-stimulatory signals (Heath *et al.* 2004). Tumors can suppress induction of proinflammatory danger signals, through mechanisms involving activated STAT3, leading to impaired DC maturation (Wang *et al.* 2004). A large amount of plasmacytoid DCs, but not functional mature myeloid DCs, can accumulate in the tumor microenvironment (Zou *et al.* 2001).

Although immunological tolerance normally exists to prevent autoimmunity, the same “tolerizing” conditions can be used by tumor cells to escape tumor immunity. Most tumor antigens are self-antigens and their expression in the thymus induces central immunological tolerance through clonal T-cell deletion. This results in a tolerized T cell repertoire with low or intermediate avidity for self-tumor antigens. Tumor cells expressing weak self-antigens can escape T cell immunity by different mechanisms of immune tolerance. Peripheral tolerance can occur through: i) anergy; ii) T cell deletion or suppression by host regulatory cells; iii) or ignorance, when naïve T cells against self peptide ignore antigen-positive cells because of inadequate affinity of self peptide for host MHC (Redmond and Sherman 2005).

There is an active process of “tolerization” taking place in the tumor microenvironment. Lack of “danger” signals, including inflammatory cytokines, molecular and cellular T-cell activating signals, has been one of major cause of poor tumor immunity. Tumors can induce anergy or deletion of tumor antigen-reactive T cells by secreting immunosuppressive cytokines (IL-10, TGF-B) and by expressing apoptosis-

inducing Fas ligand, resulting in apoptosis of tumor-reactive T cells (Khong and Restifo 2002).

## **1.12 Regulatory T cells (Tregs) and Immunological Tolerance to Tumor Antigens**

Regulatory T cells are functionally defined as T cells that inhibit an immune response by influencing the activity of another cell type (Shevach 2004).

Naturally occurring thymus-derived CD25<sup>+</sup>CD4<sup>+</sup>FOXP3<sup>+</sup> regulatory cells (Tregs) have been extensively studied and are known to play a key role in maintaining immunologic self tolerance and in controlling pathologic, as well as physiologic immune responses. Several other identified phenotypically distinct regulatory T-cell populations can mediate immunosuppression, including “adaptive” Treg cells. These can be induced in the periphery from naïve T cells that convert to Tregs, *in vivo*, upon antigen stimulation and under certain conditions (Roncarolo *et al.* 2001; Weiner 2001; von Herrath and Harrison 2003; Apostolou and von Boehmer 2004; Curotto de Lafaille *et al.* 2004).

Tregs involvement in peripheral tolerance was first demonstrated by experiments where reduction in their number or attenuation of their suppressive activity resulted in severe or even fatal immunopathologies, including autoimmune and inflammatory diseases. In mice, transfer of CD25<sup>+</sup> cell-depleted T cell or thymocyte suspensions from normal mice into syngeneic T cell-deficient nude mice results in various autoimmune diseases in recipient mice. However, transfer of CD25<sup>+</sup> CD4<sup>+</sup> T cells or thymocytes together with the CD25<sup>+</sup> cell-depleted population can prevent those diseases (Sakaguchi *et al.* 1995; Itoh *et al.* 1999). Moreover mice thymectomized (2-4 days after birth) spontaneously develop a wide spectrum of autoimmune diseases that can be prevented by transfer CD25<sup>+</sup> CD4<sup>+</sup> T cells or thymocytes from normal mice. Thus, natural Treg can actively suppress the activation and expansion of potentially pathogenic self-reactive T cells normally present in the immune system.

Thymus-derived Treg cells can also link central and peripheral mechanisms of self-tolerance. In the thymus, central tolerance is responsible for both negative selection of self-reactive T cells and production of natural Treg, which control in the periphery self-reactive T cells that have escaped thymic selection. IL-2 is an essential cytokine for thymic generation and peripheral maintenance of suppressor Treg.

T regulatory suppression seems to involve several distinct mechanisms, including cell-cell contact and soluble factors, as IL-10 and TGF- $\beta$  (Shevach 2002; von Herrath and Harrison 2003; Sakaguchi 2005). Treg and DC interaction can lead Tregs to expand and suppress. DCs also seem to be targets of this suppressive Treg activity. The effects of

Treg on DC can be direct (cell-cell contact), or indirect, through cytokines. *In vitro* studies have shown that TGF- $\beta$  and IL-10 can downregulate DC function by altering DC maturation or modulating cell surface expression of co-stimulatory molecules important for T cell-activation (Cederbom *et al.* 2000; Misra *et al.* 2004). In mouse tumor models, Tregs can mediate suppression through the actions of IL-10 and TGF- $\beta$  *in vivo* (Green *et al.* 2003; Peng *et al.* 2004; Chen *et al.* 2005; Ghiringhelli *et al.* 2005). However, since these immunosuppressive cytokines can be produced by different cell types in the tumor microenvironment, Treg cells might not be the only source of IL-10 and TGF- $\beta$ .

### 1.13 Tregs in Tumor Immunity

Recent studies have focused on the role of “natural” Tregs in the suppression of tumor immunity in cancer-bearing hosts. CD25<sup>+</sup>CD4<sup>+</sup> TCR repertoire is as diverse as that of CD25<sup>-</sup>CD4<sup>+</sup> cells, but more skewed toward recognizing self peptide–MHC complexes expressed in the thymus and periphery (Takahashi *et al.* 1998; Hsieh *et al.* 2004). Tregs can recognize normal self-antigens targeted in autoimmune diseases, tumor-associated antigens and allogeneic transplantation antigens (Klein *et al.* 2003; Nishikawa *et al.* 2003; Reddy *et al.* 2004). Upon stimulation by their antigens they can suppress autoimmunity, reduced tumor immunity and suppress graft rejection.

Sehon and colleagues were the first ones to suggest that regulatory T cells could regulate tumor immunity and contributed to tumor growth in mice (Fujimoto *et al.* 1975). The role of Tregs in mouse tumor immunity was later demonstrated in studies where systemic depletion of CD25<sup>+</sup>CD4<sup>+</sup> T cells *in vivo* before tumor challenge induced rejection of different immunogenic tumors in multiple strains of mice (Onizuka *et al.* 1999; Shimizu *et al.* 1999; Golgher *et al.* 2002; Jones *et al.* 2002). In support of these findings, depletion of total CD4<sup>+</sup> T cells was found to improve tumor immunity and induce tumor rejection (Sutmuller *et al.* 2001; van Elsas *et al.* 2001; Yu *et al.* 2005). This enhanced tumor immunosurveillance was mediated at least in part by tumor-specific CD8<sup>+</sup> cytotoxic T lymphocytes, CD4<sup>+</sup> T cells and NK cells. Depletion of CD4<sup>+</sup>CD25<sup>+</sup>T cells can also synergistically enhance vaccine induced anti-tumor responses. Experiments where anti-CD25 treatment was given together with GM-CSF transfected tumor cells or anti-CTLA4 antibody improved vaccination efficacy (Sutmuller *et al.* 2001). Additionally, IFN- $\alpha$  transfected B16 tumor vaccine given anti-CD25 treatment induced long-lasting protective immunity against B16 (Steitz *et al.* 2001).

Association of Tregs and reduced tumor immunity was also shown by additional experiments with adoptively transferred human and mouse Treg (Curiel *et al.* 2004; Turk *et al.* 2004; Antony *et al.* 2005). In the B16 melanoma model, it was shown that tumor

specific CD8<sup>+</sup> T cells transferred with Treg cells, but not with CD4<sup>+</sup>CD25<sup>-</sup> cells, could abolish CD8<sup>+</sup> T-cell mediated tumor immunity, suggesting that Treg cells inhibit mouse TAA-specific immunity (Turk *et al.* 2004; Antony *et al.* 2005).

Recent evidence has demonstrated that regulatory T-cell-mediated immunosuppression is a key tumor immune evasion mechanisms and one of the main obstacles in tumor immunotherapy (Sakaguchi 2005). They can strongly suppress IL-2 production and proliferation of antigen-specific T cells and, in animals, can prevent tumor regression. Suppressive T cells, some of them specific for tumor antigens, can be found in a variety of human cancer. Tregs mediate peripheral tolerance by suppressing self-antigen reactive T cells (Shevach 2002; von Herrath and Harrison 2003; Zou 2005). As most tumor antigens are self-antigens, Treg-cell-mediated suppression of TAA-reactive lymphocytes has been proposed as a potential mechanism to explain the failure of antitumor immunity (Khong and Restifo 2002; Curiel *et al.* 2004; Sakaguchi 2005).

In humans, a higher frequency of Treg cells was found in the peripheral blood and in tumor sites of patients with different cancers (Ichihara *et al.* 2003; Wolf *et al.* 2003; Ormandy *et al.* 2005). These studies showed that peripheral Tregs have potent suppressive activity *in vitro* and also that a high frequency of these cells could reduce TAA-specific immunity in patients with cancer. A correlation between increased numbers of Treg in cancer patients and poor prognosis or survival was also demonstrated (Sasada *et al.* 2003; Curiel *et al.* 2004). Moreover, Treg with specificity for antigens expressed by human tumors have recently been identified and vaccination of mice with similar tumor antigens has shown to expand Treg (Wang *et al.* 2004; Nishikawa *et al.* 2005; Wang *et al.* 2005).

Accumulation of Treg at the tumor site balances the system towards immunosuppression. Thus, successful immunotherapy relies on combinatorial approaches able to overcome normal and tumor-induced tolerogenic mechanisms, as well as immune escape.

In this work, we identified new humoral targets induced by a protective immune response, in the RENCA murine tumor model. Our findings highlight the role of these proteins in carcinogenesis and possible mechanisms of their immunogenicity. In addition, by using different antigen-based vaccines, our studies suggest that these antigens may be involved in tolerance by activating an immunoregulatory pathway.

## MATERIAL AND METHODS

### 2.1 Mice

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.

### 2.2 Tumor Models

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). Splenocytes were cultured *in vitro* in RPMI 1640 media supplemented with 10% (v/v) IFS, 50 $\mu$ M  $\beta$ -mercaptoethanol, 10 mM HEPES buffer, 2 mM L-glutamine, 100 units/ml penicillin/ streptomycin and 1 mM nonessential aminoacids. All cell lines were grown at 37°C, with 5% (v/v) CO<sub>2</sub>.

### 2.3 RENCA cDNA Library Construction

To construct a cDNA expression library from RENCA cells, 5 $\mu$ g of polyadenylated mRNA was prepared with a messenger RNA (mRNA) isolation kit (Stratagene). Briefly, the cell culture was homogenized by using guanidine isothiocyanate (GIT) and  $\beta$ -mercaptoethanol and the clear lysate was hybridized to the oligo(dT) cellulose resin that specifically binds the 3'-polyadenylated tail of mRNA, at room temperature. After several washes to remove unwanted components of the crude lysate from the poly(A)<sup>+</sup>mRNA, the oligo(dT) cellulose was loaded into a column, and mRNA was eluted at 65°C, with elution buffer.

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. Briefly, purified mRNA was reversed transcribed with Moloney Murine leukemia virus reverse transcriptase and first strand synthesis was performed using an oligo(dT) linker primer with an internal Xho I site and 5'-methyl dCTP. The 5'-methyl dCTP leads to methylation of the first strand, protecting it from digestion with Xho I. To generate the second cDNA strand, Rnase H is used to nick

the RNA strand and dCTP (un-methylated) was used, so that the Xho I sites in the linker were accessible for digestion. The cDNA is then blunted with Pfu DNA polymerase (Stratagene) and EcoR I adaptors are ligated (adaptors were phosphorylated only on the blunt side so that they inefficiently anneal to one another). A kinase reaction was then performed on the ligated adaptors so that the cDNA would be able to be cloned in the vector. Xho I digestion was carried out, resulting in fragments with 5' EcoR I and 3' Xho ends. The cDNA was size fractionated on a Sephacryl S-500 column and fragments (with 1200 base pairs or larger) were cloned directionally into the UniZap bacteriophage expression vector (Stratagene) and packaged into phage particles using GigapackIII gold extracts. The library consisted of  $10^6$  primary recombinants and was amplified to  $10^9$  plaque forming units.

## 2.4 Phage Library Immunoscreening

Serum was collected and pooled from each group of 8 mice, one week after last immunization, and stored at  $-80^{\circ}\text{C}$ . To remove antibodies reactive against antigens related to the vector system, pooled serum from 10 mice was preabsorbed four times against bacteria lysed by nonrecombinant ZAP Express phages. The preabsorbed serum mix was diluted in 1x TBST (Tween Tris buffered saline: 200 mM Tris, 110 mM NaCl, 0.05% (v/v) Tween 20) and 0.01% (w/v) Na-azide to a final concentration of 1:300.

Immunologic screening of our RENCA cDNA expression library was done according to the manufacturer's instruction (picoBlue Immunoscreening Kit, Stratagene). In brief, *Escherichia coli* XL1 Blue MRF' (XL1 Blue) bacteria were transfected with the expression library and this solution was mixed with top agar and poured onto NZY plates. Plated phages ( $5 \times 10^4$  plaques per 150 cm dish) were propagated at  $42^{\circ}\text{C}$  for about 3.5 hours until a dense bacterial lawn could be seen. Expression of recombinant protein was induced by incubation with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG 10 mM in ddH<sub>2</sub>O, Invitrogen) - treated nitrocellulose membranes (Schleicher and Scheull), placed onto the plates and then incubated for another 3.5 hours, at  $37^{\circ}\text{C}$ . After marking the membranes orientation in relation to the plate, membranes were then washed extensively in TBST and subsequently left overnight at  $4^{\circ}\text{C}$ , in blocking solution, 5% (w/v) non-fat dry milk (NFDM) in Tris buffered saline (TBS). Next day, membranes were washed in TBST and incubated with precleared mouse serum overnight, at  $4^{\circ}\text{C}$ . The membranes were washed several times before probed with an alkaline phosphatase-conjugated polyclonal anti-mouse pan IgG antibody (Jackson ImmunoResearch, diluted 1:2000 in TBST). Antigen-antibody complexes were visualized with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt and nitro-blue tetrazolium chloride (BCIP, NBT from Promega) color development solution (developing buffer: 100 mM Tris-Cl, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, pH 9.5). Positive

phage plaques were cored out and stored in SM buffer (100 mM NaCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 50 mM Tris·Cl, pH 7.5), at 4°C. Selected clones were purified through secondary and tertiary screenings until single plaques were isolated.

## **2.5 Plasmid Excision**

Isolated serum-reactive clones were converted into phagemids by *in vivo* excision using the ExAssist Interference-Resistant Helper Phage (Stratagene) according to the manufacturer's instructions. Briefly, Phage stock was incubated with XL1-Blue MRF' bacteria and ExAssist helper phage at 37°C, for 15 minutes. After heating up for 20 minutes at 65-70°C, the mixture was centrifuged. In the final step, SOLR cells were transformed with the excised plasmid and incubated on ampicilin (Sigma) LB bacterial plates.

## **2.6 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.

## **2.7 Sequence Analysis of Positive Clones**

Plasmid DNA from positive clones were isolated using commercially available kits (QIAGEN). The length of DNA inserts was determined after double EcoRI 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](http://www2.licr.org/cancerimmunomeDB)) was also analyzed for representation of human orthologs of our cloned mouse antigens.

## **2.8 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 MgCl<sub>2</sub>, 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.

## **2.9 Polymerase Chain Reaction (PCR)**

The cDNA preparations were done as described above. One-tenth of the RT reaction mixture was used for PCR amplification of specific products, with oligonucleotides flanking the open-reading frames of identified cDNAs. Amplification reactions were performed in a MiniCycler (MJ Research) with Expand High Fidelity PCR System (Roche) according with manufacturer's recommendations. PCR mixtures were heated up to 94°C for 2-5 minutes, followed by 30-40 thermal cycles (denaturation at 94°C for 1 minute, annealing at 50-60°C for 1 minute, and primer extension for 1 minute at 72°C). For GC rich templates, we used 95°C for 3 minutes in the first step. Elongation step was performed at 72°C for 2-5 minutes (depending on the fragment length). Amplification products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. PCR primers specific for select SEREX-defined RENCA antigens were designed based on their published sequence (NCBI).

## **2.10 Total RNA Isolation**

Total RNA was isolated from tumor cells or normal tissues with TRizol (Gibco/BRL) (a 4 M guanidine thiocyanate and phenol solution) 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 ddH<sub>2</sub>O and stored at -80°C.

## **2.11 Northern Blot**

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 [1g agarose, 10 ml 10x MOPS running buffer (10x MOPS running buffer: 0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA), 5.4 ml of 37% (v/v)

formaldehyde and 85 ml of sterile water] and electrophoresed in 1x MOPS running buffer. After confirming the RNA integrity under the UV light, a picture was taken and the gel was rinsed in RNase free water for 5 minutes before transfer (see below).

