A DENDRITIC CELL VACCINE FOR MURINE RENAL CELL CARCINOMA

by

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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of
Doctor of Philosophy

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ABSTRACT

Renal Cell Carcinoma (RCC) has a very high rate of mortality since it does not respond to conventional therapies such as chemotherapy and radiation therapy. Furthermore, in the majority of cases, metastases are already present at the time of diagnosis. The objective of our study is to develop a novel treatment for RCC, using a dendritic cell (DC) vaccine. An animal model of RCC, RENCA, was used to develop the vaccine.

We have demonstrated the ability of RENCA antigen-pulsed DCs to reduce tumor growth in animals with low tumor burden. Furthermore, tumor growth was shown to negatively impact on spleen cells and T cell proliferation, interferon gamma production, NK cell activity and decrease the translocation of the transcription factor NF-kB to the nucleus. In order to overcome RENCA-induced immunosuppression, DCs were activated with CpG-ODNs. We demonstrated that RENCA-pulsed CpG-treated DCs were able not only to significantly reduce tumor growth, but to prevent tumor implantation in 60% of animals. In addition, successfully treated animals can sustain a tumor challenge and the protection conferred by the vaccine can be adoptively transferred to naïve animals by splenic T cells. Successful implantation of a colon carcinoma (CT-26) in resistant animals demonstrated the specificity of our DC vaccine.

This data demonstrates that RENCA impacts negatively on the immunity and that DC therapy, in conjunction with CpGs, can overcome this problem. Furthermore, this regimen helps to develop long-term and specific immunity. These experiments also emphasize the importance of an optimal host immune response for the positive outcome of a therapy for cancer.

Overall, the data presented in this thesis pave the way for the development of a novel therapy for human RCC specifically but other cancers could be treated using the same strategy.

RÉSUMÉ

Le cancer du rein a un taux très élevé de mortalité puisqu'il ne répond pas aux thérapies conventionnelles telles que la radiothérapie et la chimiothérapie. En outre, dans la majorité de cas, les métastases sont déjà présents au moment du diagnostic. L'objectif de notre étude est de développer un nouveau traitement pour le cancer du rein, en utilisant un vaccin composé de cellules dendritiques (CDs). Un modèle animal, RENCA, a été employé pour développer le vaccin.

Nous avons démontré la capacité des CDs pulsées avec des antigènes de RENCA à réduire la croissance tumorale chez l'animal avec un faible fardeau tumoral. En outre, nous avons démontré que la croissance tumorale avait un impact négatif sur la prolifération de cellules spléniques et des lymphocytes T, la production d'interféron-gamma, sur l'activité des cellules NK et diminuait la translocation du facteur de transcription NF-κB au noyau. Afin de surmonter l'immunosuppression induite par RENCA, les CDs ont été activées avec des oligonucléotides contenant des motifs CpG (CpG-ODNs). Nous avons démontré que les CDs traitées aux CpG-ODNs et pulsées avec des antigènes de RENCA pouvaient non seulement réduire de manière significative la croissance de la tumeur, mais pouvaient aussi prévenir l'implantation de tumeur chez 60% des animaux. En outre, les animaux traités avec succès peuvent résister à l'implantation d'une seconde dose tumorale et la protection conférée par le vaccin peut être transférée à des animaux sains par les cellules T spléniques. L'implantation réussie d'un cancer du colon (CT-26) chez les animaux résistants a démontré la spécificité de notre vaccin composé de cellules dendritiques.

Ces données démontrent que RENCA a un impact négatif sur le système immunitaire et que la thérapie à base de CDs traitées aux CpG-ODNs peut surmonter ce problème. En outre, ce régime aide à développer une immunité spécifique et de longue durée. Ces expériences soulignent également l'importance d'une immunité optimale chez l'hôte pour l'obtention de résultats positifs d'une thérapie pour le cancer.

De façon générale, les données présentées dans cette thèse préparent le terrain pour le développement d'une nouvelle thérapie pour le cancer du rein plus

spécifiquement mais d'autres formes de cancer chez l'humain pourraient également être traités en utilisant la même stratégie.

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LIST OF ABBREVIATIONS

ADCC	antibody dependant cellular cytotoxicity
Ag	antigen
APC	Antigen presenting cell
BCG	bacillus Calmette Guerin
CCR	chemokine receptor
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
EMSA	electromobility shift assay
GM-CSF	granulocyte/macrophage colony-stimulating factor
HSP	heat shock protein
IFN	Interferon
IL	Interleukin
IP	intraperitoneal
IV	intravenous
KLH	keyhole limpet hemocyanin
LPS	lipopolysaccharide
MHC	major histocompatibility complex
NF-κB	nuclear factor κB
NK	natural killer
NKT	natural killer T cell
ODN	oligodeoxynucleotide
PMA	phorbol 12-myristate 13-acetate
PO	phosphodiester
PS	phosphorothiorate
PSA	prostate specific antigen

RCC	Renal Cell Carcinoma
RNA	ribonucleic acid
TAA	tumor associated antigen
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TIL	tumor infiltrating lymphocyte
TIR	toll/ interleukin-1 receptor
TLR	toll like receptor
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated nick-end labeling
VHL	Von Hippel-Lindau
WT	Wilm's tumor

PREFACE

In accordance with the "Guidelines concerning thesis preparation" of the Faculty of Graduate Studies and Research, manuscripts of paper which have been published or which have been submitted for publication have been incorporated in the thesis. This format for the thesis preparation has been approved by the Division of Experimental Medicine, Department of Medicine.

This thesis is written in a form that includes manuscripts submitted (or to be submitted) for publication, according to the instructions to authors of thesis that can be found on the McGill University Graduate and Post-doctoral Studies Faculty web site, Each manuscript presented in this thesis (chapter 2, 3 and 4) possesses its own Abstract, Introduction, Materials and Methods, Results, Discussion and References. Prefaces, used to connect the chapters, are present at the beginning of each chapter. Furthermore, an Abstract (p. v), Introduction (chapter 1), and General Discussion (Chapter 5) sections are included in the thesis in order to present an overall view of the subject matter contained in the thesis. Contributions from each author to these manuscripts are also clearly indicated on the following page.

CONTRIBUTION OF AUTHORS

The studies described in the thesis have been carried out by the author of the thesis under the guidance and supervision of Drs. Simon Tanguay and Mario Chevrette and the guidance of Dr LuAnn Thompson-Snipes who was a close collaborator for Chapter 2 and 3. All studies were performed at the Montreal General Hospital Research Institute. Dr. Mostafa Elhilali has provided financial assistance for all the studies presented in this thesis.

Studies presented in Chapter 2 have been published in the British Journal of Urology[*]. The author of the thesis is first author on the paper although the second author (Dr LuAnn Thompson-Snipes) and the first author participated equally in the design and execution of all experiments, as well as the writing of the manuscript.

The results presented in Chapter 3 have been submitted for publication in Clinical Immunology[+]. The author of the thesis is first author on the paper.

Although most experiments were conducted by the first author, Meng Guan contributed to Figure 4 and data not shown and Levent Ozdal contributed to Figure 2. The results presented in Chapter 4 will be submitted to the British Journal of Urology[#]. All experiments have been designed and executed by the first author, with technical help from Jean-Sébastien Ripeau and O. Levent Ozdal.

[*] **Chagnon, F.**, Thompson-Snipes, L., Elhilali, M.M., Tanguay, S. Murine renal cell carcinoma: evaluation of a dendritic cell tumour vaccine. BJU International (2001) 88: 418-424.

[+] **Fanny Chagnon**, Simon Tanguay, O. Levent Ozdal, Meng Guan, Mario Chevrette, Mostafa M. Elhilali and LuAnn Thompson-Snipes. Potentiation of a Dendritic Cell Vaccine for Murine Renal Cell Carcinoma by CpG Oligonucleotides. (2003) Submitted to Clin. Immunol.

[#] **Fanny Chagnon**, Mario Chevrette, O. Levent Ozdal, Jean-Sébastien Ripeau, Mostafa M. Elhilali and Simon Tanguay. Long term memory and immunity conferred by a CpG-ODN-treated DC vaccine for murine renal cell carcinoma. (2003) Will be submitted to BJU International.

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor Dr. Simon Tanguay for his support and guidance. It was not always easy to put up with me and Simon has always be there to encourage me to go on in down moments. Simon, despite your busy schedule, I always felt I was a priority for you and you always had time for me, whether by email, phone or in person.

I also wish to thank my co-supervisor, Dr. Mario Chevrette who, as a non-immunologist, always had a very interesting perpective on my results. Mario, you might not realize that your multiple comments at my Journal Club presentations gave me many ideas and kept my thoughts alert!

I wish to thank Dr. LuAnn Thompson-Snipes who helped me develop a critical, independent scientific mind and who provided marvellous ideas for the pursuit of this project.

I would like to thank all the members of the Tanguay laboratory, past and present, but especially Frédéric Bergeron who was with me for the very beginning of this adventure. My gratitude also goes to all the members of the Urologic Oncology Research Group who provided technical help, moral help and most of all, physical help for the tasks I could not accomplish!

I would also like to acknowledge the help and judicious advice of my Ph.D. committee members: Drs. Danuta Radzioch, Marianna Newkirk, Albert Descôteaux and Patricia Tonin, with the additions of Drs. Nicole Bernard and Joyce Rauch who have been my academic advisors in the past.

I would like to acknowledge the support of McGill's Office for Students with Disabilities, especially Nissim Louis as well as the financial support from FRSQ-FCAR Santé, the McGill's Urology Departement and the MUHC Research Institute.

Last but not least, I would like to thank my husband Dr. Pierre Allard, who worked very hard to revise this thesis, as well as my family and friends for their encouragement and support. I would not have started and certainly not finished my Ph.D. without all of you.

CLAIMS TO ORIGINALITY

Chapter 2 established the validity of the RENCA model to study DC vaccines in human RCC.

1) DC cultures were established in our hands.
2) The DC vaccine can prevent tumor growth as long as tumor burden is minimal.
3) DCs enhance splenocyte proliferation *in vivo*, but RENCA antigen treated DCs abrogate splenocyte proliferation and this phenomenon is even more pronounced when DCs are put in contact with splenocytes from RENCA-bearing animals.

Chapter 3 demonstrates that CpG-ODN treatment of DCs potentiates the DC vaccine enough to overcome RENCA-induced immunosuppression.

1) The immune response to RENCA changes over time; it is enhanced at first (for 3-6 days) and then decreases to or below normal (around day 9).
2) NF-κB translocation to the nucleus is greatly diminished at day 7 after RENCA injection.
3) DC treatment with CpG-ODNs enhances their production of Th1 cytokines in our hands.
4) CpG-ODN-treated DCs can prevent RENCA tumor growth, whether the DCs are treated with RENCA antigens or not.
5) The tumors of the animals vaccinated with CpG-ODNs-treated DCs have a marked lymphocyte infiltration when compared with controls.

Chapter 4 demonstrates the capacity of CpG-ODN-treated DCs to establish a long-term, specific immunity to RENCA.

1) Animals vaccinated with CpG-ODNs-treated DCs that remain tumor free after 22 days are able to sustain a tumor challenge.

2) The splenic T cell of the animals that sustain a tumor challenge can transfer immunity to a naïve animal only if they were vaccinated with DCs that were treated with CpG-ODNs and RENCA antigens.
3) The immunity conferred by the T cell transfer is specific as the animals are not able to sustain challenge of an unrelated tumor, in this case the BALB/c colon carcinoma CT-29.

CHAPTER 1: INTRODUCTION

1.1 Renal Cell Carcinoma (RCC)

Although relatively uncommon, (only 4,000 new cases diagnosed in Canada in 2002 [Canadian Cancer Society, 2002]), Renal Cell Carcinoma (RCC) remains an important health problem because of its significant mortality. In fact, it accounts for 2.2% of newly diagnosed cancer and for 1.8% of deaths from cancer in 2002. It is predicted that 36% of patients presenting with a new diagnosis of RCC will succumb to their disease (Canadian Cancer Society, 2002). RCC is often characterized by an advanced stage at diagnosis, and not surprisingly, a high proportion of patients already have evidence of metastatic disease. RCC is also accompanied by diverse clinical manifestations as well as resistance to both radiotherapy and chemotherapy. However, many studies have suggested that RCC, like many other cancers, is partly controlled by the immune system (1).