### **2.11.1 Northern Blot Transfer**

After electrophoresis, the gel was placed on top of sponges soaked in 10x SSC buffer (20x SSC buffer: 3.0 M NaCl, 0.3 M sodium citrate). A pre-wetted positively charged nylon membrane (Hybond-XL, Amersham Biosciences) was placed onto the gel, followed by several layers of gel blot paper (Schleicher Schuell) and a stack of paper towels. After overnight transferring, the membrane was removed and rinsed in 2x SSC for 5 minutes. The RNA was covalently bound to the membrane by UV-crosslinking (UV Stratalinker 2400, Stratagene). The membrane was then stored at -80°C until hybridization was performed.

### **2.12 Hybridization**

Multitissue (Stratagene) or mouse tumor mRNA blots were incubated for 1 h in the appropriate amount of hybridization solution (ExpressHyb, Clontech), with continuous shaking, at 68°C, in a hybridization oven. A 5 ml aliquot of the hybridization solution was also placed in oven. For the probe preparation, 25 ng of template DNA ranging from 500 to 1500 nucleotides was labeled with [ $\alpha^{32}$ ]P-dCTP (NEN/Perkin Elmer Life Sciences) according to the manufacturer's instructions (Prime-It II Random Primer Labeling Kit, Stratagene). The non-incorporated radioactive dCTP was removed with a sepharose column (Probe Quant G-50 micro column, Amersham Biosciences). After checking for incorporation above 25% of the total radioactivity, the probe was boiled for 5 minutes, chilled on ice for 30 seconds and then mixed with the 5 ml of pre-heated aliquot of hybridization solution. This probe solution was added to the pre-hybridized membrane and incubated for one hour to overnight.

The radioactive hybridization solution was then discarded and the membrane washed at progressive higher stringency. Briefly, the membrane was incubated twice using 2x standard saline citrate (SSC buffer) with 0.1% (w/v) SDS at room temperature, for 10 minutes, followed by a final washing step at 60°C, with 0.1x SSC (w/v) buffer/ 0.1% SDS for 30 minutes. Autoradiography was conducted at -80°C for 1-5 days, by exposing the membrane to film (Kodak X-OMAT-AR) and an intensifying screen. Thereafter, the filters were stripped and rehybridized with 18S ribosomal RNA or GAPDH (Glycerol 3-phosphate dehydrogenase) as a loading control.

### **2.13 Whole cell lysates**

Whole cell lysates were prepared by washing cells in PBS followed by 30 minutes incubation at 4°C, with agitation, in a lysis buffer containing the detergent NP-40 and protease inhibitors [(PBS with 0.5% (v/v) NP-40/IGEPAL CA-630, 1 µg/ml pepstatin, 10 µg/ml leupeptin, 174 µg/ml PMSF, 100 µg/ml soybean trypsin inhibitor, 65.5µg/ml aminocaproic acid, all Sigma reagents)]. Samples were then centrifuged and the supernatant stored at -80°C, after protein concentration was determined with a BioRad protein assay.

### **2.14 SDS polyacrylamide gel electrophoresis (SDS PAGE)**

Gel electrophoresis was performed on polyacrylamide gels [8% to 12% resolving gels prepared in 4x Tris·Cl/SDS resolving buffer: 1.5 M Tris·Cl, 0.4% SDS; and 3.9% stacking gel prepared in 4x Tris·Cl/SDS stacking buffer, pH 6.8: 0.5 M Tris·Cl, 0.4% SDS; 30% acrylamide/0.8% bisacrylamide; 5x electrophoresis buffer: 0.125 M Tris base, 0.96 M glycine, 0.5% SDS].

Each lane was loaded with an appropriate amount of protein diluted in PBS and 6x denaturing buffer [70% (v/v) 4x Tris·Cl/SDS, pH 6.8, 30% (v/v) glycerol, 10% (w/v) SDS, 0.6 M DTT, 0.012% bromophenol blue]. Samples were boiled for 5 minutes and then loaded on a denaturing polyacrylamide gel. A stained protein ladder was used for determining the weight of protein bands (Invitrogen).

### **2.15 Immunoblotting (Western)**

After electrophoresis, proteins from the gel were transferred into a polyvinylidene fluoride membrane (PVDF) membrane (Millipore) with a wet transfer system (BioRad) according to the manufacturer's instructions (10x transfer buffer: 25mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol). The membrane was blocked with 5% (w/v) NFDM/ PBS overnight at 4°C, or 2 hours at room temperature. The appropriate first antibody was diluted in 5% (w/v) NFDM/TTBS and incubated at room temperature for 1 hour.

After washing with TTBS the 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).

If necessary, blots were stripped by incubation in a stripping solution (100 mM β-mercaptoethanol, 62.5 mM Tris·Cl, pH 6.8, 2% (w/vol) SDS) at 65°C, in a hybridization oven.

## 2.16 FACS Analysis

Fluorescent staining of RENCA cells with sera was performed by using PE-conjugated goat anti-mouse IgG. Fluorescent staining of splenocyte populations was performed by using FITC- or phycoerythrin-, conjugated mAbs to CD3, CD8, CD4, CD11c, CD80 obtained from PharMingen. Stained cells were analyzed on a FACScan cytometer (Becton Dickinson).

## 2.17 Vector Construction

The cDNAs for the murine GM-CSF and RENCA tumor associated antigens (TAA) were amplified by reverse transcription PCR and subcloned into pMFG.S, a replication-deficient retroviral vector (pUC19/MMLV-based). 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. Resulting constructs (pTA) were introduced into 293GPG cells to generate recombinant virus with amphotropic range.

Green Fluorescent protein (GFP) and TAA cDNAs were subcloned into pCDNA3.1 (-) (INVITROGEN) under the T7 RNA polymerase promoter, for IVT (see below). Large scale preparations of each construct were generated using Maxi Prep Kits (QIAGEN).

## 2.18 Production of High Titer VSV-G-pseudotyped Retroviral Particles and Infection

The production of amphotrophic retroviral particles was done according with Ory *et al.* by using 293 GPG cells that express MMLV gag.pol constitutively and VSV-G protein under a tetracycline-repressed promotor (Ory *et al.* 1996). In brief, the 293 GPG packaging cells were plated in tetracycline containing media. Next day cells were washed with serum free media (Opti-MEM, Invitrogen) and incubated with a suspension of the plasmid, Lipofectamine 2000 (Invitrogen) and Opti-MEM. 6 hour post transfection DMEM (10% fetal calf serum) is added and 24 hours after, the mix was replaced with regular DMEM. The viral supernatant was harvested 72h after, filtered through a 0.45µm filter (Pall Gelman) and stored at 80°C and replaced with regular DMEM. The procedure was repeated for about 5 consecutive days until most of the 293 GPG cells were dead. Viral supernatants were thawed and concentrated by ultracentrifugation at 50.000 g for 1.5 hours, at 4°C. After discarding the supernatant, the viral pellet was resuspended in a small volume of 10% Hanks balanced saline solution (HBSS) in PBS. The tubes were

incubated overnight at 4°C and on the next day, the concentrated viral solution was aliquot and stored at -80°C.

For the retroviral infections,  $2 \times 10^5$  target cells were plated for 24 hours in 6cm Petri dishes. Diluted viral supernatants in the appropriate media were added for 4-6 hours in the presence of 8 g/ml hexadimethrine bromide (Polybrene, Sigma). Target cells could go through a second round of infection in order to be transduced with more than one gene. Two murine tumor cell lines of H-2d background, CMS5 and CT-26 were exposed to viral supernatants and transduced cells were characterized for expression or secretion of the gene product.

### **2.19 Enzyme-Linked Immunosorbent Assays (ELISAs)**

GM-CSF secretion from transduced CT26 and CMS5 cell lines was measured by an ELISA kit as indicated by the manufacturer's instructions (mouse GM-CSF BD OptEIA ELISA Set). 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.

### **2.20 Antibody Purification**

Anti-murine CTLA-4 antibody 9H10 (hamster) was isolated from hybridoma culture supernatant previously described (Krummel and Allison 1995). 9H10 was purified using a protein G Sepharose column (MabTrap Kit, Amersham) followed by desalting using a matrix Sephadex column (HiTrap desalting, Amersham). The concentration was measured by Elisa using control hamster IgG (Jackson ImmunoResearch laboratories) and adjusted with sterile PBS.

### **2.21 In vivo Studies**

For vaccination experiments, survival was assessed by monitoring mice twice a week. Evidence of progressive tumor growth was done by palpation and inspection for a period of 60 days (after challenge). Otherwise, they were sacrificed when tumors reached 1.5-2 cm in longest diameters. Mice were bled from the ocular area usually 7 days after the last immunization and sera was pooled from each group. After centrifuging for 15 minutes, supernatant was collected and kept at -70°C.

## **2.21.1 “Naked” DNA Vaccines**

### **2.21.1.1 Intramuscular Injection**

Mice were immunized 1-3 times with the indicated dose of pTA constructs, in PBS, into quadriceps muscle in the rear leg. DNA inoculations were given 1 week apart and when indicated challenge was administered 2 weeks later. The maximum volume used per inoculation was 200 $\mu$ l.

### **2.21.1.2 Gene Gun Delivery of DNA**

Plasmid DNA was affixed to gold particles by adding 10 mg of 0.95- $\mu$ g gold powder (Bio-Rad) and an appropriate amount of plasmid DNA (amplified using Endotoxin Free Plasmid purification kit, QIAGEN) to a 1.5-ml centrifuge tube containing 50  $\mu$ l of 0.1 M of spermidine. Plasmid DNA and gold beads were coprecipitated by the addition of 50  $\mu$ l of 2.5 M CaCl<sub>2</sub> during vortex mixing, after which the precipitate was allowed to settle for 5-10 minutes at room temperature. After washing 3 times in cold ethanol, the precipitate was resuspended in 1.0 ml of ethanol. Then, 100  $\mu$ l of gold/DNA suspension was layered onto 1.8 cm X 1.8 cm Kapton sheets and allowed to settle for several minutes until were dried. The total amount of DNA per sheet was a function of the DNA/gold ratio. Animals were shaved in the abdominal area and DNA-coated gold particles were delivered into abdominal skin using helium pressures of 300-500 psi with a Helium Gene Gun.

## **2.21.2 DC Vaccination**

### **2.21.2.1 DC Generation from Bone Marrow Cultures**

Murine DCs were generated from bone-marrow progenitors as previously described (Ashley *et al.* 1997). In brief, bone marrow was flushed from the long bones of the limbs and depleted of red cells with ammonium chloride Tris buffer for 3 minutes in a 37°C water bath. Cells were then washed twice in cold RPMI 1640 supplemented medium. Supernatant of CMS5/GM cell line was used as a source of GM-CSF for generation of murine BMDC. GM-CSF-containing supernatant from these cells was harvested after 24h, centrifuged at high speed to eliminate cell debris and used at a final dilution 1/10.

Three days later, the floating cells (mostly granulocytes) were removed and the adherent cells were replenished with fresh GM-CSF containing medium. Four days later, non-adherent cells were harvested (immature day 7 DC), washed, and replated at 10<sup>6</sup>/ml

in GM-CSF-containing medium. After 4-5 days the non-adherent and loosely adherent cells were harvested as DC (mature day 12 DC), washed and transfected.

### **2.21.2.2 *In Vitro* Transcription (IVT) of cDNA**

Plasmids for transcribing GFP and RENCA TAA were generated by cloning the corresponding cDNAs into pcDNA3.1(-) plasmid (Invitrogen) under the T7 RNA polymerase promoter and large scale preparations were generated using Maxi Prep Kits (QIAGEN).

The plasmids were then linearized and after phenol/chloroform extraction and ethanol precipitation, 1µg of cDNA was placed in a standard *in vitro* transcription reaction using a mMessage mMachine T7 Ultra Kit (Ambion). The reaction was carried out at 37°C for 2 hours, followed by Dnase I incubation for 15 minutes. A poly(A) tail of 50-100 base pairs was added to the RNA transcripts by *E. coli* Poly(A) Polymerase (E-PAP), at 37 °C, for 45 minutes. Ammonium acetate was added, and RNA was isolated by phenol/chloroform extraction and isopropanol precipitation. After centrifugation, the RNA pellet was resuspended in RNase-free water, and the quantity and purity were determined by UV spectrophotometry. An aliquot was electrophoresed on an agarose/formaldehyde gel to determine the size range of the products.

### **2.21.2.3 RNA Transfection of Murine DCs**

DC were collected on day 12, washed twice in serum-free Opti-MEM medium (Life Technologies) and resuspended about  $1 \times 10^6$ /ml in Opti-MEM medium containing 0.1µg/ml of LPS in 15-ml polypropylene tubes (Beckton Dickinson).

The cationic lipid DOTAP (Roche) was used to deliver RNA into the cells. In brief, an appropriate amount of *in vitro* transcribed RNA and DOTAP were mixed in a total volume of 500 µl of Opti-MEM at room temperature for 20 minutes. The RNA-lipid complex was added to the DCs in a total volume of 1 ml and incubated, with occasional agitation, for about 3 hours at 37°C in a water bath. The cells were washed twice and resuspended in PBS for intraperitoneal or subcutaneous immunizations (0.5- $1.5 \times 10^6$  RNA-pulsed DCs in 500 µl of PBS per mouse). RNA-pulsed DCs were used for FACS analysis before vaccination.

### **2.21.3 Whole Tumor Cell Vaccines**

GM-CSF secreting cell lines (CMS5/GM and CT26/GM) transduced with RENCA antigens (pTA) were used in our whole tumor cell-based vaccines. The level of GM-CSF

secretion was determined using a GM-CSF specific enzyme-linked immunosorbent assay detection system (see 2.19).

Transduced tumor cells were treated with trypsin and washed twice in serum free Hank's balanced saline solution (HBSS) (GIBCO) before inoculation. Trypan blue-resistant cells were resuspended to the appropriate concentrations and injected in 0.5 ml of HBSS. Mice were injected subcutaneously (s.c.), on the abdominal wall, with  $5 \times 10^5$  irradiated (35Gy) tumor cells. Unless specified otherwise animals were immunized twice, one week apart and challenged 2 weeks later with  $5 \times 10^6$  live, WT RENCA cells injected s.c. on the back.

## **2.22 Purification of CD4<sup>+</sup> CD25<sup>+</sup> and CD4<sup>+</sup> CD25<sup>-</sup> T cells**

Spleen cells were fractionated into CD25<sup>-</sup> and CD25<sup>+</sup> using CD4 CD25<sup>+</sup> regulatory T cell isolation kit (Miltenenyi Biotec). Briefly, for the isolation of CD4<sup>+</sup> T cells, non-CD4<sup>+</sup> T are indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies and anti-biotin microbeads. In parallel, cells are labeled with CD25-PE. The cell suspension was loaded onto a MACS column which was placed in the magnetic field of a MACS separator. The magnetically labeled non-CD4<sup>+</sup> T cells were retained in the column, while the CD4<sup>+</sup> T cells run through. For the isolation of CD4<sup>+</sup>CD25<sup>+</sup> cells, the CD25<sup>+</sup> PE-labeled cells in the enriched CD4<sup>+</sup> T cell fraction were magnetically labeled with anti-PE microbeads. The cell suspension was loaded onto a column which was placed in the magnetic field on a MACS separator. The magnetically labeled CD4<sup>+</sup>CD25<sup>+</sup> cells were retained in the column, while the unlabelled cells run through (this corresponds to the CD4<sup>+</sup>CD25<sup>+</sup> T cells fraction). After removal of the column from the magnetic field, the retained CD4<sup>+</sup>CD25<sup>+</sup> cells were eluted as the positively selected cell fraction and the process of separation was repeated over a new column, to achieve high purities. FACS analysis was performed by staining with FITC-anti-CD4 and PE-anti-CD25 to confirm that purity of CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cell populations was > 95%.

## **2.23 Generation of RENCA-specific Effector T Cells**

Splenocytes were obtained from animals vaccinated twice s.c., one week apart, with irradiated R-WT cells and harvested 7-10 days after last immunization. Upon lysis of erythrocytes with ammonium chloride, cells were washed twice and resuspended in supplemented 10% FCS in RPMI. Wild-type RENCA cells were treated with 200U/ml IFN- $\gamma$  for 24 h to increase expression of MHC class I and II molecules on their surface, washed twice, and irradiated (100 Gy). These stimulator cells were then added to  $5 \times 10^5$

splenocytes and incubated *in vitro* for 5 days in the presence of IL-2 10 U/ml. These effector cells were collected and used for T-cell proliferation assay.

### **2.24 T-cell Proliferation Assay**

For the measurements of T-cell proliferation to RENCA cells,  $5 \times 10^4$  splenic T cells (previously stimulated *in vitro*) were plated in 96 flat-bottomed plates and cultured for 72h with  $1 \times 10^5$  RENCA stimulators.  $5 \times 10^4$  CD4<sup>+</sup> CD25<sup>-</sup> or CD4<sup>+</sup> CD25<sup>+</sup> T cells were added to these cultures. Proliferation was evaluated by pulsing with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine for the last 15-20 hours. Proliferation was determined on a 1205 Betaplate reader (Wallac, Turku, Finland).