1.1.1 Etiology

The incidence and mortality of RCC have increased dramatically over the past twenty years. The reasons for that increase remain unclear. RCC occurs almost twice as often in males as in females which could be due to an hormonal contribution, and the incidence is equivalent among blacks and whites (1). Studies have suggested that cigarette smoking (2), obesity (1), and many other factors such as hypertension, high-protein diet and occupational exposure to petroleum products, heavy metals and asbestos (3, 4) could have a causative role for RCC. In most cases of sporadic RCC, there is no known recognizable hereditary pattern (1) although specific gene mutations have been identified (1).

1.1.2 Diagnosis

Patients with small and localized RCC rarely present with clinical symptoms, contributing to the frequent delay in diagnosis (see section 1.1.4) (1). The most common clinical signs and symptoms are hematuria (50 to 60% of patients), abdominal pain (40%) and a palpable mass (30 to 40%) (1). The other signs are relatively nonspecific and include fever, night sweats, malaise and weight loss (1). Although there has been an earlier diagnosis in part due to the liberal use of abdominal ultrasound leading to incidental diagnosis of renal mass, 25 to 30% of patients are diagnosed with metastasis. The most frequent site of metastasis is the lung, followed by liver, bone and brain (1).

1.1.3 Molecular genetics

Patients with the Von Hippel-Lindau (VHL) syndrome have an extremely high incidence of RCC. This syndrome is known to be transmitted in an autosomal dominant fashion and associated with deletions or mutations of VHL tumor suppressor gene located on chromosome 3p25 (5). VHL disease can also be associated with tumors of the pancreas, adrenal, cerebellum, spine, inner ear, retina and epididymis (6). This syndrome is rare and renal tumors often appears at a early age (5, 7). The gene coding sequence contains three exons spanning 712 nucleotides (8). Tumors may arise after inactivation, e.g., deletion or hypermethylation, of the remaining wild-type allele in a cell (i.e., loss of heterozygosity) (9-11). The VHL gene product (pVHL) contains 213 amino acid residues (12, 13). The apparent molecular weight of pVHL is 28 to 30 kD (12, 13). The VHL gene product binds to two transcription factors, elongin B and elongin C and its binding site in often mutated in disease (14-16). It seems that the normal gene product is involved in inhibition of transcription elongation (14, 15). Loss of this function allows the unregulated growth of vascular tumors in multiple tissues (5). Furthermore, introduction of wild-type, but

not mutant, VHL protein into clear cell RCC cell lines lacking functioning VHL genes suppressed their ability to form tumors in nude mouse xenograft assays, confirming that the VHL gene product functions as a tumor suppressor (13, 17).

The WT1 (Wilms tumor) gene has also been implicated in the etiology of RCC. It is also a tumor-suppressor gene and may regulate the activity of two key oncogenes, namely *bcl-2* and *c-myc*. This gene has been found to be constitutively active in RCC (18). The *WT1* gene encodes a 3.5-kb transcript (19-21), alternatively spliced to give 4 protein isoforms (22). The amino terminal half of the protein contains a high concentration of proline and glutamine residues divided into 2 domains responsible for transcriptional activation and repression as measured by *in vitro* assays (23). The carboxy terminus of the protein contains 4 contiguous zinc finger domains that have a high degree of homology to the zinc finger domains of the EGR-1 and SP3 proteins (19) (24). All of the isoforms of WT1 are capable of binding specific sequences within the promoter regions of a number of different genes, including insulin-like growth factor-II (IGF2), platelet-derived growth factorA (PDGF-A) chain, the IGF-I receptor (IGF-IR) and the *WT1* gene itself (25). Transient transfection experiments have shown that WT1 can either activate or repress transcription from chimeric gene constructs containing WT1 binding sites depending on the promoter and cell type utilized (25). In addition to its ability to bind DNA and modulate transcription, the WT1 protein also interacts with the p53 protein (26). Given its properties as a transcriptional regulator it appears likely that induction of expression of this wild-type *WT1* gene in an inappropriate cell type could perturb cellular phenotype to a similar extent as loss of expression in normally expressing cells.

1.1.4 Stages of disease and prognosis.

The pathological stage remains one of the most important determinants of prognosis in RCC (1). Other prognostic factors, such as histologic pattern, nuclear grade, and deoxyribonucleic acid (DNA) content are much less established and influential than the pathological stage (27). Today, "TNM" is the most commonly

used staging system (28). It was published in 1997 by the International Union Against Cancer (29), and it based mostly on tumor size and level of invasion as seen in Table 1.

Table 1. UIC TNM Classification of Renal Cell Carcinoma (1997)

T--Primary Tumor	TX Primary Tumor cannot be assessed	
	T0 No evidence of primary Tumor	
	T1 Tumor 7.0 cm or less in greatest dimension, limited to the kidney	
	T2 Tumor more than 7.0 cm in greatest dimension, limited to the kidney	
	T3 Tumor extends into major veins or invades adrenal gland or perinephric tissues but not beyond Gerota fascia	
		T3a Tumor invades adrenal gland or perinephric tissues but not beyond Gerota fascia
		T3b Tumor grossly extends into renal vein(s) or vena cava below diaphragm
		T3c Tumor grossly extends into vena cava above diaphragm
	T4 Tumor invades beyond Gerota fascia	
N--Regional Lymph Nodes	NX Regional lymph nodes cannot be assessed	
	N0 No regional lymph node metastasis	
	N1 Metastasis in a single regional lymph node	
	N2 Metastasis in more than one regional lymph node	
M--Distant Metastasis	MX Distant metastasis cannot be assessed	
	M0 No distant metastasis	
	M1 Distant metastasis	

When detected early, RCC can be cured with surgical resection. In fact, T1 RCC have a 5 year disease free survival of 95%. However, higher stages have a much lower survival rate. In fact, close to 40% of the patients treated for local tumors will relapse with metastatic disease (6), and the prognosis of such untreated patients is highly unfavorable, with a 3-year survival rate of less than 5% (6).

1.1.5 Treatment

Treatment of RCC remains surgical resection of the affected kidney in most patients (30). Hormonal and chemotherapeutic approaches have demonstrated little or no effect in patients with metastatic renal cancer (27). This resistance to medical treatment is in part due to the expression of the multidrug resistance gene (*MDR-1*), whose main function is to prevent entry of drugs in the cell, and favor export of the drug if it does enter the cell (31). Furthermore, radiation therapy has not shown to have therapeutic benefit, even after nephrectomy, for patients with stage T2, T3, and T4 RCC and its use is limited mostly to palliation of bone pain secondary to metastatic disease (32). The importance of the patient's immune system in tumor regression was suggested from the late relapses after nephrectomy, prolonged stabilization of disease in the absence of treatment or spontaneous regression of metastatic disease (1, 30). The exact mechanism allowing these patients to develop a successful response against their tumor is still poorly understood. However, many strategies have been developed and adopted to strengthen the immune system. The most successful ones will be described below.

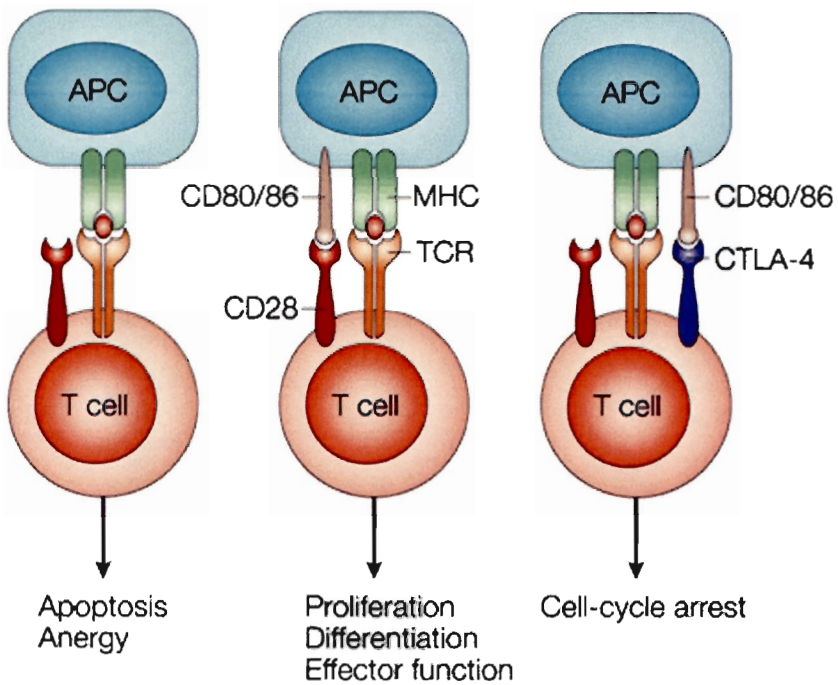
1.1.5.1 Overview of immune system

Cytokines activate the immune system in general. The ultimate goal of an immune-based therapy would be to avoid T cell anergy and stimulate quiescent CTLs to eliminate the tumor. If an antigen (Ag) is presented to a T cell by an antigen presenting cell (APC) (macrophage, B cell or DC) that lack the appropriate co-activation molecules, anergy will occur, and the T cell will not be activated (33). The

concept of anergy is similar to self-tolerance. However, if the same Ag is presented by an APC and co-activation molecules are present (eg Cluster of Differentiation (CD)-40, B7-1 (CD80) and B7-2 (CD86)) and bind CD28 on the T cell, the T cell will be activated (34). On the other hand, if CD80/CD86 bind CTLA-4 (an inhibitory molecule) on the T cell, cell cycle arrest will be induced. It is one of the signals for stopping an immune reaction and the most relevant in the context of cancer immunotherapy. (35) Other signals include production of anti-idiotypic antibodies, secretion of inhibitory cytokine (such as IL-10) and induction of T cell apoptosis (137). This is illustrated in figure 1.

Figure 1- *T-cell fate under different conditions of TCR engagement.* Simultaneous recognition of a specific MHC–peptide complex by the T-cell receptor (TCR) and of CD80 or CD86 by the co-stimulatory receptor CD28 results in T-cell activation, cytokine production, proliferation and differentiation. In the absence of CD28 ligation, T cells undergo apoptosis or become anergic. After T-cell activation and upregulation of cytotoxic T-lymphocyte antigen 4 (CTLA-4; CD152), co-ligation of the TCR and CTLA-4 results in cell-cycle arrest and termination of T-cell activation.

From Alegra *et al.*, 2001



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1.1.5.2 Biological response modifiers

The strategy based on biologicl response modifiers is centered on the modulation of the immune system to eradicate tumors. The first attempts were made with cytokines. It was known that the interferons (IFNs) had a direct antiproliferative effect on renal tumor cells *in vitro* (36) and that they enhanced Ag presentation through MHC (Major Histocompatibility Complex) molecules (37). Many clinical trials have been performed using IFNs in the treatment of RCC. Interferon-γ was shown to provide no benefit over placebo in patients with metastatic RCC (38). Studies have also been performed with recombinant Interleukin-2 (rIL-2). The use of high doses intravenous bolus rIL-2 resulted in objective response rate reaching approximately 20%. In addition, a large proportion of the responders to high dose IL-2 will maintain prolonged clinical response (6). Unfortunately, significant toxicity is associated with the use of rIL-2 (6, 30). Consequently, biological response modifiers such as either rIL-2 or IFN-α are still used but the need to find new and effective alternatives is pressing. Given the limited success of those two individual cytokines, attempts have been made to see if they have a synergistic effect. Once again, the clinical response rate was limited with only 5% of patients achieving a complete response (6). Furthermore, the combination does not lead to improved survival but increases toxicity (39). IL-6 being elevated in RCC, its use was tested in a phase I clinical trial. Although IL-6 was well tolerated, its use in the treatment of metastatic RCC was not recommended because of the poor response rate observed (30). IL-12 is known to enhance T helper 1 (Th1) cytotoxic responses and is being tested alone or in conjunction with IL-2 in the RENCA mouse model (40, 41).