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## RESULTS

### 3.1 Humoral Response Induced by Vaccination with GM-CSF Secreting RENCA cells

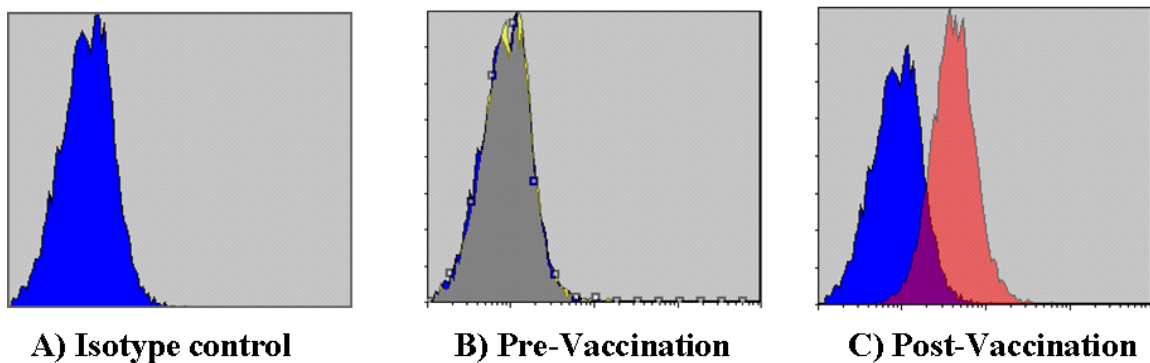
Tumor cells express a variety of gene products that can be recognized by the host's immune system (Boon and van der Bruggen 1996). Innate and adaptive immune recognition of these tumor-associated antigens (TA) can be used to activate the immune system to mount an effective, tumor-specific immune response that may ultimately lead to tumor regression.

Renal Cell Carcinoma (RENCA) is an inherently immunogenic tumor cell line when inactivated by irradiation. Vaccination with irradiated wild-type RENCA cells (R-WT) can induce some tumor protection in mice. Nonetheless, previous findings by our group have shown that upon GM-CSF transduction (R-GM) this vaccine can promote higher levels of tumor protection *in vivo* (Dranoff *et al.* 1993). To assess if this immunogenicity was associated with the induction of a humoral response, pooled sera collected from non-immunized mice (Pre) or mice vaccinated ten times with irradiated R-GM (Post) cells were compared by flow cytometry. After incubation with sera, a secondary anti-mouse IgG antibody was used to determine antibody titers recognizing surface proteins on RENCA cells. As shown in Figure 3.1, tumor cells were strongly positive with serum from vaccinated mice. In contrast, Pre serum or staining with isotype control antibody showed no reactivity. Moreover, FACS analysis demonstrates no reactivity with sera collected after one or two vaccinations, suggesting that the number of immunizations may contribute to increased antibody reactivity against RENCA determinants (data not shown). These data support the notion that tumor rejection observed *in vivo* in this tumor model is associated with induction of a humoral response.

### 3.2 RENCA cDNA Library Construction and Immunoscreening

In our study, we were interested in examining in more detail the immunogenic targets of this humoral response induced upon vaccination. We used a serologic analysis by a phage-based expression screening system (SEREX) in order to identify tumor-associated antigens mediating GM-CSF improved tumor protection *in vivo*. This approach has been shown to be a powerful tool to identify tumor antigens associated with concomitant T and B cell response in cancer patients (Jager *et al.* 2000; Jager *et al.* 2000; Ayyoub *et al.* 2002). A cDNA expression library was constructed in the Lambda Zap phage vector using

mRNA derived from RENCA cells. A primary cDNA library with  $2 \times 10^6$  independent clones was established and used for the immunologic screening.



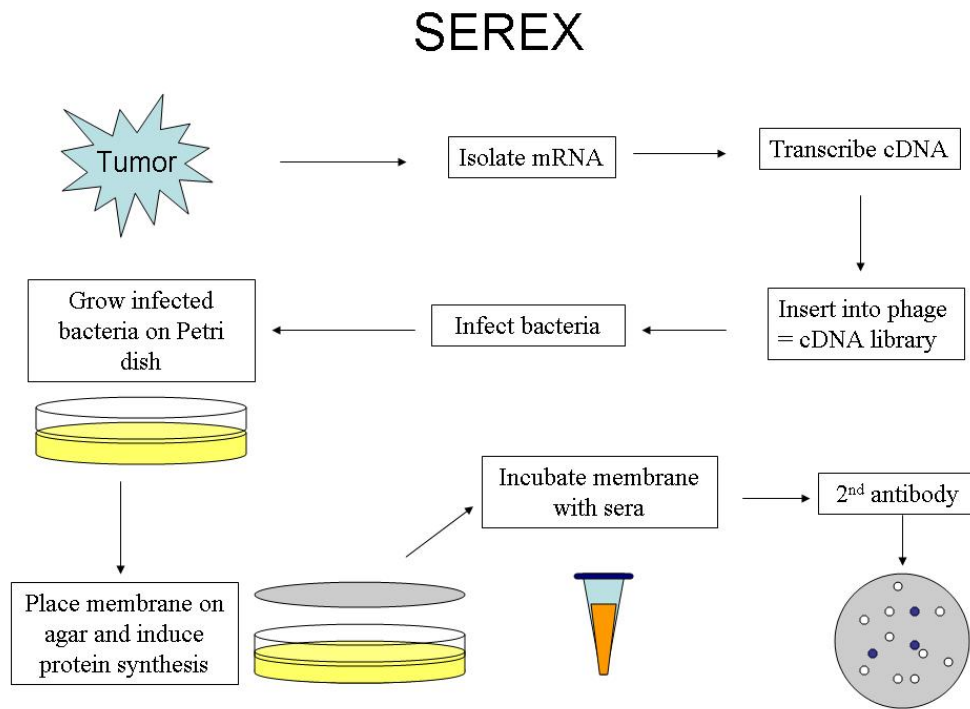
**Figure 1: Humoral response to cell surface antigens induced by vaccination with irradiated GM-CSF secreting RENCA cells.** Flow cytometry analysis of RENCA cells after treatment with A) isotype control antibody or pooled sera diluted 1/100 from B) naïve (Pre) or C) mice vaccinated 10 times with R-GM (Post), were tested against cell surface antigens using a secondary PE-labelled goat anti-mouse IgG antibody.

Two groups of BALB/c mice were vaccinated either with irradiated WT or with irradiated GM-CSF secreting RENCA cells. Sera collected after 10 immunizations were pooled from each group of vaccinated mice and used at 1:300 dilutions to screen the library. Figure 3.2 provides a schematic representation of the library screening. An initial immunoscreening, using pre-cleared serum, was performed to determine if reactivity to the library was present. Positive plaques were isolated by the reactivity of the recombinant proteins with high-titer IgG antibodies present in the sera from vaccinated mice. Positive plaques were re-plated for a secondary and tertiary screening until clonality was reached.

### **3.3 Sequence Analysis of RENCA-associated Tumor Antigens: Serologic Differences Induced by GM-CSF-transduced Tumor Vaccines**

cDNA inserts from positive clones, detected with sera from vaccinated mice, were isolated, restriction enzyme digested and their DNA sequence aligned against the GeneBank and SEREX database. Two clones were identified with sera from wild-type RENCA cells versus 177 clones identified with sera from GM-CSF secreting RENCA cell vaccines (Table I). Out of 180 immunoreactive clones, sequence analysis and homology

search revealed that they represent a total of 28 unique antigens, 21 of which corresponding to proteins with known function (Table I). Table II lists all gene products with known function that were identified during our serologic analysis. Database search indicates that these genes represent a diversity of antigens that range from intracellular to membrane localization and include secreted proteins.



**Figure 3.2: Schematic representation of serological identification of antigens by recombinant expression cloning (SEREX).**

Irradiated GM-CSF-secreting RENCA cells are known to be more efficient than wild-type cells alone in inducing tumor protection against live tumor cells (Dranoff *et al.* 1993). We then addressed the question if these differences, between vaccination with wild-type and transduced tumor cells, were associated with immune recognition of different antigenic targets. Comparison of serum reactivity showed 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 against R-WT sera (Table I). These results confirm that GM-CSF-transduced RENCA cells induce a quantitatively different humoral response when compared with wild-type tumor cells, which is characterized by a more diverse antibody repertoire.

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

	<b>Sera*</b>	
	<b>RENCA-WT<sup>i</sup></b>	<b>RENCA-GM<sup>ii</sup></b>
<b>Positive clones</b>	<b>3</b>	<b>177</b>
<b>Unique Antigens</b>	<b>2</b>	<b>26</b>
<b>Gene Products with known function</b>	<b>1</b>	<b>20</b>
<b>Gene Products with unknown function</b>	<b>1</b>	<b>6</b>

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  $5 \times 10^5$  irradiated wild-type RENCA cells.

ii) serum from mice vaccinated with  $5 \times 10^5$  irradiated, GM-CSF-secreting RENCA cells.

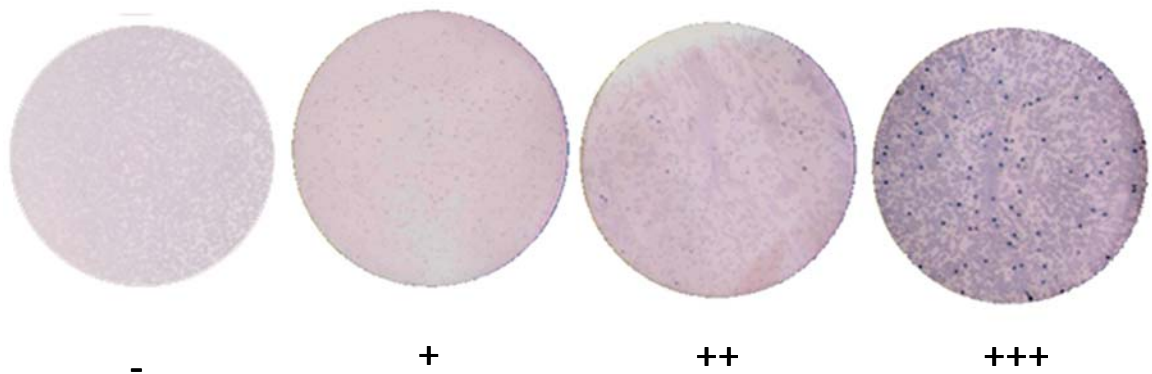
### **3.4 Antibody Response Against RENCA-associated Antigens is a Result of Vaccination**

Once clones identified by serologic screening 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, differences in the intensity of reactivity can be clearly observed (Figure 3.3). 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, strong antibody reactivity to each of the isolated gene products was detected in Post-immunized sera. In contrast, no reactivity was observed using sera from non-vaccinated mice (Pre). These data show that the immune response observed against these antigens is a result of vaccination and, for the concentrations of sera tested, this antibody repertoire was not present in naïve mice.

**Table II: Functional characterization of RENCA gene products identified by serologic screening.**

<b>Function</b>	<b>Abbreviation</b>	<b>Gene products Identity/Homology</b>	<b>Serum*</b>	<b>Localization</b>
<b>Protein synthesis/Turnover</b>	<b>EIF4A</b>	Translation initiation factor 4	GM	Intracellular
	<b>RPL15</b>	Ribosomal protein L15	GM	Intracellular
	<b>PDI/Erp59/Ph4b</b>	Protein disulfide isomerase	GM	Intracellular Membrane Secreted
	<b>PSMB5</b>	Proteasome subunit, beta 5	GM	Intracellular
<b>DNA/RNA binding</b>	<b>TCEA1/TFIIS</b>	Transcription elongation factor A1	GM	Intracellular
	<b>H1(0)</b>	H1 Histone family, member 0	GM	Intracellular
	<b>HnRNP C1/C2</b>	Heterogeneous ribonuclear protein C1/C2	GM	Intracellular
	<b>SSRP1</b>	Structure specific recognition protein 1	GM	Intracellular
<b>Metabolic pathway</b>	<b>FDS</b>	Farnesyl diphosphate synthase	GM	Intracellular
	<b>AR</b>	Aldose Reductase	GM	Intracellular
	<b>ACAT2</b>	sterol O-acyltransferase 2	GM	Intracellular
	<b>F1F0 ATPsynthase</b>	ATPsynthase, mitochondrial F1F0 complex	GM	Intracellular
	<b>Cytokine</b>	<b>PBEF / Visfatin</b>	Pre-B colony enhancing factor	GM
<b>Cytoskeleton</b>	<b>ROCK2</b>	Rho kinase 2	WT	Intracellular
	<b>GNB2</b>	Guanine-nucleotide binding protein	GM	Membrane Intracellular
	<b>IQGAP1</b>	IQ motif containing GTPase activating protein 1	GM	Intracellular
	<b>CD44 ARF4</b>	Cell adhesion molecule CD44 ADP-ribosylation factor 4	GM GM	Transmembrane Intracellular
<b>Stress Inducible</b>	<b>HRP12</b>	Heat Responsive Protein12	GM	Intracellular
<b>Cell death</b>	<b>Apg3</b>	Autophagy-related 3 (yeast)	GM	Intracellular
	<b>Apg12l</b>	Autophagy-related 12 (yeast)	GM	Intracellular

\* Serum obtained from mice vaccinated with irradiated wild type RENCA cells (WT) or GM-CSF secreting cells (GM), diluted 1:300.



**Figure 3.3: 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.

### **3.5 Antibody Reactivity Against RENCA Antigens Changes with the Number of Vaccinations**

The phage plate assay allows a simple and rapid semi-quantification of antibody response. Using this approach, we determined if the number of immunizations could induce differences in the antibody repertoire. Sera collected after 1, 2, 3 or 10 inoculations (W1, W2, W3, W10 respectively), were compared at 1:300 dilution in a phage plate assay by measuring intensity of antibody response to the same target antigens. After incubation with replica-plated phages, all antigens tested showed weaker to no reactivity with sera from early time points, W1 and W2 (Table IV). Evidence of antibody reactivity could only be detected after the third immunization, W3. The strongest antibody response to this panel of antigens was observed with the latest time point corresponding to sera collected after 10 vaccinations (W10). Taken together, these observations show that a more potent antibody response is evoked by increasing immunizations.

**Table III: Comparison of serum reactivity against a panel of identified RENCA associated proteins.**

<b>Clone Name</b>	<b>Pre-vaccination</b>	<b>Post vaccination</b>
<b>PDI</b>	-	+++
<b>HnRNP1/C2</b>	-	+++
<b>SSRP1</b>	-	+++
<b>AR</b>	-	+++
<b>HRP12</b>	-	+++
<b>Apg3pl</b>	-	+++
<b>ARF4</b>	-	+++
<b>EIF4A</b>	-	+++
<b>ACAT2</b>	-	+++
<b>PBEF</b>	-	+++
<b>IQGAP1</b>	-	+++
<b>TCEA1</b>	-	+++
<b>Apg12</b>	-	+++
<b>F1F0 ATPsynthase</b>	-	+++
<b>RPL15</b>	-	+++
<b>CD44</b>	-	+++
<b>H1</b>	-	+++
<b>GNB2</b>	-	+++
<b>FDS</b>	-	+++
<b>PSMB5</b>	-	+++
<b>R2</b>	-	+++

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.

### **3.6 Reactivity of RENCA Associated Antigens with Sera from Cancer Patients**

Several of the immunogenic antigens that we pulled out from our library have been previously identified in patients with other tumors and have their human orthologues represented in the SEREX database ([www.licr.org/SEREX](http://www.licr.org/SEREX)) (Table V). For example, antibodies against ROCK were identified in immunologic screenings using sera from patients with different types of cancer, including human squamous cell lung carcinoma, breast cancer, fibrosarcoma, multiple myeloma, and human renal cell carcinoma (Scanlan

*et al.* 1999; Diesinger *et al.* 2002; Bellucci *et al.* 2004). In addition, a member of this family was also identified as a humoral target in the B16 melanoma mouse model by our group (Park *et al.*, in preparation). Since human and mouse ROCK2 proteins share about 95% homology, we decide to test our mouse clone against sera from melanoma patients that had been vaccinated with GVAX – an autologous GM-CSF-secreting melanoma vaccine (Soiffer *et al.* 1998; Nemunaitis 2005). As a control, we also tested this clone against sera from normal donors. Reactivity toward mouse ROCK2 clone was detected in sera samples from 10 out of 11 melanoma patients (Table VI). In contrast, only 2 out of 5 normal donors were positive against this protein.

**Table IV: Antibody repertoire increases with the number of vaccinations.**

<b>Clone Name</b>	<b>W1</b>	<b>W2</b>	<b>W3</b>	<b>W10</b>
<b>PDI</b>	-	-	+	+++
<b>HnRNP</b>	-	-	+	+++
<b>SSRP1</b>	-	-	+	+++
<b>AR</b>	-	-	+	+++
<b>HRP</b>	-	-	+	+++
<b>Apg3p</b>	-	-	+	+++
<b>ARF4</b>	-	-	+	+++
<b>EIF4A</b>	-	-	+	+++
<b>ACAT2</b>	-	-	+	+++
<b>PBEF</b>	-	-	+	+++
<b>IQGAP1</b>	-	-	+	+++
<b>TCEA1</b>	-	-	+	+++
<b>Apg12</b>	-	-	++	+++
<b>ATPsynthase</b>	-	-	++	+++
<b>RPL15</b>	-	-	+	+++
<b>CD44</b>	-	-	+++	+++
<b>H1</b>	-	-	+	+++
<b>GNB2</b>	-	-	+	+++
<b>FDS</b>	-	-	++	+++
<b>PSMB5</b>	-	-	+	+++
<b>R2</b>	-	-	-	+++

Time course of antibody reactivity was determined by phage-plate assay. 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 - sera collected after one, two, three or 10 vaccinations with irradiated,  $5 \times 10^5$  GM secreting RENCA cells, respectively.

**Table V: RENCA Antigens and their human orthologs identified in the screening of other tumor libraries.**

<b>RENCA Antigens</b>	<b>Homology in other tumor library screening</b>
<b>ROCK-II</b>	<b>RCC, MM,B16, lung carcinoma, Sarcoma</b>
<b>HnRP c1/c2</b>	<b>Colorectal cancer, gastrointestinal cancer, Head and Neck cancer, Lung cancer</b>
<b>SSRP1</b>	<b>stomach cancer/ColorectalACC</b>
<b>AR</b>	<b>RCC, Cutaneous T-cell lymphoma, non-small cell lung carcinoma</b>
<b>EIF</b>	<b>Lung carcinoma, SCLC, sarcoma</b>
<b>ACAT2</b>	<b>Breast cancer</b>
<b>IQGAP</b>	<b>Esophageal cancer</b>

PDI has been shown to be present in the cell membrane of B cells from B-CLL patients and involved in the regulation of surface expression of thiols and drug sensitivity of these cells (Tager *et al.* 1997). Changes in this protein level also correlated with patient outcome. Murine PDI and its human ortholog share about 93% homolog, thus we decided to test our isolated clone isolated from the RENCA cDNA library against sera from B-CLL patients (kindly provided by Dr Gribben lab). Table VII shows that, 5 out of 9 B-CLL patients were reactive against this immunogenic protein. Testing of normal donors for reactivity to PDI is currently underway.

Together, these findings highlight common immunoreactive antigens found in multiple tumor malignancies in both murine and human models. Furthermore, they raise the possibility that these genes contribute to tumorigenesis, thereby suggesting their potential role as targets for immunotherapy.

**Table VI: Reactivity of a murine RENCA Antigen – ROCK2 - against sera from melanoma patients and normal donors.**

<b>Melanoma Patients</b>	<b>ROCK2 reactivity</b>	<b>Normal Donor</b>	<b>ROCK2 reactivity</b>
<b>M34</b>	+++	<b>51</b>	-
<b>M8</b>	-	<b>52</b>	-
<b>M9</b>	+++	<b>55</b>	-
<b>M15</b>	+++	<b>58</b>	+++
<b>M17</b>	+++	<b>59</b>	+++
<b>K008</b>	+++		
<b>M014</b>	+		
<b>K011</b>	+		
<b>K014</b>	+++		
<b>K18</b>	++		
<b>K20</b>	++		

Sera from melanoma patients and control donors, diluted at 1:300 were tested against murine ROCK2 isolated by serologic screening in a RENCA cDNA library.

**Table VII: Reactivity of PDI clones against sera from B-CLL patients.**

<b>Serum B-CLL Patient</b>	<b>Reactivity to PDI clone</b>
<b>M</b>	+++
<b>W</b>	++
<b>Y</b>	+
<b>C</b>	+
<b>L</b>	-
<b>B</b>	++
<b>K</b>	+++
<b>R</b>	-
<b>E</b>	-
<b>X</b>	-

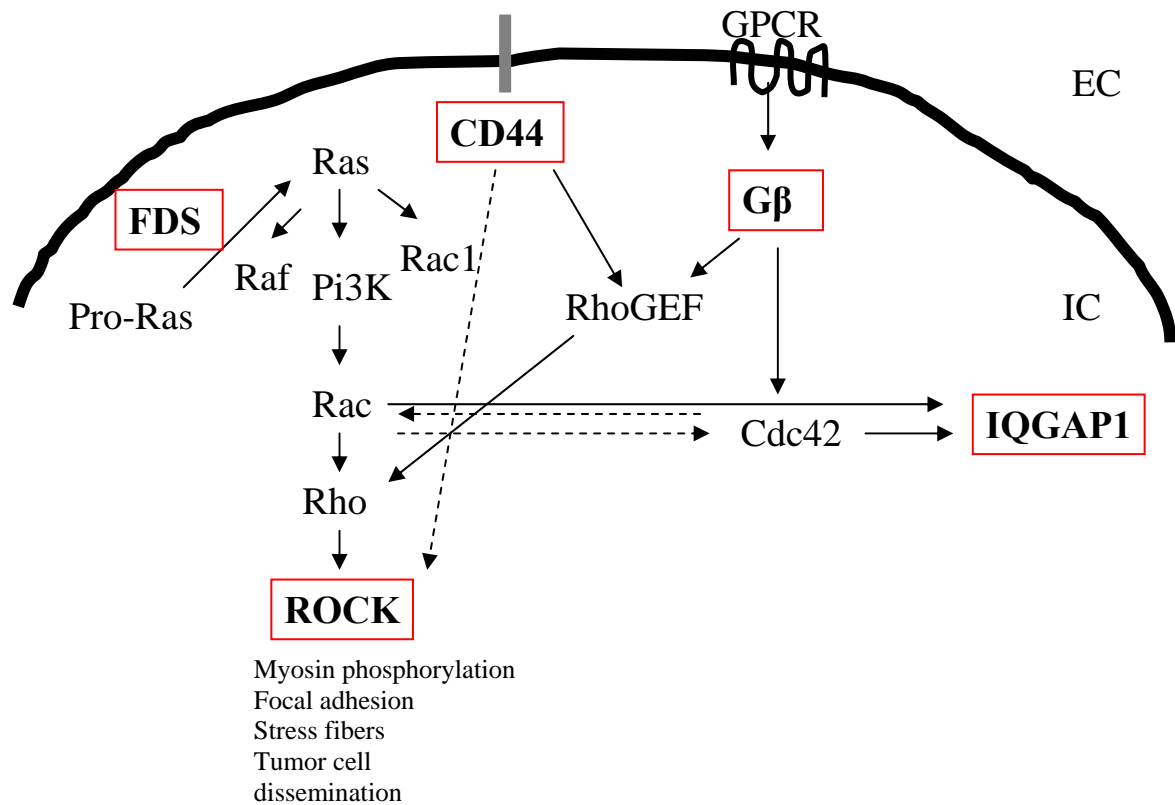
Serum from B-CLL patients diluted at 1:300 was tested against murine PDI isolated by serologic screening in a RENCA cDNA library.

### **3.7 Functional Characterization of Serologic defined RENCA Antigens: Key role in Cancer**

As summarized in Table II, database search shows that this immunologic screening led to the discovery of a large spectrum of broadly expressed antigens 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.

One major group that we were particularly interested in, included proteins directly or indirectly involved with the Ras/Rho signaling pathway: ROCK2, FDS, GNB2, IQGAP1, CD44 and ARF4. Some of these proteins act as molecular switches directing upstream signals to multiple downstream effectors, as schematically represented in Figure 3.4. This pathway plays a pivotal role in the regulation of numerous cellular functions associated with malignant transformation. These proteins are key regulators of actin reorganization, cell-motility, cell-cell and cell-extra-cellular matrix adhesion, as well as cell cycle progression, gene expression, apoptosis, tumor invasion, and metastasis (Kuroda *et al.* 1998; Itoh *et al.* 1999; Okamoto *et al.* 1999; Bishop and Hall 2000). 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 (Sugimoto *et al.* 2001; Okamoto *et al.* 2002).

Interestingly, one antigen identified in our immunologic screen was detected repeatedly among the isolated clones, 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, correspond to the same protein – Protein Disulfide Isomerase (PDI). PDI was first identified as a physiological catalyst of native disulfide bond formation of nascent peptides in cells (Freedman *et al.* 1989). *In vitro*, it catalyzes the oxidative formation, reduction, or isomerization of disulfide bonds depending on the redox potential of the environment (Freedman *et al.* 1994). In eukaryotic cells, this chaperone is part of the quality control system for the correct folding and disulfide bonding of proteins in the ER.



**Figure 3.4: Serologic identification of antigenic components of the Ras/Rho signalling pathway.** Proteins identified by serologic screen are represented in red boxes; Extracellular (EC); Intracellular (IC).

### 3.8 Potential mechanisms of immunogenicity of SEREX-defined RENCA antigens in tumor cells

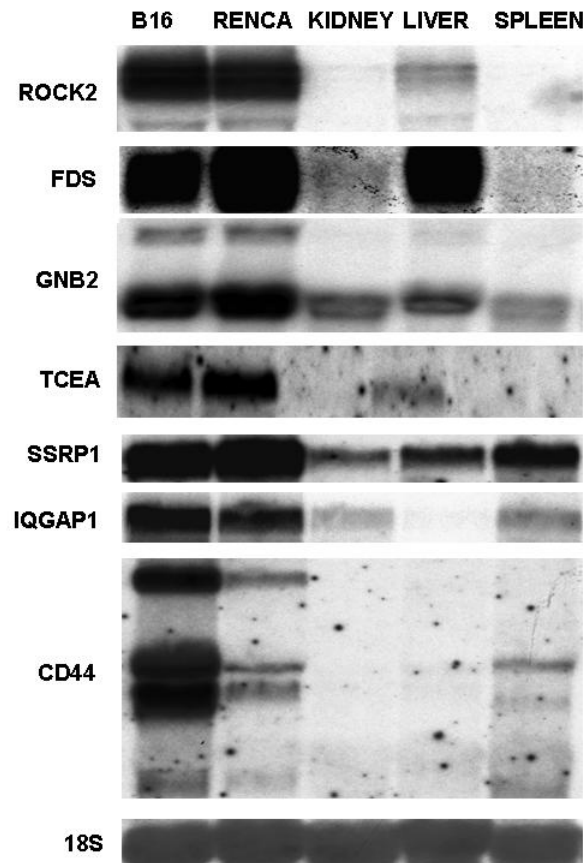
In tumor cells, one mechanism that can result in the generation of antigenic epitopes recognized by the immune system relates to mutations. Several examples, including the mutated ras oncoprotein and the p53 tumor suppressor protein have been shown in the literature (Abrams *et al.* 1996; Fedoseyeva *et al.* 2000). Surprisingly, we did not find mutations in any of the genes isolated by library screening when their nucleotide sequence was compared against NCBI database. Nevertheless, previous work indicates that the immunogenicity of non-mutated cancer antigens might be related to increased expression in tumor cells [(e.g. gp100 and Mart1 in melanoma or prostate-specific antigen (PSA) in prostate cancer)] (Bakker *et al.* 1994). In order to address this question, we characterized mRNA and protein levels of a panel of identified genes, to evaluate if their upregulation in RENCA tumor cells 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 3.5, proteins involved in the Ras/Rho signalling pathway, including ROCK2, FDS, GNB2, IQGAP1 and CD44 show increased transcript levels in the two tumor cell lines B16 (melanoma) and RENCA. In contrast, absent or low mRNA transcript levels were found in the normal tissues tested, including kidney, spleen and liver; the only exception being high levels of FDS in the liver, which is explained by the essential role of this enzyme in the cholesterol synthesis in this organ.

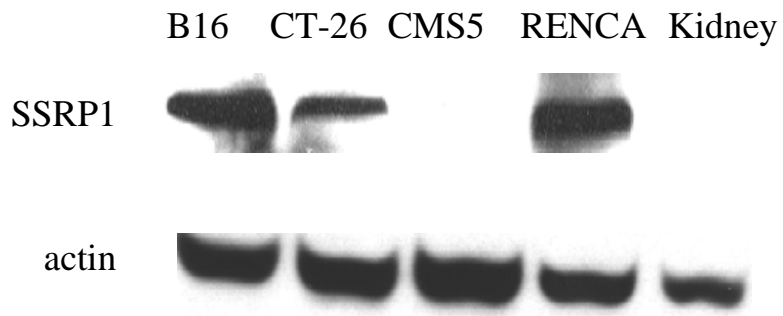
A similar pattern of upregulation in RENCA and B16 tumor cell lines was observed for two transcription activators SSRP1 and TCEA1, when compared with normal tissues (Figure 3.5). Furthermore, we confirmed overexpression of SSRP1 protein by western blot analysis. Figure 3.6 shows that this protein is highly expressed in RENCA cells but, on the contrary, it is low or undetected in kidney. High levels of SSRP1 expression were also confirmed in two other tumor cell lines B16 and CT-26 (colon carcinoma), but not CMS5 (fibrosarcoma).

A third mechanism 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. Some of these isoforms have been associated with tumor progression (Wielenga *et al.* 1993). 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. Further studies are necessary to assess the functional significance of these differences.

Overall, these data show that upregulation of genes involved in two key carcinogenic pathways - Rho/Ras signalling pathway and transcriptional activation - may account for their immunogenicity observed in RENCA vaccines. Moreover, alternatively spliced variants shown to be present in these tumor cells suggest another possible mechanism of immunogenicity associated with these gene products.



**Figure 3.5: 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 3.6: Western blot analysis of SSRP1 shows increased expression in tumors.** 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.

## Summary

In this part of our study, we show that the improved anti-tumor immunity by vaccination with irradiated GM-CSF secreting RENCA cell versus irradiated wild-type tumor cells is associated with induction of a more diversified antibody repertoire. High titer IgG antibodies recognizing RENCA antigens were found to be present in Post-vaccination sera, as revealed by FACS analysis. To further examine in more detail the targets of this antibody repertoire, a phage library was constructed from cDNA of RENCA tumor cells. Library screening using Post-vaccination serum led to the serologic discovery of immunogenic antigens associated with tumor rejection in this model. We identified a total of 28 unique proteins, including 21 with known function. Comparison of serum reactivity shows that all proteins are recognized by GM-CSF vaccines. In contrast, only 2 are detected in wild-type vaccination. Moreover, we demonstrate that antibodies against this panel of antigens are induced upon vaccination, with the antibody repertoire increasing with the number of vaccinations. Nevertheless, none of these proteins is recognized with serum from naïve mice.

The array of genes detected represent intracellular, transmembrane as well as secreted antigens, and analysis of their coding sequences revealed no mutations. Database search revealed that these proteins play key roles in the process of carcinogenesis, and some are autoantigens also found in patients with different cancers. We show that a panel of these broadly expressed self-antigens is specifically upregulated in tumor cell lines. This increased expression may represent a possible mechanism of immunogenicity for these self, non-mutated proteins. Furthermore, some of these murine antigens proved to be immunologic targets in cancer patients.

### **3.9 Uncovering the immunologic role of RENCA associated Antigens in Protective Antitumor immunity *versus* tolerance**

Protective anti-tumor immune responses involve multiple components of the immune system, in particularly effector T cells, capable of destroying tumor target cells. A major goal is to understand how does one activate these effector cells and induce a state of effective tumor immunity. However, multiple mechanisms of immune tolerance are likely to inhibit effective therapy of cancer. There are a number of mechanisms by which tumors can evade and or suppress immune responses. A major limiting factor is the fact that tumors express mainly self, non-mutated antigens to which T cells have already been tolerized. Furthermore, T-cell responses to tumor antigens may be further reduced by immunosuppressive cell populations, such as CD4+ CD25+ T cells. These regulatory T cells are crucial for maintaining tolerance to self-antigens, and can also suppress effector T-cells immunity to tumor-associated antigens, thus compromising successful immunotherapy.

In this part of the work, we explored the immunologic role of serologically defined antigens identified as targets of protective GM-CSF-transduced RENCA tumor cell vaccines. We specifically wanted to address the question if active immunization with these molecules is able to induce protective anti-tumor immunity, and if not, to examine how these antigenic targets are involved in tipping this delicate immunologic equilibrium towards tolerance. Understanding the mode of action of these proteins is a powerful tool to help us learn more about the mechanisms associated with protective immune responses observed with whole tumor cells, and how this successful immunotherapeutic approach works.

### **3.10 Immunotherapeutic Potential of Serologically-defined RENCA Tumor Antigens: In Vivo studies**

Using an immunologic screening, we identified a variety of humoral targets induced by GM-CSF-secreting RENCA cells. Given the potential of these whole tumor cell-based vaccines to induce tumor protection, it is reasonable to postulate that these proteins might function as tumor rejection antigens in the RENCA tumor model. If this hypothesis is correct, then we should be able to recapitulate the same vaccination activity seen with the whole tumor cell approach with these defined molecules.