1.1.5.3 Exogenous lymphocyte infusion

The use of tumor infiltrating lymphocytes (TILs) has been another promising approach in the treatment of RCC. In fact, *ex vivo* expansion of lymphocytes obtained from the tumor with recombinant IL-2 and reinfusion in conjunction with cytokine

therapy generated a 33% response rate in a Phase II trial (42). However, a randomized controlled trial using nephrectomy, blinded TIL/placebo infusion and IV IL-2 therapy did not demonstrate a benefit in using TILs over IL-2 alone (43).

1.1.5.4 Vaccines

In the context of cancer, it is very interesting to think in terms of vaccine strategies, given the very unpleasant side effects of current therapies. Currently, the use of vaccines is mostly prophylactic and their goal is centered in the prevention of infectious diseases such as rubella and diphtheria. For cancer, a therapeutic vaccine (e.g. a vaccine administered as a cure) would be ideal but considering the different types of cancer, it would be impossible to manufacture an antigen that would be common to all cancer types. The use of cancer vaccines will be discussed in details in another section (see section 1.2.7).

Right now, there are no licensed vaccines for RCC (30), although several interesting approaches have been developed and many clinical trials are being performed. As an example, a trial is being performed in patients with VHL mutations. It involves vaccination of patients with a peptide containing a mutation particular to the tumor (30). Another vaccine based approach therapy for RCC is making use of heat shock protein (HSP) 96 isolated from the tumor. This HSP carries tumor-specific peptides that are hopefully immunogenic. There are currently two randomized phase III trials going on and results are awaited (30, 44). The third approach for which clinical trials are underway for RCC treatment is to transduce tumor cells with co-stimulatory molecules such as CD80. A Phase I clinical trial was performed where transduced cells were administered in conjunction with the administration of IL-2. Four of nineteen patients responded but the discrimination between the effect of IL-2 and the vaccine could not be achieved in such a small cohort (45). Further studies are needed. As mentioned earlier, the vaccine approach is extremely interesting in RCC for two reasons: firstly, as no efficient treatment is available, it could represent an interesting alternative; secondly, in general, the side effects of DC vaccination are

relatively minor. For example, in the case of DC therapy against melanoma (46), patients experimented redness at the site of injection, fever and vitiligo.

1.1.6 Immunosuppression

1.1.6.1 T cell apoptosis in RCC

The relative poor response rate of therapeutic immune trials directed against RCC may be at least partially explained by inactivation signals resulting in immunosuppression. It is thought that the inactivation signals may originate from the tumor itself (47). In accord with this immunosuppression hypothesis, it has been demonstrated that T cell apoptosis reduces the immune response against tumor cells. It has been observed *in vitro* that T lymphocytes from patients with RCC undergo massive apoptosis and have altered signaling pathways, leading to nuclear factor-κB (NF-κB) inactivation (47). As will be described later, NF-κB is a transcription factor controlling many genes involved in the immune response and the protection against apoptosis (48) When examined *ex vivo*, the TILs from renal tumors exhibit impaired proliferation and cytotoxicity (49). This could be explained by the large portion (15 to 20%) of these TILs undergoing apoptosis as demonstrated by terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay. Furthermore, T lymphocytes harvested from healthy donors will undergo apoptosis when cultured in the presence of RCC tumor supernatant (50). This tumor-induced T cell apoptosis is thus an obstacle to the generation of an efficient immune based therapy. It has also been demonstrated that patients treated with IFN-α that have undergone nephrectomy have an increased survival compared with patients treated with IFN without having a nephrectomy (51). This is one more indication that an immunosuppressive molecule is secreted by the tumor and that its removal is beneficial for treatment. A better understanding of the mechanism resulting in this immunosuppression is necessary in order to develop more successful therapies.

1.1.6.2 NF-κB signalling pathway

NF-κB is a transcription factor regulating the expression of many genes. In mammals, NF-κB exists as hetero and homodimers formed by different combinations of these five proteins: NF-κB1 (p50), NF-κB2 (p52), c-Rel, RelA (p65) and RelB (48). All these proteins have a Rel homology region which is responsible for nuclear localization, and also for DNA binding (52). Under normal, unstimulated conditions, NF-κB is present in the cytoplasm in an inactive form where it is bound by the I-κB family of inhibitors. The I-κB proteins bind NF-κB through the Rel sequence, thus hiding its nuclear localization signal and preventing its nuclear translocation. Under these conditions, NF-κB cannot regulate the transcription of targeted genes (53). Activation of the NF-κB pathway is mediated by cell surface receptors interaction with their ligand (Figure 2), such as cytokines, LPS (lipopolysaccharide) and TCR or by physicochemical stress such as hypoxia or UV light. These signals lead to the activation of the IKK kinases, which phosphorylate the inactive I-κB/NF-κB complex. Once phosphorylated, I-κB is ubiquitinilated and degraded by the 26S proteasome. In the absence of I-κB, NF-κB is then free to go to the nucleus and activate gene transcription (53). Among the many genes activated by NF-κB, there are genes involved in the immune and inflammatory response such as cytokine, cytokine receptor and MHC class I genes, as well as genes involved in cell survival and prevention of apoptosis, like Bcl-2 and Bcl-XL (48). This cellular signaling pathway is present in almost every cell, but the rapid onset of NF-κB activation makes it ideal in the immune system where a rapid response is needed to initiate an immune response (48).

Figure 2- *Proposed mechanisms for induction of NF-κB proteins.* NF-κB molecules exist in the cytoplasm of most cells in an inactive form. NF-κB homo- and heterodimers are retained in the cytosol by the family of inhibitory molecules IκB. Extracellular inducers creating stress or indicating infection cause the activation of IκB kinase family of inhibitors through poorly defined pathway(s). Activation of NF-κB occurs predominantly through the phosphorylation and degradation of IκB proteins. Phosphorylation of IκB proteins is followed by polyubiquitination, and they are degraded as part of their respective ternary complexes. "Free" NF-κB dimers translocate to the nucleus, where it upregulates expression from many genes involved in the immune and inflammatory responses.

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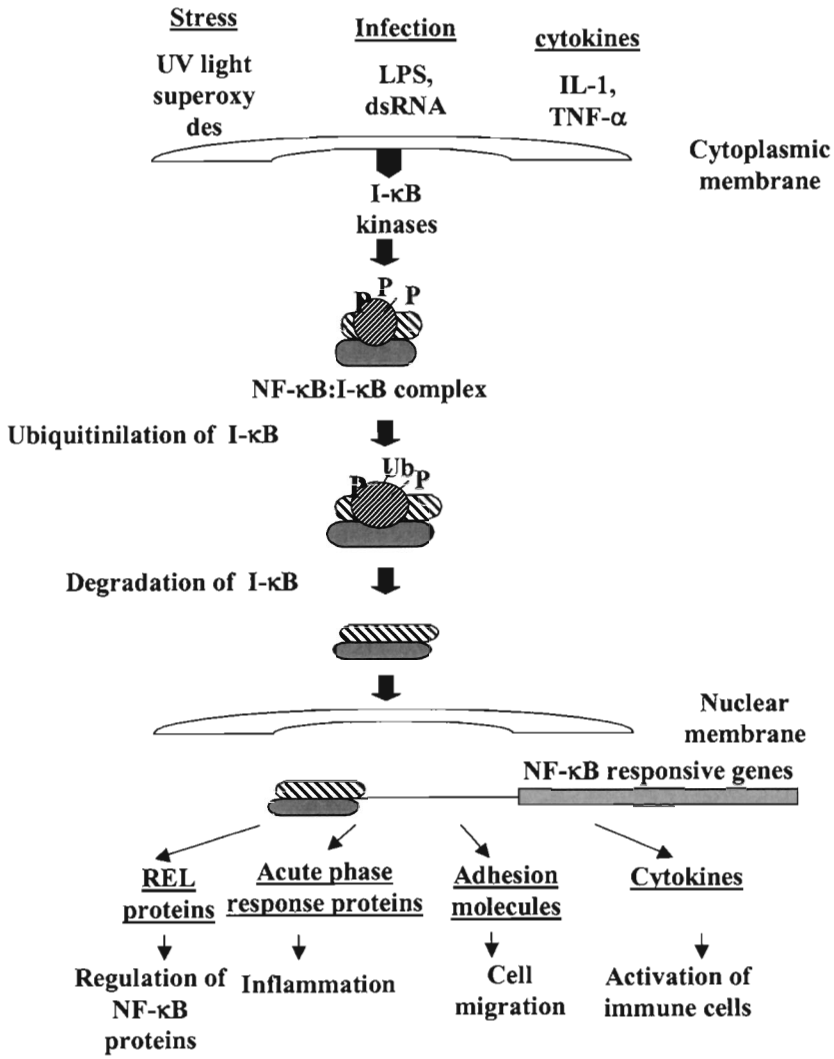
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Regulation of
NF-κB
proteins
Inflammation
Cell
migration
Activation of
immune cells

1.1.6.3 Suppression of NF-κB activation in RCC

Since NF-κB plays such a major role in the transcription of many cytokines important in T cell activation and since TIL from renal tumors were found to be dysfunctional (49), NF-κB was examined as being potentially involved in this process (47). Indeed, as mentioned earlier in section 1.1.6.1, the NF-κB pathway is suppressed in peripheral blood T cell from RCC patients. There is considerable evidence suggesting that this inactivation is due to soluble factors released by tumor cells (54-56). Some factors, such as IL-10, transforming growth factor (TGF) -β and prostaglandins may also play a role in tumor induced immunosuppression (47). In recent years, several teams have suggested that gangliosides might play an even more important role in the immunosuppression observed (57-60). Gangliosides are acidic glycosphingolipids that are present on the outer leaf of the plasma membrane (61). In normal cells, their role include regulation of cell differentiation and growth, cell adhesion, cell to cell interactions and membrane receptor modulation (61). Many solid tumors, including RCC, melanoma and neuroblastoma, aberrantly overexpress gangliosides and release them in the microenvironment (47). More specifically, GD_{1a}, GM_1 and GM_2 are overexpressed in RCC (62) and these gangliosides have been known to interfere with Ag presentation and overall T cell function (47). RCC-derived gangliosides have been shown to inhibit NF-κB activation (63) and induce apoptosis (64) in normal T cells. The exact mechanism by which gangliosides exert this action is not fully understood, however, since I-κB is normally degraded, it is believed that the defect is directly affecting the free activated NF-κB (47). Although recently discovered, these inhibitory mechanisms, as well as immune based therapy approaches needs to be studied further. To do so, *in vivo* studies animal models are needed.

1.1.7 Animal models of RCC

Based on the properties of human RCC, the characteristics of an ideal animal model for RCC would be a) spontaneous origin, b) histologically proven to be

adenocarcinoma, c) predicable growth rate and d) similar ability to metastasize to human RCC (65). There are three models that fulfill these criteria: the rat kidney carcinoma in Wistar-Lewis rats (65), human RCC tumor xenografts in athymic mice (65) and the murine renal adenocarcinoma RENCA syngeneic to BALB/c mice (65). The rat model is not very easily reproducible, so although useful, this model is less used. The xenograft model is extremely useful to study the responsiveness of human RCC to therapeutic agents (65). The disadvantage of working with nude mice is the lack of T cells preventing any immunological studies. Since the avenue of choice for future RCC treatment is immunotherapy, this model is unfortunately not very useful (65). Therefore, although not perfect, the most suited and used animal model remains RENCA.

1.1.7.1 The RENCA model

RENCA is a tumor which arose spontaneously in the kidney of a BALB/c mouse (65). It can be cultured either *in vitro* or passaged *in vivo* by subcapsular renal or intraperitoneal injection in BALB/c mice (65). Histological analysis hasconfirmed that RENCA is an adenocarcinoma, and is thus very similar to human RCC (66). Just as in RCC, when RENCA is implanted under the kidney capsule, it forms a primary tumor and metastasizes to the lymph nodes, lung and liver (67). Its immunogenicity is relatively low, although natural killer (NK) cells have been demonstrated to have an important role in the control of metastasis (68, 69). Another of its remarkable features is that similar to RCC, RENCA does not respond to either hormonal, radiation or chemotherapy. Some evidence suggest the presence of tumor-induced immunosuppression in RENCA (70, 71). This immunosuppression involves mainly splenocytes, which have a defective NK activity as well as a failure to generate LAK activity in the presence of IL-2 (70, 71). All those characteristics showing similarities with human RCC make RENCA a very relevant model to study human RCC.