Successful vaccination strategies using defined antigen have been reported. These include DNA vaccines, Ag-transfected dendritic cells, xenogeneic vaccines, and engineered whole tumor cells. Since the relative potency of these immunization strategies still remains to be defined, we initially chose DNA vaccines, given the relative simplicity of

this approach. We evaluated different immunization schedules, routes of antigen delivery, and the role of several immunologic adjuvants with this approach.

### **3.10.1 Naked DNA Vaccines**

DNA vaccines consist of a bacterial plasmid, engineered for optimal expression in eukaryotic cells, containing the target gene of interest. The ability to rapidly screen a large number of TA, and to design specific types of expression constructs, makes this strategy a suitable approach for cancer immunotherapy.

Potent and long-lived cell-mediated and humoral immunity in several antigen systems have been demonstrated after injection of naked plasmid DNA into muscle tissue or dermis of mice (Gurunathan *et al.* 2000). Intramuscular injection of plasmid predominantly leads to transfection of myocytes, whereas bombardment of the epidermis with plasmid coated onto gold microbeads directly transfects epidermal keratinocytes and Langerhans cells, which then migrate rapidly to regional lymph nodes.

#### **3.10.1.1 Amplification and Cloning of RENCA Antigens in the pMFG vector**

In order to test the genes of interest as DNA vaccines, their coding sequence has to be cloned in a plasmid vector. Thus, DNA sequences corresponding to a panel of SEREX-defined RENCA antigens (TA) (Table VIII) were amplified from either the purified recombinant phage DNA or from RENCA cells (if full length or not, respectively) using reverse transcriptase polymerase chain reaction (RT-PCR). Full-length cDNAs were then cloned into the plasmid pMFG, a PUC19/MoML (Moloney murine leukemia virus)-based vector (Figure 3.7). Protein coding sequences were inserted between the Nco I and Bam HI restriction sites so that the position of the initiator ATG was maintained. The expression of inserted sequences is controlled by the MoML promoter/enhancer in the viral LTR (long terminal repeat). The resulting constructs (pTA) make it possible to screen in a rapid and efficient way the large number of immunogenic antigens for their role in tumor rejection as “Naked” DNA vaccines.

By sequence analysis, we confirmed that there were no mutations in the coding region of these genes. Recombinant plasmid vectors coding for each RENCA antigen were then selected for further studies of tumor protection *in vivo*.

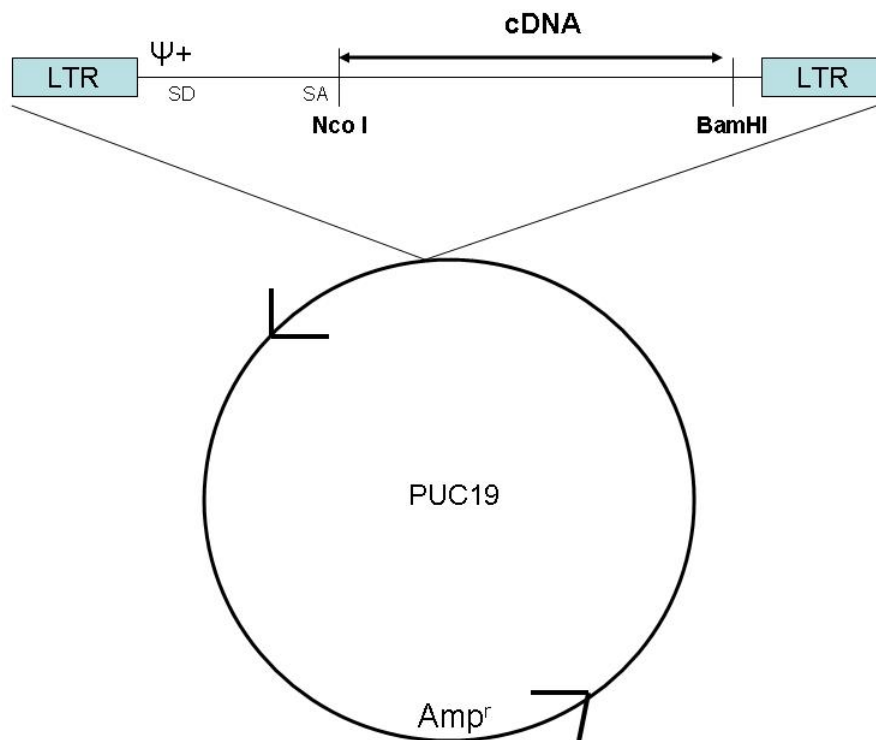
**Table VIII: List of pMFG-TA constructs (pTA) derived by SEREX-defined RENCA Tumor Antigens (TA).**

<b>pTA constructs</b>	<b>Insert Size (bp)</b>
pPDI	1530
pAR	951
pApg3p	945
pARF4	543
pEIF4A1	1221
pPBEF	1476
pIQGAP1	4974
pTCEA1	906
p ATPsynthase	507
pCD44	1092
pH1F0	585
pGNB2	1023
pFDPS	1062
pROCK2	4167

Full length cDNAs corresponding to selected RENCA tumor antigens (TA) were cloned in the pMFG vector.

### **3.10.1.2 Intramuscular Immunization**

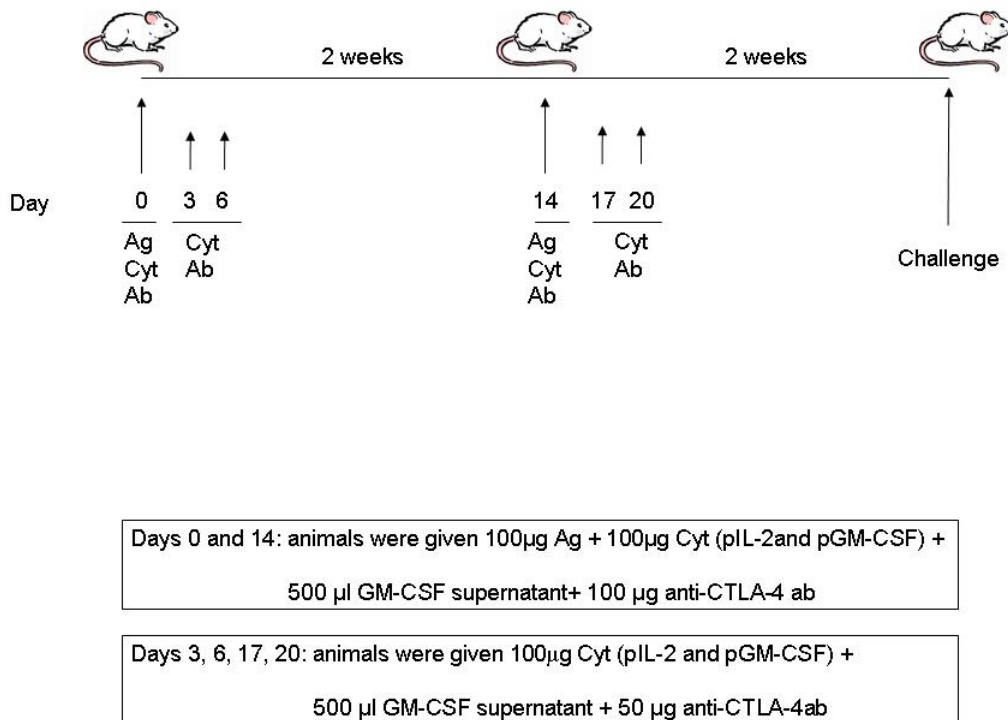
Even though naked DNA vaccines have the ability to screen the immunogenicity of TA rapidly, without any special formulation, their application for tumor immunity has not been optimized. The incorporation of additional immunostimulatory molecules with antigen encoding plasmids can enhance the potency of immune responses elicited against weak tumor antigens. Thus, in order to maximize our opportunity for revealing tumor protection in the RENCA system, we elected to combine multiple antigens instead of a single antigen. Moreover, we also evaluated the use of IL-2, GM-CSF and anti-CTLA-4 antibody (CTLA-4 ab) blockade as adjuvants. IL-2 and GM-CSF are two immunostimulatory cytokines whose administration has proven to induce tumor regression in patients and murine tumor models (Dranoff 2004). These cytokines can induce systemic immunity through a coordinated host immune response including lymphocytes, macrophages, DCs and NK cells. CTLA-4 is a key factor in limiting the magnitude of an immune response. Upon engagement of this receptor with its ligands, B7-1 and B7-2, it delivers an inhibitory signal to T cells. Thereby, removal of this potential inhibitory checkpoint is the rationale for administering a blocking CTLA-4 ab. Allison and colleagues have shown that administration of this antibody blocking CTLA-4/B7 interactions increased the anti-tumor effects of naked DNA vaccines (Gregor *et al.* 2004).



**Figure 3.7: Schematic representation of the pMFG recombinant vector.** pMFG recombinant constructs encoding RENCA antigens and cytokines. The MFG retroviral backbone contains the Moloney murine leukemia virus (MMLV) long terminal repeat (LTR) sequences used to generate both a full-length viral RNA (for encapsidation into viral particles) and an mRNA that is responsible for expression of inserted sequences (cDNA). Protein coding sequences are inserted at the initiation codon of the viral *env*, between NcoI/XbaI and BamHI sites. The plasmid backbone contains the ampicillin resistance gene (Amp<sup>r</sup>).

In the first set of experiments, immunizations were performed intramuscularly (i.m.) with a mix of DNA constructs coding for 13 different RENCA antigens. A schematic representation of the protocol is summarized in Figure 3.8. Two groups of mice were immunized twice, two weeks apart, with DNA plasmids coding for tumor antigens and the cytokines IL-2 and GM-CSF. To further increase the effective local concentration of GM-CSF, we also included supernatant from GM-CSF transduced cells (400 ng/ml). Finally, we also administered anti-CTLA-4 blocking antibody. On day 3 and day 6 after each

immunization, all animals received additional boosts of cytokines and anti-CTLA-4 antibody. Mice were challenged subcutaneously with live RENCA tumor cells two weeks after the second vaccination, and animals were then monitored for tumor development. As shown in Table IX, this vaccination schema was unable to elicit tumor protection. Additional studies involving intravenous tumor challenges similarly failed to demonstrate protective immunity (not shown).



**Figure 3.8: Schematic representation of intramuscular (i.m.) DNA immunizations.** On day 0 and 14, animals were given plasmid antigens (Ag) and cytokines (Cyt), and anti-CTLA-4 antibody (Ab). On day 3, day 6, day 17 and day 20 animals were boosted with cytokines and anti-CTLA-4 antibody. Arrows represent immunizations. Two weeks later, mice were challenged with live RENCA cells.

### **3.10.1.3 Gene-Gun delivery of DNA**

To examine whether alternative routes of DNA immunization might evoke more potent responses, we next evaluated gene-gun-based acceleration of DNA-coated gold beads into the epidermis. These beads deliver DNA into keratinocytes, Langerhans cells and dermal dendritic cells, where the DNA can be expressed. In these studies, we investigated different DNA doses, the use of multiple antigens or a single target (PDI), and the inclusion of GM-CSF expressing plasmids, as an adjuvant. As shown in Table X, this approach similarly failed to elicit tumor protection. Taken together, these experiments suggest that serologically-defined tumor antigens administered as naked DNA vaccines do not recapitulate the immunologic activity of GM-CSF secreting RENCA cells.

### **3.10.2 DCs Vaccines**

#### **3.10.2.1 Bone-Marrow derived DC (BMDC) pulsed with Tumor RNA**

In view of the limited efficacy of naked DNA immunizations, we next investigated the use of gene modified dendritic cells as vaccines. Dendritic Cells (DCs) are key players in the initiation of immune responses and in the induction of T and B cell immunity *in vivo*. Moreover, immunizing mice with DCs engineered to express specific antigens can prime a CTL response that is tumor-specific and capable of mediating tumor protection. Indeed, Gilboa and colleagues showed that DCs transfected with whole tumor *in vitro* transcribed RNA (IVT RNA) are nearly equivalent to GM-CSF secreting tumor cells in inducing tumor protective immunity (Ashley *et al.* 1997). Moreover, the route by which Ag-pulsed DCs are injected into the body leads to differences in their distribution in lymphoid tissue (Mullins *et al.* 2003). Subcutaneous (s.c.) immunizations are able to induce memory T cells in spleen, as well as in lymph nodes and improve protection against subcutaneously growing tumors. We thus sought to determine if vaccination with DCs transfected with RNA derived from our identified RENCA TA were able to induce a protective antitumoral response.

#### **3.10.2.2 Phenotypic Characterization of BMDC**

When considering the use of mRNA-transduced DCs as a vaccine modality in cancer immunotherapy, it is important for the TA to be efficiently processed and presented in the context of MHC class I and II molecules and that the injected dendritic cells are functionally mature. Thus, we generated dendritic cells from bone marrow, by culture in GM-CSF, transfected the cells on day seven with *in vitro* transcribed RNA loaded into liposomes, and then matured the cells with lipopolysaccharide (LPS) before vaccination. In order to optimize the system, we first investigated the transfection efficiency of BMDC

using *in vitro*-transcribed GFP mRNA. Flow cytometry was used to monitor reporter gene expression, as well as the maturation status of DCs in response to LPS. Staining of CD11c, CD11b+ dendritic cells showed high surface expression of B7-1, consistent with the acquisition of a mature phenotype (data not shown). Moreover, GFP expression was detected a few hours after transfection in 15 to 20% of the mature dendritic cells.

**Table IX: Naked DNA Immunizations (intramuscular Injection)**

<b>Group</b>	<b>Antigens</b>	<b>Adjuvants</b>	<b>Challenge Live Renca cells</b>	<b>Tumor Protection<sup>iii</sup></b>
Ag+Adjuv	pTA <sup>i</sup>	Cyt <sup>ii</sup> + Ab	5X10 <sup>5</sup>	0/5
Adjuv	None	Cyt + Ab	5X10 <sup>5</sup>	0/5
control	None	None	5X10 <sup>5</sup>	1/5

Groups of BALB/c mice were immunized with antigens plus adjuvants or adjuvants alone as indicated in Figure 3.8. Animals were immunized twice two weeks apart with a mix of plasmids encoding the RENCA associated antigens plus cytokines (IL-2 and GM-CSF) and anti-CTLA-4, as adjuvants (Ag+adjuv). Control groups were given adjuvants alone (Adjuv) or PBS (control). Two weeks after last immunization mice were challenged s.c. with live 5X10<sup>5</sup> RENCA cells and monitored for tumor development. i) mix of pMFG constructs coding for 13 RENCA Tumor Antigens (Table VIII); ii) cytokines were given i.m. as DNA (pMFG constructs coding for murine IL-2 or GM-CSF) or i.p. (GM-CSF was also given as supernatant from transduced B16 cells). All DNA constructs were given i.m.; B16 supernatant and anti-CTLA4 blocking antibody (Ab) was given i.p.; iii) Tumor Free mice/ total number mice, 60 days after challenge.

**Table X: Gene Gun Protocol I (GGP1) Immunizations.**

<b>Group</b>	<b>Immunization</b>	<b>Amount DNA/Ag (<math>\mu</math>g)</b>	<b>Challenge</b>	<b>Tumor Protection**</b>
Ag 1	TA*+GM-CSF	2	$8 \times 10^5$	0/5
Ag 2	TA+ GM-CSF	10	$8 \times 10^5$	0/4
PDI	PDI + GM-CSF	10	$8 \times 10^5$	0/5
Control	None	None	$8 \times 10^5$	0/7

Mice were vaccinated twice with gene gun, one week apart, and challenged one week later with live tumor RENCA cells. Groups Ag 1 and Ag 2 were immunized with a mix of RENCA antigens (2 or 10  $\mu$ g per antigen, respectively) plus GM-CSF coding plasmid. PDI group was immunized with plasmids coding for PDI plasmid (10  $\mu$ g) and GM-CSF. After challenge, mice were kept under observation for tumor development. \* A mix of DNA constructs each coding for 13 different RENCA antigens (Table VIII). \*\* Tumor Free mice/ Total number mice, 21 days after challenge.

### **3.10.2.3 Vaccination with BM-derived DC pulsed with PDI**

Having established the system, we next tested whether vaccination using RENCA antigen loaded DCs could induce tumor protection. The cDNAs of the tumor antigens PDI, ARF4, Histone 1 and GNB2 were used as template for RNA transcription using T7 RNA polymerase in the presence of a 5'-cap analogue. RNA was analyzed, before and after amplification, by agarose gel. BMDC grown in the presence of GM-CSF were transfected with the IVT RNA in the presence of the cationic lipid DOTAP.

BMDC were RNA transfected and LPS matured immediately before injection. Mice were immunized with equal amounts of mature nontransfected and GFP or TA-transfected DCs twice at two week intervals. Challenge with live tumor cells was performed 2 weeks after last immunization.