Based on the similar properties between human RCC and RENCA, and their suppressive effects on the immune system, we hypothesized that there should be some ways to increase the immune response against these tumors by increasing the amount

of putative antigens presented to T cells. Among the most potent APC is the DC, which will be described in detail in the following sections.

1.2 Dendritic cells (DCs)

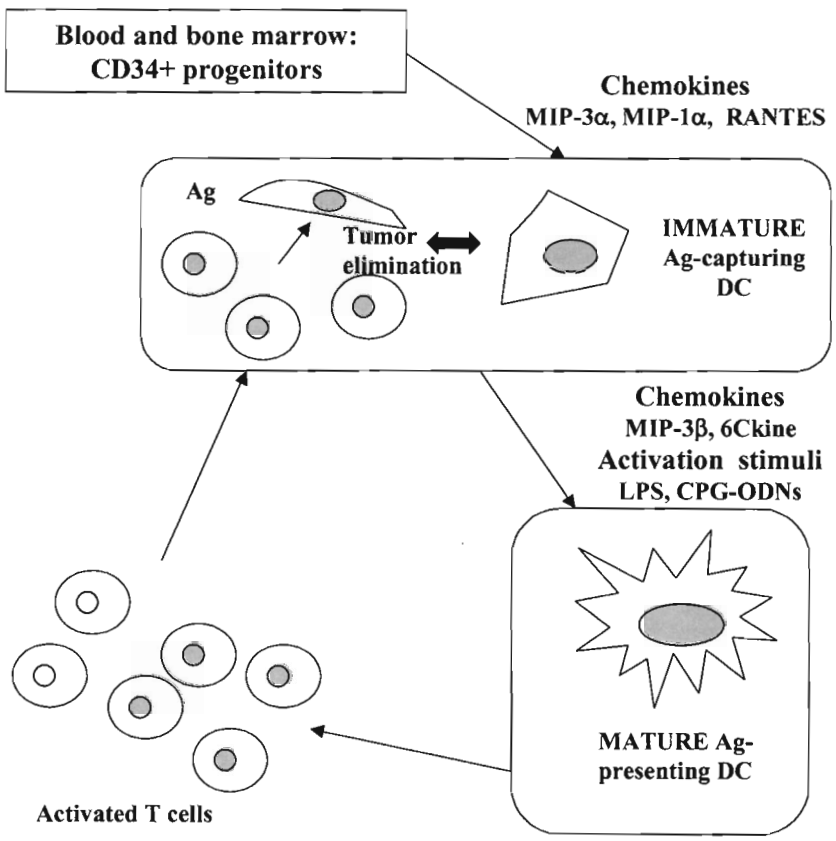
1.2.1 Dendritic cell function

DCs are APCs originating from the hematopoietic progenitors of the bone marrow (72). In fact, they are the most efficient APCs because, in addition to MHC class I and II molecules, they bear co-stimulatory molecules such as CD80 and CD86 (73). Other APCs, such as macrophages and B cells, need to be activated to express co-stimulatory molecules, whereas DCs already express these molecules even in the resting state (72). As all the other APCs, DCs have phagocytic properties allowing them to capture, process and present Ags at the cell surface via MHC class II molecules (72). In new therapeutic approaches for cancer treatments, DCs were recently used to generate tumor vaccines (74). By using this strategy, the patient's own resources are "educated" to fight tumor without the toxic side effects of radiation or chemotherapy (74).

One of the most remarkable features of DC is their mobility which can be observed at all stages of differentiation (75). DCs originate from bone marrow; migrate to peripheral tissues, where they reside in their immature state before they encounter Ags. That encounter, combined with the presence of chemokines, triggers their migration to secondary lymphoid organs. During migration, they undergo a maturation process that renders them more efficient APCs because of the up-regulation of co-stimulatory molecules expression. There, Ag-bearing DCs select the Ag-specific lymphocytes and elicit a strong immune response (72). All these properties make them on many aspects more potent than other APCs, thus justifying their use for immune based therapies. Their life cycle is pictured in figure 3 and further detailed in the next sections (76)

Figure 3- *The life cycle of human dendritic cells (DCs)*. DCs originate from the bone marrow. Circulating precursors will encounter Ag and secrete IFN-α, which in turn can activate macrophages and NK cells. Following antigen-capture, immature DCs migrate to lymphoid organs, guided by chemokines expressed in lymphatic vessels and expressed in T cell zone. After maturation, DC display peptide-MHC complexes, allowing selection, expansion and differentiation of rare circulating antigen-specific lymphocytes. Activated T lymphocytes migrate and reach the injured tissue. Th cells secrete cytokines, which allow activation of macrophages and NK cells. CTLs eventually lyse the infected cells.

Adapted from Nouri-Shirazi *et al*, 2000.


Blood and bone marrow:
CD34+ progenitors
Chemokines
MIP-3α, MIP-1α, RANTES
Ag
Tumor
elimination
IMMATURE
Ag-capturing
DC
Chemokines
MIP-3β, 6Ckine
Activation stimuli
LPS, CPG-ODNs
MATURE Ag-
presenting DC
Activated T cells

1.2.1.1 Antigen capture

As mentioned earlier, immature DCs are already very efficient at capturing Ag. The capture is done by different pathways: macropinocytosis ("cellular drinking" of large volumes of liquid) (77), receptor-mediated endocytosis (77) and phagocytosis of larger particles such as bacteria or viruses (78). In fact, DCs constantly sample their environment by macropinocytosis of extracellular fluids (79), and they are the only APC able to do so without stimulation (80). The macropinocytosis is done through water channels called aquaporins (81). Once Ags are captured (by one of the mechanisms described above), DCs undergo maturation, which is very closely linked to migration. The maturation process involves up-regulation of expression of molecules, such as CD80 and CD86, which renders the DCs better at presenting Ag, as well as upregulation of the expression of different chemokine receptors expression, which enables DCs to migrate to different areas (82). In the process, their capacity to capture Ag is greatly decreased, mostly because of the loss of endocytic receptors (72), but also because the overall levels of phagocytosis and macropinocytosis are reduced in mature DCs (79).