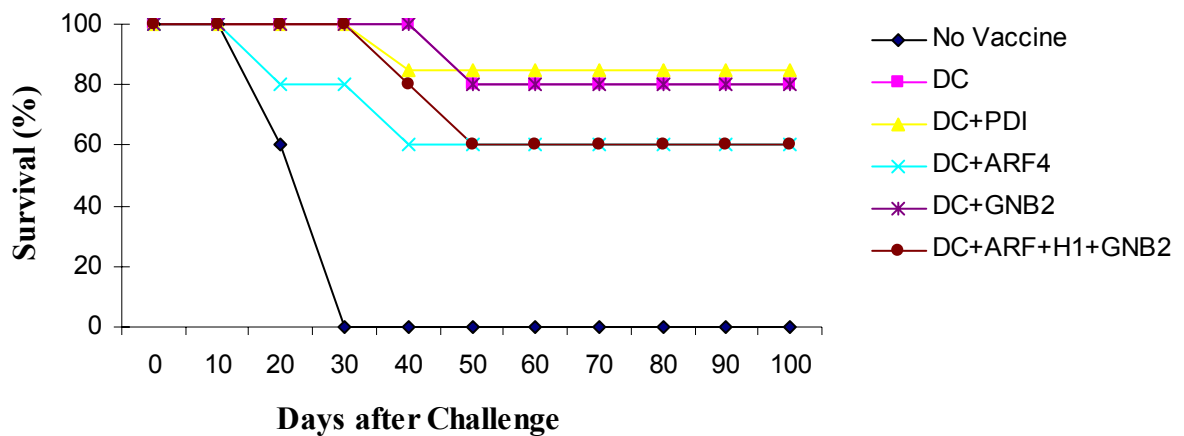
As shown in Figure 3.9, we unexpectedly observed strong vaccination activity with non-transfected dendritic cells compared to control, non-immunized mice. The efficiency of protection was similar to mice vaccinated with dendritic cells pulsed with different serologically defined RENCA antigens. Additional experiments, in which smaller numbers of dendritic cells were injected or only single vaccinations were administered resulted in diminished protective immunity for both non-transfected and RENCA antigen expressing dendritic cells. While the mechanisms underlying the vaccination activity of unmodified dendritic cells in this system remain to be clarified, it is important to note that both the bone marrow derived dendritic cells and RENCA challenge cells were cultured in fetal calf serum. This raises the possibility that proteins present in the media might be presented by dendritic cells and elicit T cell and / or antibody responses to these proteins that remain associated with RENCA cells, despite extensive washing. Additional experimental using different culture media need to be tested, in order to address the immunotherapeutic potential of RENCA antigen-loaded DCs.

### **3.10.3 Xenogeneic Immunization**

The presentation of altered self to the immune system is an additional strategy to prime adaptive immunity. Xenogeneic vaccination can induce immunity against self-antigens by breaking immune tolerance to self. Houghton and colleagues showed that vaccination of mice with human melanosomal antigens could elicit protective immunity in the B16 melanoma model shows that xenogeneic immunization with orthologous melanoma antigens can induce tumor immunity (Hawkins *et al.* 2002). Furthermore, in this system CTLA-4 blockade increased T-cell response and tumor protection (Gregor *et al.* 2004).

To evaluate the application of xenogeneic vaccination in the RENCA model, we selected PDI for study, as human PDI (hPDI) shares 95% homology with the mouse protein. We administered human PDI with incomplete Freund's adjuvant (IFA), a water-oil-emulsion that provides continuous release of antigen, which is necessary to induce a strong and persistent immune response. Moreover, we included oligodeoxynucleotides (ODN) containing unmethylated cytosine-guanine motifs (CpG) as additional immunostimulants. CpGs have shown powerful immunomodulatory activity in murine and human vaccine experiments (Jakob *et al.* 1998; Shirota *et al.* 2000). CpG-ODN can bind to Toll-like receptors (TLR9) expressed by dendritic cells, resulting in functional maturation comparable to CD40 ligation. Thus, we evaluated the tumor protection efficacy of human PDI (hPDI) protein using IFA and CpG-ODN as adjuvants. Mice were vaccinated twice, subcutaneously, with 100 µg of protein in the presence of 250 µl of IFA and 100 µg of CpG ODN (PDI group). Control groups include nonvaccinated mice or mice

injected with adjuvants (IFA and CpG). Nonetheless, as shown in Table XI, protection against RENCA challenge was not achieved, suggesting that this immunization strategy was not sufficiently potent to break tolerance against this self protein.



**Figure 3.9: Efficacy of DCs transfected with RENCA antigens as therapeutic tumor vaccines.** Groups of 5 animals were vaccinated twice, 2 weeks apart with  $1.5 \times 10^6$  or  $0.5 \times 10^6$  RNA transfected DCs, respectively. RNA pulsed DCs were administered by subcutaneous injection. 50  $\mu\text{g}$  of IVT RNA from PDI (DC+PDI), ARF4 (DC+ARF4), GNB2 (DC+GNB2) or 10  $\mu\text{g}$  of each antigen ARF4, H1 and GNB2 (DC+ARF+H1+GNB2) were used. Animals were challenged 2 weeks after with live RENCA tumor cells. These results are representative of three experiments.

**Table XI: PDI Xenogeneic Vaccination.**

<b>Treatment</b>	<b>Number of tumor-free mice 30 dayst after RENCA challenge</b>
None	1/5
IFA+CpG	0/5
IFA+CpG+hPDI	0/4

Mice were treated twice, one week apart, with 250  $\mu$ l of IFA, 100  $\mu$ g of CpG ODN, with or without human PDI recombinant protein (hPDI). The mix was given s.c. as a homogeneous solution, in a total volume of 500  $\mu$ l. One week after, mice were challenged with  $4 \times 10^6$  RENCA live cells. The number of tumor-free mice is shown. Similar results were obtained in two separate experiments.

#### **3.10.4 Whole Tumor-Cell Vaccines genetically modified to express GM-CSF and RENCA Tumor Antigens (GM/TA vaccines)**

Since none of the vaccination strategies tested above revealed the immunogenicity of serologically defined RENCA antigens, we wondered whether the presentation of these targets might depend on specific characteristics of tumor cells. In the RENCA-GM model, tumor antigens are presented in the context of dying cells and an immunostimulatory microenvironment. This raised the possibility that tumor cells engineered to express candidate RENCA antigens might provoke a stronger antigen specific response.

As a first step in testing this idea, we examined whether other Balb/c derived tumors might serve as an appropriate vehicle for presenting RENCA antigens. A key requirement for this approach is that the vaccinating tumor cell line must show limited cross-protection against RENCA challenge. We studied both CT-26 colon carcinoma cells and CMS-5 fibrosarcoma cells, as previous work indicated that GM-CSF transduction increased vaccination potency in each system. As shown in Figure 3.10, vaccination with GM-CSFsecreting CMS-5 cells resulted in efficient protection against challenge with RENCA cells, whereas GM-CSF secreting CT-26 cells failed to evoke protective immunity under the conditions tested. These results indicated that CT-26 cells could serve as a platform for delivering RENCA antigens.

To engineer GM-CSF secreting CT-26 cells to express serologically defined RENCA antigens, we employed retroviral mediated gene transfer. The MFG retroviral vector used exploits the Moloney Murine Leukemia Virus long terminal repeat to regulate expression of both a full length transcript (for encapsidation into viral particles) and a spliced transcript (analogous to env) containing the inserted cDNAs (Figure 3.7).

Taking advantage of the MFG retroviral constructs coding for the RENCA tumor antigen (pTA), previously prepared for the naked DNA vaccine experiments, we introduced each construct into 293GPG packaging cells to generate recombinant virus with amphotropic host range (schematic represented in Figure 3.11) and then infected CT26 cells with retroviral supernatants. While mRNA transcripts for each of the antigens were detected following transduction into CT26 cells (not shown), we confirmed high level protein expression for both ROCK and PDI by western analysis (Figure 3.12). Moreover, significant levels of PDI were detected in cell supernatants, consistent with previous work, indicating that transformation altered the subcellular distribution of the protein, resulting in membrane and secreted forms.

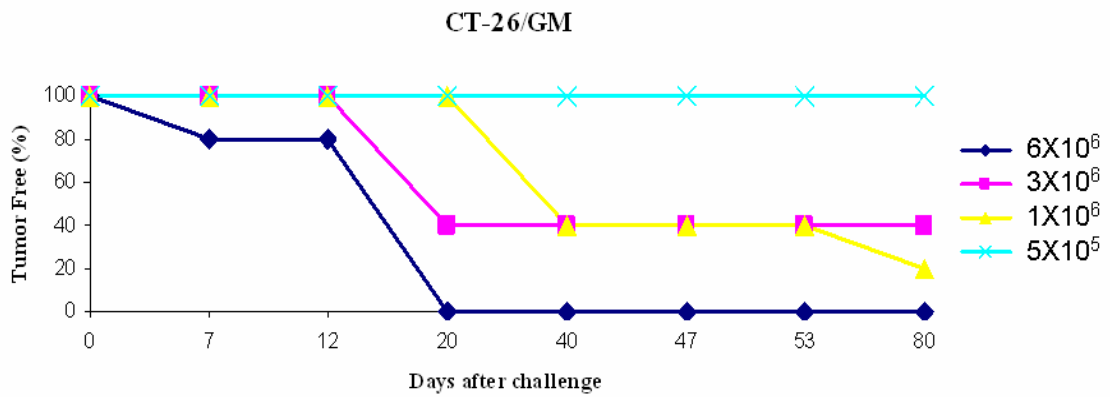
After a variety of recombinant retroviruses encoding different RENCA tumor antigens (TA) were generated, the vaccination properties of irradiated transduced tumor cells (CT26/GM/TA) were compared with the parental cell line CT26/GM. As shown in Figure 3.13, initial experiments suggested that PDI expressing tumor cells, but not other engineered lines, might improve vaccination activity. However, subsequent studies failed to confirm these findings and even the addition of CTLA-4 antibody blockade did not improve protection (not shown).

### **3.11 Potential Role of RENCA self-antigens in immunosuppression**

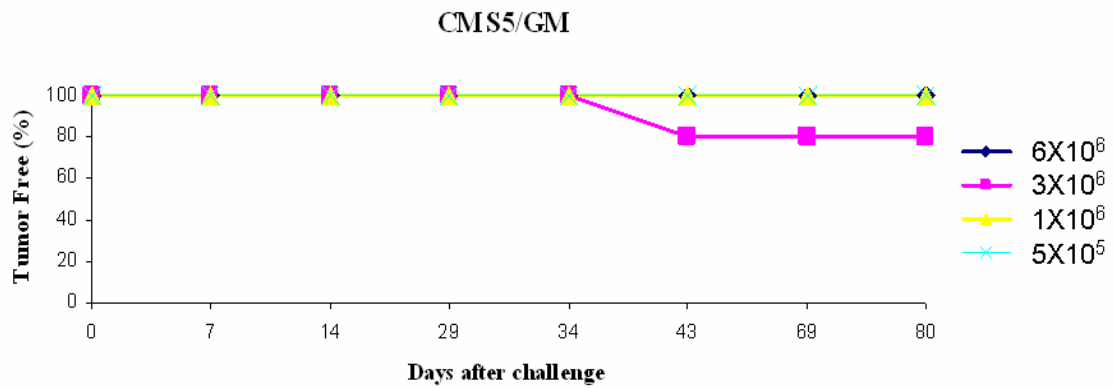
Since none of the vaccination strategies tested demonstrated the capability of the serologically defined RENCA antigens to function in tumor protection, we considered the possibility that these antigens might alternatively generate tolerance. Indeed, several studies have shown that the potency of GM-CSF secreting tumor cell vaccines can be enhanced by the inhibition of negative immune regulation mediated by regulatory T cells or CTLA-4 blockade. These results suggest that although whole tumor cell vaccines can elicit protective anti-tumor responses, they might also evoke tolerizing responses that limit their overall potency.

The SEREX-defined molecules identified in our screening were mainly self-proteins, without evidence of mutations. Recent work suggests that these proteins might, under some conditions, trigger Treg responses.

A)



B)



**Figure 3.10: Tumor Protection efficacy of GM-CSF transduced tumor cell lines.** Two BALB/c derived cell lines CT-26 and CMS5, transduced with a plasmid coding for GM-CSF, were evaluated for their tumor protection efficacy against a challenge with live RENCA cells. Mice were vaccinated twice, one week apart, with irradiated (35Gy)  $5 \times 10^5$  CT26/GM (A) or CMS5/GM (B). Two weeks after last immunization, mice were challenged with different doses of live RENCA cells ( $5 \times 10^5$ ,  $1 \times 10^6$ ,  $3 \times 10^6$  or  $6 \times 10^6$ ).

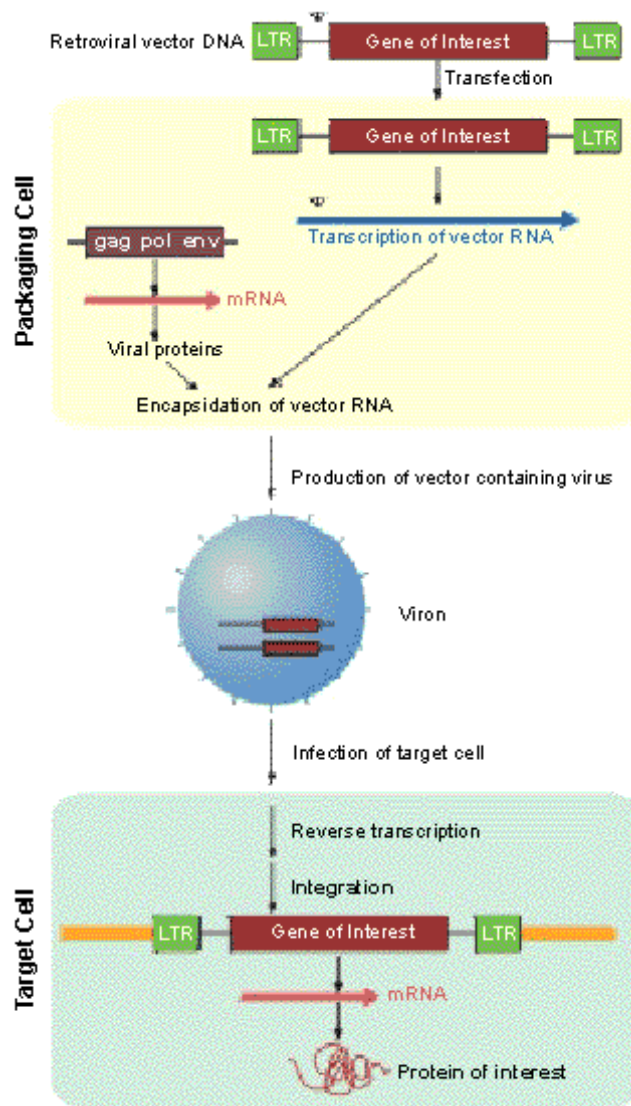
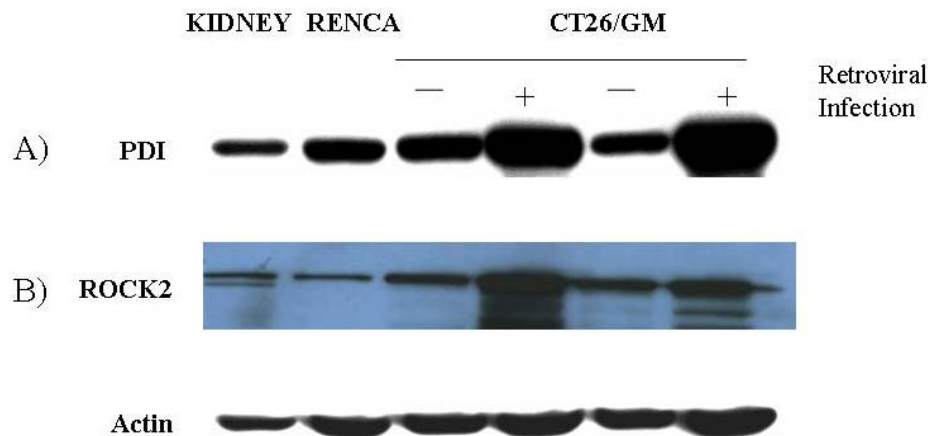


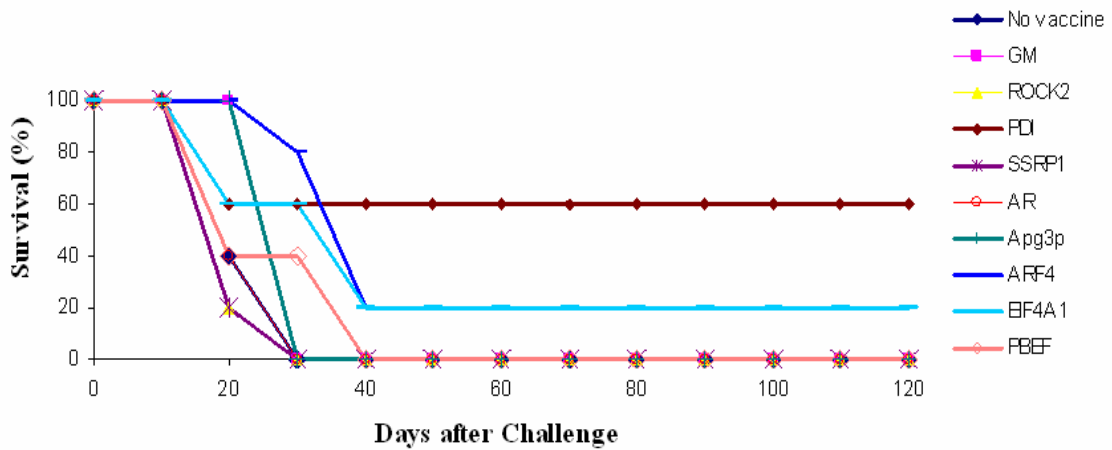
Figure 3.11: Schematic overview of retroviral transduction of target cells.



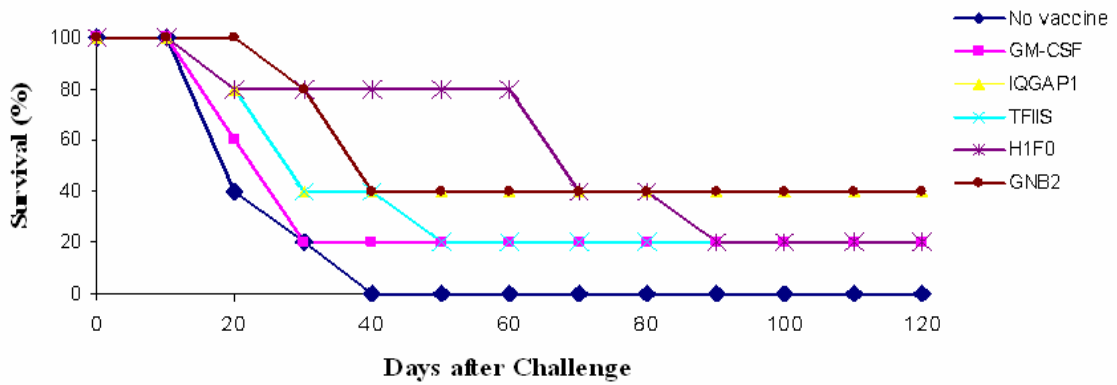
**Figure 3.12: PDI and ROCK2 overexpression upon retroviral transduction of BALB/c syngeneic cell lines CMS5/GM and CT26/GM.** Western blot analysis was performed using similar amounts of total protein lysates loaded on each lane and probed with anti-mouse PDI or anti-mouse ROCK2. The membranes were rehybridized with anti-actin antibody as control. Detection was performed as described in material and methods.

Shiku and colleagues uncovered a potential dual role of self-antigens in inducing protective tumor therapy or suppressing it (Nishikawa *et al.* 2001; Nishikawa *et al.* 2003). In these studies, antibody based expression cloning was used to identify the targets of high titer antibodies in mice injected with a chemically-induced sarcoma. Consistent with our results, Shiku and associates identified a number of broadly expressed, non-mutated self antigens as antibody targets. Interestingly, they showed that co-immunization of these autoantigens with a tumor-specific, mutated epitope presented to class I restricted cytotoxic T cells enhanced CD8+ T cell responses, resulting in a much higher degree of protection. In contrast, immunization with these SEREX-defined autoantigens alone resulted in antigen-specific Treg responses and increase susceptibility to tumor challenge.

A)



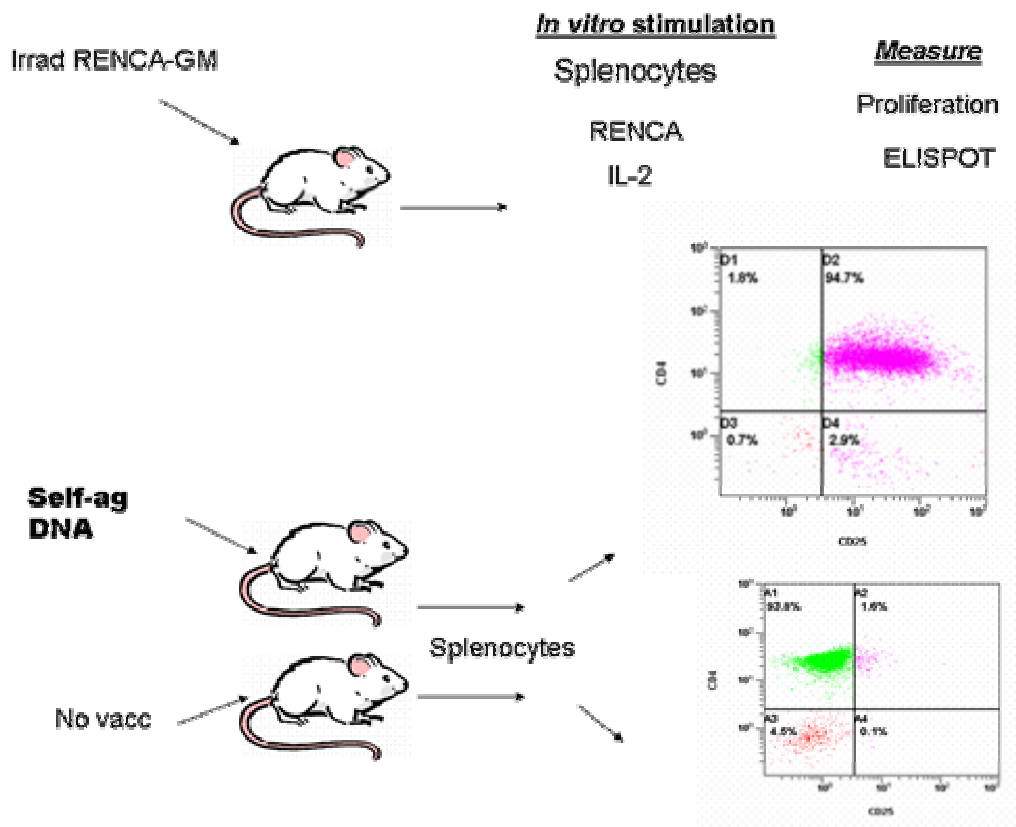
B)



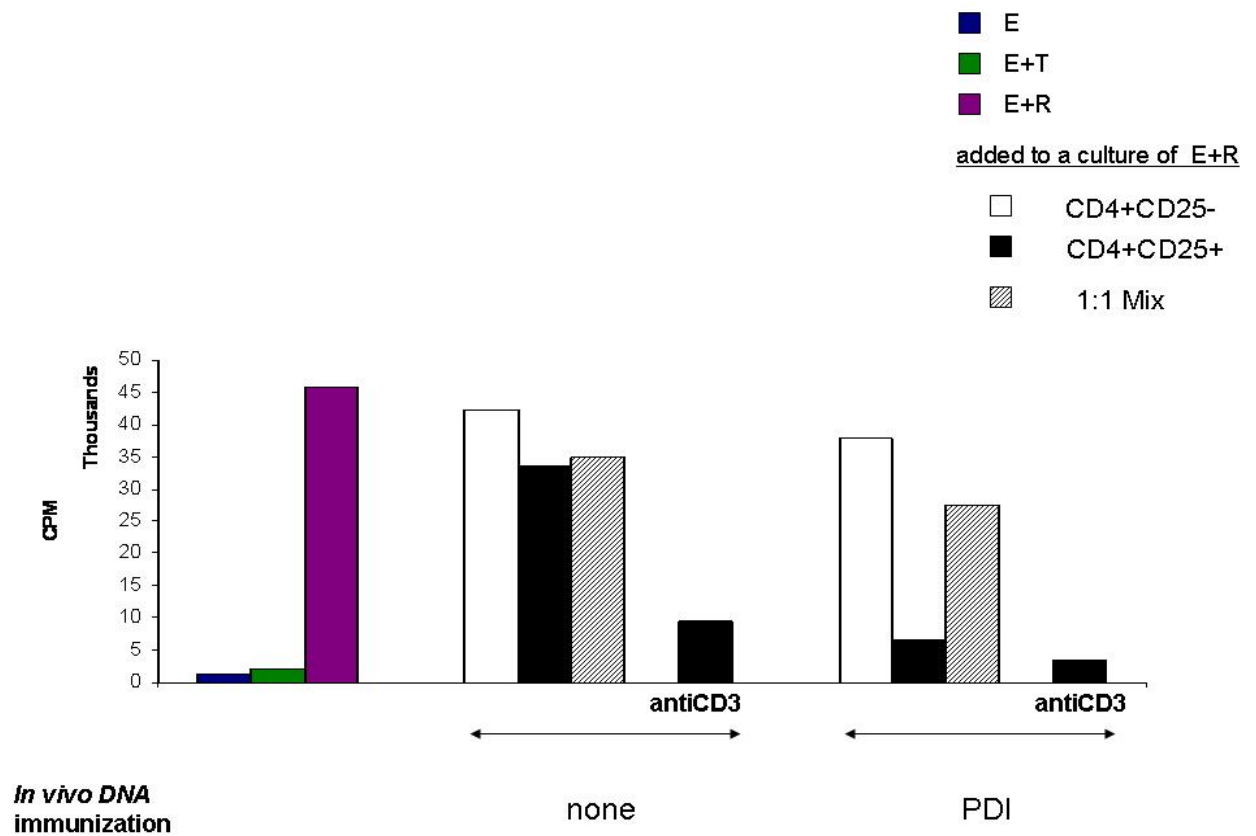
**Figure 3.13: Tumor efficacy of whole cell vaccines transduced with RENCA antigens and GM-CSF.** GM-CSF secreting CT26 cell lines alone or transduced with the following antigens: ROCK2, PDI, SSRP1, Aldose reductase (AR), Apg3p, ARF4, EIF4A1 or PBEF (A) and IQGAP1, TFIIS, H1F0 or GNB2 (B) were used for subcutaneous immunization in these experiments. Mice were vaccinated twice, one week apart. Challenge with  $6 \times 10^6$  live RENCA cells was performed 2 weeks after last vaccination.

To examine whether a similar mechanism of tolerance was operative in the RENCA model, we investigated whether vaccination with serologically-defined antigens triggered Treg responses. A schematic of the experimental design is presented in Figure 3.14. Wild type mice were vaccinated with irradiated, GM-CSF secreting RENCA cells. Single cell suspensions were prepared from harvested spleens, and mixed lymphocyte-tumor cell cultures established with irradiated RENCA cells and IL-2. Lymphocytes were collected after one week of *in vitro* stimulation and used as effectors for Treg suppression assays. Another cohort of mice was immunized with PDI as naked DNA. Spleens were then harvested and CD4<sup>+</sup>CD25<sup>+</sup> Tregs and CD4<sup>+</sup>CD25<sup>-</sup> T cell populations were isolated using antibody-based magnetic sorting. We then compared the proliferation responses of the effector T cells to RENCA targets in the presence or absence of Tregs harvested from PDI vaccinated or naïve mice (Figure 3.15). Anti-CD3 antibodies were used as a control to trigger maximum suppression of Tregs, regardless of prior immunization. As shown in Figure 3.15, CD4<sup>+</sup> CD25<sup>+</sup> T cells from PDI immunized mice induced significant suppression of RENCA effector T cell responses in the absence of anti-CD3 antibody stimulation. This implies that the Tregs were specifically activated *in vivo* in response to the PDI vaccination. In contrast, CD4<sup>+</sup> CD25<sup>+</sup> T cells isolated from naïve BALB/c mice showed immunosuppression only following anti-CD3 stimulation.

Overall, these preliminary results suggest that immunization with PDI might elicit antigen specific CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells. Additional experiments are necessary to confirm these results and extend them to other SEREX-defined antigens.



**Figure 3.14: Schematic representation of Tregs isolation and immunosuppressive activity assessment.** RENCA specific T cells (Effectors) are obtained from splenocytes of mice vaccinated twice, with irradiated (irrad) RENCA-GM and stimulated *in vitro* for about 7 days in the presence of irradiated RENCA cells (Targets) and IL-2. CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from DNA vaccinated (self-ag DNA) or wild-type mice (no vacc) are added to a mix of Effectors plus Targets; vaccination (vacc). Both populations purity is confirmed by FACS analysis. Proliferation or interferon- $\gamma$  secretion (ELISPOT) can be performed to assess immunosuppressive activity.



E -Effectors (Figure 3.14)  
 T -irradiated thymocytes  
 R -irradiated RENCA cells (100Gy)

**Figure 3.15: Tregs suppressive activity by PDI DNA vaccines.**  $5 \times 10^4$   $CD4^+CD25^-$  and  $CD4^+CD25^+$  T cells isolated from wild-type or BALB/c mice, immunized i.m. twice with plasmid DNA coding for PDI, were added to a mix of *in vitro* stimulated splenocytes (Effectors) and irradiated RENCA cells (Targets). Proliferation was evaluated by pulsing with [ $^3$ H]thymidine for 20 hours. As control, we added 1  $\mu$ g/ml of anti-CD3 antibody to control wells.

## **Summary**

In this part of our study, we evaluated several antigen-based and whole tumor cell vaccines to test the immunogenic potential of serologically identified RENCA-associated antigens. Naked DNA vaccines, transduced dendritic cells, xenogeneic proteins, and engineered tumor cells, also failed to stimulate protective immunity. The inclusion of cytokine adjuvants and CTLA-4 antibody blockade did not improve therapeutic efficacy. In contrast, preliminary experiments raise the possibility that vaccination with these non-mutated, self antigens alone stimulates immunosuppressive regulatory T cell responses. Additional studies are required to define the mechanisms that determine the balance between tumor protective and regulatory responses elicited with GM-CSF secreting tumor cells.

## DISCUSSION

### 4.1 Diversified Antibody Repertoire Induced by GM-CSF Secreting RENCA Cell Vaccines: Mechanisms of Immunogenicity

Tumor cells can be genetically modified to produce cytokines and /or costimulatory molecules to improve their immunogenicity, thus providing better cellular vaccines. Previous studies from our group have demonstrated that GM-CSF is one of the most potent stimulatory molecules in augmenting tumor immunity in multiple murine tumor models, including RENCA (Dranoff *et al.* 1993). Vaccination with irradiated tumor cells engineered to secrete GM-CSF was shown to generate a potent, specific and long-lasting immunity in these tumor models. This vaccination requires the participation of both arms of the immune system, specifically T lymphocytes and plasma cells, as well as improved antigen presentation by macrophages and DC recruited to the immunization site.

Identification of tumor antigens able to elicit an immune response leading to tumor destruction in tumor-bearing hosts has been a long-term goal in the field of tumor immunology (Boon and Old 1997). In this study, we examined the humoral response induced by GM-CSF-secreting RENCA vaccines, to better understand the role of this cytokine in the enhanced tumor immunity observed in this model. Using a serologic approach, we performed an immunoscreening of a cDNA library derived from RENCA cells with sera from mice vaccinated with wild-type and GM-CSF-secreting RENCA cells. Our analysis led to the identification of 28 distinct antigens, 22 representing a diversity of cellular proteins with well-known functions. Immunoreactivity analysis to this panel of antigens showed that a more potent antibody response was evoked by increasing immunizations and, for the concentration of tested sera, these high titer IgG antibodies titers were not present in naïve mice. Moreover, comparison of sera reactivity confirmed that GM-CSF-secreting vaccines induced a quantitatively different humoral response when compared with wild-type cells, which was characterized by a more diverse antibody repertoire. These results suggest that a broader immune response may be responsible for the enhanced antitumor effect observed in GM-CSF secreting vaccines.

Tumor antigens' immunogenicity 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 (Ichiki *et al.* 2004; Lennerz *et al.* 2005). To investigate whether this was the case for any of the antigens found in our 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 immunogenicity of these proteins. To further explore the mechanism of immunogenicity of these broadly expressed self-antigens, we performed a series of studies to define tissue expression. Using Northern blot analysis, we found that several of the identified antigens including ROCK II, TFIIS, FDS, SSRP1, CD44, IQGAP1 and GNB2 were upregulated in tumor cells (RENCA and B16, a murine melanoma cell line) when compared to normal 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 additional DNA mutations (Fukuchi-Shimogori *et al.* 1997). This has been suggested as a mechanism by which self-proteins are recognized by the immune system (Brass *et al.* 1997). 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 the mechanism of immunogenicity.

#### **4.2 Key Biological Role of Upregulated RENCA Antigens in Tumor Progression**

A large proportion of the proteins found in our immunoscreening have been identified as immunologic targets in different murine and human tumor models (Table V). This suggests their association with antitumor immunity and a potential use in the clinic for immunotherapy of different tumor malignancies. Characterization of these immunogenic targets showed that we uncovered a multitude of tumor-associated antigens, some of which upregulated in tumor cells, sharing common biologic pathways implicated in carcinogenesis. These include transcription, translation, metastasis, and stress response.

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. Alterations in the mevalonate pathway are known to be associated with malignant cell growth (Goldstein and Brown 1990). 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. ROCK II or protein serine/threonine kinase is a downstream effector of Rho, a GTPase of the Ras superfamily (Hunter 1997; Van Aelst and D'Souza-Schorey 1997).

Antibodies against proteins of the ROCK family have been found, not only in our screening, but also in the B16 murine melanoma model by our group, as well as in patients with squamous cell lung carcinoma, sarcoma, renal cell carcinoma and multiple myeloma (Scanlan *et al.* 1999; Diesinger *et al.* 2002; Lee *et al.* 2003; Bellucci *et al.* 2004). Moreover, this protein was recognized by several melanoma patients that had undergone GVAX vaccines (Soiffer *et al.* 2003; Nemunaitis 2005). These observations point out to the validation of murine models in identifying human tumor rejection antigens.

G protein-coupled receptors (GPCRs) are integral membrane proteins that respond to specific extracellular signals by activating G protein within the cell. Upon GPCR activation, heterotrimeric G proteins can signal to different effector molecules through their  $\alpha$  and  $\beta\gamma$  subunits (G-dimers) (Gilman 1987; Birnbaumer 1992). Recent studies have indicated that activation of these proteins can lead to the oncogenic transformation of different cell types. GNB2 is a subunit of heterotrimeric G-proteins that function as downstream effectors of G-protein coupled receptors (GPCR) on the surface. As shown in Figure 3.4, they function upstream of RhoGTPases as well as other GTPases regulatory proteins (e.g. RhoGEF). In addition, their aberrant expression has been associated with tumor proliferation.

IQGAP1 and CD44 members of the Rho-signalling 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 adhesion. This scaffolding protein participates in multiple cellular functions, including  $\text{Ca}^{2+}$ -calmodulin signaling, cytoskeleton architecture, CDC42 and Rac signaling, E-cadherin-mediated cell-cell adhesion and  $\beta$ -catenin-mediated transcription (Hart *et al.* 1996; Ho *et al.* 1999). IQGAP1 has a fundamental role in cell motility and invasion (Mataraza *et al.* 2003). Overexpression of IQGAP1 in mammalian cells enhances cell migration in different cell types in a Cdc42- and Rac1-dependent manner. Knock down of endogenous IQGAP1 using small interfering RNA (siRNA) and by transfection of a dominant negative IQGAP1 construct can significantly reduce cell motility. Cell invasion can similarly be altered by manipulating intracellular IQGAP1 concentrations. Stable overexpression of IQGAP1 also led to a significant increase in cell invasive capacity (Mataraza *et al.* 2003).

CD44 is a type I transmembrane glycoprotein and functions as the major cellular adhesion molecule for hyaluronic acid (HA), a component of the ECM. This protein is expressed in most human cell types and is implicated in a wide variety of physiological and pathological processes, including lymphocyte homing and activation, wound healing, cell migration, and tumor growth and metastasis (Aruffo *et al.* 1990; Gunthert *et al.* 1991; Okamoto *et al.* 1999; Okamoto *et al.* 2001; Nagano *et al.* 2004). CD44 is encoded by a

single gene, but multiple forms are generated by alternative RNA splicing. Different posttranslational modifications, including glycosylation, generate additional structural diversity of CD44. CD44 has been shown to interact with ROCK protein promoting tumor cell migration (Bourguignon *et al.* 2003). CD44 is proteolytically cleaved at the ectodomain through MMPs in various cancer cell lines. This ectodomain cleavage was found to play a critical role in CD44-mediated tumor cell migration by regulating the dynamic interaction between CD44 and the extracellular matrix (Okamoto *et al.* 1999; Kajita *et al.* 2001). Increased levels of soluble CD44 (sCD44) have been detected in plasma from patients with some tumors (Okamoto *et al.* 2002). This may reflect the increase in proteolytic activity and matrix remodeling that is associated with tumor growth and metastasis. Highly aggressive melanoma cell lines were found to shed significant amounts of CD44 from the cell surface and show increased CD44 synthesis as compared to other cell lines and melanocytes (Goebeler *et al.* 1996).

Another gene product identified in our screen found 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, through its middle domain (Spencer *et al.* 1999). It can also heterodimerize with Spt16 to form FACT, a complex initially shown to facilitate chromatin transcription (Orphanides *et al.* 1999). 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. Interestingly, antibodies (Abs) against the structure specific recognition protein 1 (SSRP1) were reported in a small series of systemic lupus erythematosus (SLE) patients, but not in other systemic autoimmune diseases (Santoro *et al.* 2002; Fineschi *et al.* 2004).

### **4.3 Intracellular Proteins as Humoral Targets of Immune Responses**

Most of the autoantigens identified in our immunoscreening were predominantly intracellular with ubiquitous expression and wide tissue distribution.

One common finding between cancer and systemic autoimmune diseases is the presence of antibodies against intracellular proteins associated with immunosurveillance or pathogenesis, respectively (Livingston *et al.* 2000). Two recent papers have shown that a combination of defects in immunoregulatory checkpoints - imperfect self-microbe discrimination by TLRs and B cell receptors - can result in antibody secretion against DNA, RNA and other intracellular self-proteins culminating in autoimmune diseases (Kumar *et al.* 2006; Pisitkun *et al.* 2006).

Moreover, data on antigen processing and presentation has shed some light on how intracellular TAA and autoimmune antigens can induce a humoral response. Protein recognition by the immune system usually happens through presentation on the cell surface. Alternatively, proteins may be released from damaged cancer cells due to tumor necrosis or apoptosis, which may result in their presentation in complex with other proteins, such as heat-shock proteins. Most cells, including tumor cells, can present endogenous antigenic peptides bound to MHC I to T cells. However, APCs are the only ones with capacity to prime an immune response. In addition, these cells have the unique ability to acquire antigens from other cells and present them via their own MHC class I molecules. This process of cross-presentation is thought to play a key role in tumor immunity (Shen and Rock 2006). Apoptotic and necrotic cells are thought to be the major source of cross-presented antigens. Even though there is some controversy about the precise mechanism that leads to antigen capture, phagocytosis of apoptotic bodies, nibbling from live cells and receptor-mediated endocytosis of HSP-chaperoned peptides are thought to play a major role in this process (Regnault *et al.* 1999; Schild *et al.* 1999; Binder *et al.* 2000). Antigen-bound antibodies, called immune complexes, can also play an important role in DC maturation and cross-priming (Regnault *et al.* 1999; den Haan and Bevan 2002). These immune complexes are taken up by DC through their Fc $\gamma$  receptors and cross-presented intracellular tumor-derived antigens can induce tolerance or immunity (cross-priming). This outcome seems to be dependent on the absence or presence of inflammatory and co-stimulatory signals.

Thus, apoptosis induced by irradiation of tumor cells following vaccination and exposure of intracellular proteins from dying cells could be one possible explanation to the immunogenicity of these intracellular antigens. These proteins become available to mature APCs able to prime an antigen-specific immune response. In addition, the inflammatory environment associated with whole GM-CSF-based vaccines is thought to play an important role. GM-CSF at the site of vaccination is known to promote recruitment and maturation of DC and macrophages. These professional APCs can, upon uptake of the exposed intracellular tumor antigens, process and present them with the right costimulatory signals and prime an immune response.

#### **4.4 Self, Non-mutated Proteins are Common Targets of Tumor Immunity and Autoimmunity**

The concept of tumor immunosurveillance was based on the existence within patients of a T-cell and/or an antibody repertoire recognizing tumor antigens specifically expressed by tumor (Boon and van Baren 2003; Boon and Van den Eynde 2003). In

addition, murine tumor models in immunodeficient mice have shown the ability of the immune system to inhibit tumor growth (Dunn *et al.* 2004).

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 (Sahin *et al.* 1995; Nishikawa *et al.* 2005). As these immunogenic molecules are detected by IgG antibodies, this method is also an indirect way to study the CD4<sup>+</sup> T cell repertoire. 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. In our study, the analysis of the antibody repertoire in mice vaccinated with irradiated wild-type or GM-CSF secreting RENCA cell vaccines led to the identification of a large panel of self, nonmutated RENCA associated tumor antigens. With few exceptions, intracellular proteins with ubiquitous expression 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) (Minota *et al.* 1991; Heegaard *et al.* 2000; Lim *et al.* 2002; Fineschi *et al.* 2004; Ludewig *et al.* 2004). Even though the basis for this self-protein immunogenicity is unknown, these data support the notion that different kind of events (e.g. viral infection, transformation) can trigger the immune system to previously ignored antigens. Upon tissue insult, these autoantigens can be released and exposed to professional APCs able to prime an immune response. A better understanding of the antigen processing pathway has uncovered new answers for self-proteins immunogenicity. Some of these pathways include cross-presentation, proteasome-mediated protein or peptide splicing, and epitope spreading (Mamula 1998; Hanada *et al.* 2004; Vigneron *et al.* 2004; van der Most *et al.* 2006). Also, the context in which tumor cells are exposed to the immune system (e.g. proinflammatory cytokines in the tumor milieu) is a key point in the generation of such an effective immune response, since the immune system is tolerant of certain tumor antigens, as they may be presented in a non-stimulatory context (Dranoff 2004).

Our findings confirm that breaking tolerance to self is a mechanism common to tumor immunity, autoimmunity and infection, and that these shared immunogenic targets can according to the immunostimulatory environment induce a protective immune response or disease.

## 4.5 Self-Antigens: Tuning the Balance Between Antitumor Immunity and Tolerance

After defining RENCA immuno relevant antigens induced by GM-CSF-secreting whole cell vaccines, our next challenge was to recapitulate protective tumor immunity observed by these vaccines using these tumor-associated antigens. In order to evaluate the potential of these proteins in tumor rejection, we used different antigen-based immunotherapeutic approaches including “naked” DNA vaccines, antigen-loaded DCs, xenogeneic proteins and transduced whole tumor cells. Immunization with antigens alone often elicits weak or no immunity, and a better immune response can be induced if antigens are administered in combination with adjuvants. Therefore, we used different immunostimulating agents such as pro-inflammatory cytokines (GM-CSF and IL-2), CTLA-4 antibody blockade, IFA and CpG dinucleotides. Our results showed that immunization of mice using these serologically-defined self-antigens was not sufficient to induce tumor protection *in vivo* against live RENCA cells.

The adaptive immune system, with its TCR and antibody diversity has developed mechanisms to discriminate self from nonself (Burnet 1961). This allows the immune system to fight nonself pathogens and at the same time avoid autoimmunity. However, cancer is not an exogenous pathogen, but rather arises from normal host cells, and the large majority of the tumor antigens recognized by T cells and antibodies in cancer bearing hosts characterized to date are unaltered nonmutated self antigens also expressed in normal cells (Boon *et al.* 1997; Rosenberg 1997). This self / nonself paradigm poses a problem for the immune system in order to achieve tumor immunity. Since the immune system is “trained” not to respond to self and most tumor-associated antigens are self proteins, these antigens are usually ineffective at triggering an immune response.

Tumor cells escape from T cell immunity can be due to: i) insufficient number of host T cells against self-Ags are present in the T cell repertoire; ii) immune tolerance of T cells through anergy, T cell deletion or suppression by regulatory cells; or iii) ignorance of T cells against self-Ag positive cells. Because high avidity, self-reactive T cells are deleted in the thymus, any residual self-reactive T cells existing in the periphery are likely to be low avidity and nonresponsive due to peripheral tolerance mechanisms. Activation of these residual T cells is critical for targeting tumors for immunotherapy.

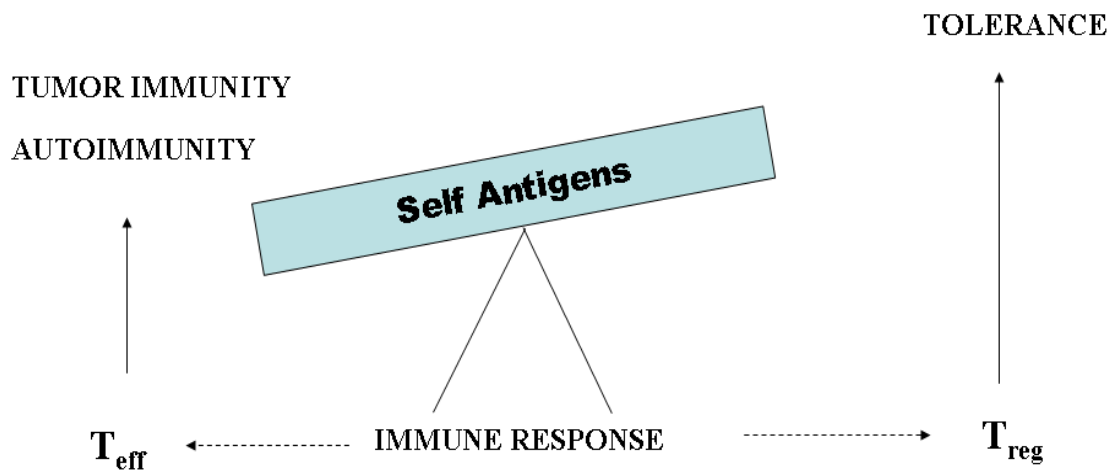
The fact that a TAA elicits a tumor-specific immune response does not necessarily mean that this immune response is accompanied by rejection of the tumor *in vivo*. As discussed above, Tregs constitute a major challenge to cancer vaccine strategies given their important role in suppressing TAA-specific immunity. Studies by Shiku and

colleagues have shown in a methylcholanthrene (MCA) tumor model, that immunization with SEREX-defined self antigens results in accelerated tumor development mediated by development of highly active CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Nishikawa *et al.* 2005). Moreover, this accelerated tumor development was abolished by antibody-mediated depletion of CD4<sup>+</sup> T cells or CD25<sup>+</sup> T cells. However, under the appropriate condition, such as copresentation of immunogenic CTL epitopes, they were able to show helper activity rather than regulatory activity of activated CD4<sup>+</sup> T cells, which led to the potentiation of specific CD8<sup>+</sup> T cell generation and increased tumor resistance, *in vivo*.

Consistent with these findings, our preliminary studies suggest that CD4<sup>+</sup> CD25<sup>+</sup> Tregs from mice vaccinated with PDI plasmid, but not wild-type mice, can suppress proliferation of effector cells *in vitro* in our RENCA tumor model. These data support the hypothesis that vaccination with self-tumor antigens can induce immunosuppressive T cells that balance the immune system towards tolerance.

Our work demonstrates that an array of autoantigenic molecules derived by tumor cells can stimulate the production of antibodies as a result of a protective immune response. Characterization of this humoral response against self-proteins highlights shared antigenic targets between tumor immunity, autoimmunity and tolerance. This study outlines the importance of the context on how these molecules are "seen" by the immune system. As represented in Figure 4.1, in the context of dying tumor cells and of an immunostimulatory environment, such as GM-CSF secreting whole cell vaccines, it is possible that these molecular targets can break tolerance to self or can induce activation of helper T cells specific for other immunogenic epitopes. These helper T cells could then be responsible for shifting this immunologic equilibrium towards protective antitumor immunity. On the contrary, without the right stimulatory environment, active immunizations with these self-antigens may not be sufficient to overcome immunoregulatory checkpoints. Thereby, these antigens can trigger Tregs-mediated suppression of TAA-reactive effector cells to induce tolerance and can be proposed as a potential mechanism to explain the failure of antitumor immunity. Our results suggest that cytokine adjuvants, CTLA-4 blockade, and engineered dendritic cells are not sufficient to overcome tolerance to these antigens. Other negative immune regulatory circuits, such as PD-1 and B-7H4, might play important roles in limiting the effector responses to these antigens.

Immune responses to self-antigens can, depending on the immunostimulatory environment, activate effector or regulatory T cells, leading to immunity (autoimmunity / tumor immunity) or tolerance, respectively.



**Figure 4.1: Tuning the immunologic balance.**

## FINAL REMARKS AND FUTURE PERSPECTIVES

Serologic-defined tumor autoantigens seem to be at a cross-road where tumor immunity, tolerance and autoimmunity meet. How T cells can be triggered to reject tumors, expressing weak self antigens, without causing autoimmunity or tolerance, has been a major challenge in the field of tumor immunology. Thereby, understanding the molecular mechanisms by which these proteins can trigger different immunologic outcomes is extremely useful, not only to develop better cancer vaccines, but also to answer fundamental biological questions. Regulatory T cells are crucial for maintaining T-cell tolerance to self-antigens. Therefore, targeting these cells by blocking their immunosuppressive mechanisms represents a new immunotherapeutic approach. In the absence of this immunoregulatory checkpoint we might be able to unveil the role of these RENCA-defined antigens in tumor rejection.

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

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