Many different signals induce DC maturation: pathogen related molecules (LPS, bacterial DNA and double-stranded ribonucleic acid (RNA)) (83-85), the balance between pro- and anti-inflammatory signals in the microenvironment (72) and T cell-derived signals, such as CD40L (72). Following those signals, the cells undergo many changes including morphological changes to increase motility, such as cytoskeleton reorganization (72) and antigen presentation, such as increase of MHC class II and co-stimulatory molecules on the cell surface (76).

1.2.1.2 Antigen processing and presentation

DCs can present Ag via MHC class II, to elicit CD4+ T cell activation, or via MHC class I, to induce CD8+ T cell activation. Their Ag presenting potential is due to the fact that DCs are equipped with molecules able to capture, process and present

Ag. DCs also contain lysosomal compartments with a very high concentration of MHC class II molecules that are ready to be loaded. Immature DCs capture Ags and target them to MHC class II compartments (86).

In immature murine DCs, newly synthesized MHC class II molecules are retained in lysosomal compartments. Upon DC maturation, the MHC class II molecules are transported to early endosomal compartments and then to the cell surface (87). The mechanism underlying this is the following: in immature DCs, the invariant chain localized in the lysosome is partly degraded and remains associated with MHC class II molecules. This fragment still contains the lysosomal retention signal. The poor degradation is due to the inhibition of a cysteine protease (cathepsin S) by cystatin C, a protease inhibitor. Upon maturation, cystatin C activity decreases, the invariant chain is degraded by cathepsin S, MHC class II is loaded with peptide and the complex is transported to the cell surface (88). Although this model is very elegant, it seems to be restricted only to a subset of MHC-Class II molecules called the I-A^b haplotype (89). For all other haplotypes, the internalization signal in the MHC class II β chain may be responsible for the lysosomal targeting and retention (90).

In humans, the mechanism is slightly different. In immature DCs, newly synthesized MHC class II molecules first appear at the cell surface complexed with the invariant chain, are internalized in the lysosome for the invariant chain degradation by cathepsin S. These class II molecules are then loaded with peptide and finally recycled to the cell surface (91). Upon DC maturation, the rate of synthesis of MHC class II and the efficiency of MHC class II-peptide complex formation are both increased. Furthermore, the internalisation is strongly reduced so the net result is an increase in the number and stabilisation of peptide-MHC class II complexes on the cell surface (92).

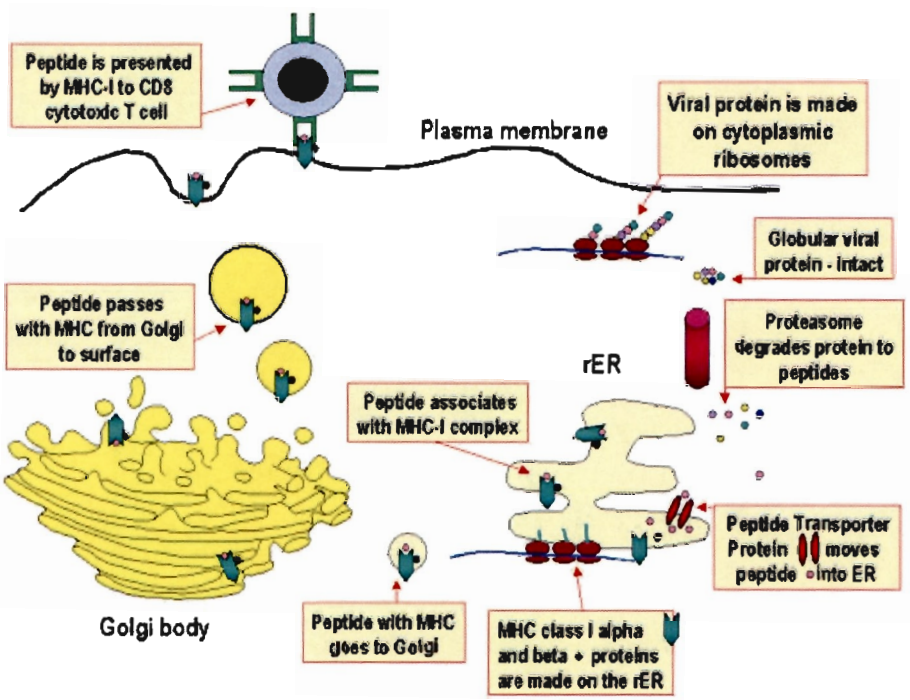
The DCs use different surface receptors to capture Ags, as well as different proteolytic machinery and those subtle differences may determine the nature of immunodominant peptides presented by MHC class II molecules (93). As a consequence, CD4+ T cells will be recruited, allowing diversity in the generated response.

In order to have an adequate immune response against any sort of invasion, CD8+ cytotoxic T cells must also be activated. This type of T cells are activated by DCs via MHC class I molecules, which can be loaded through both endogenous and exogenous pathways (93). In the endogenous pathway, cytosolic proteins are degraded by proteasomes and then loaded onto newly synthesized MHC class I molecules (72). The exogenous pathway involves a process called cross priming. This means that DCs have an alternative MHC class I pathway that can present peptides derived from exogenous Ags. This pathway is probably involved in immune responses against transplantation Ags, particulate Ags, tumors and viruses but represent a minor way to process and present Ag (93). The MHC class I pathway is very useful to activate cytotoxic CD8+ T cells to kill virus-infected cells. Briefly, the exact mechanism of MHC class I antigen degradation and presentation is: classical class I molecules assemble in the endoplasmic reticulum (ER) with peptides mostly generated from cytosolic proteins by the proteasome. The activity of the proteasome can be modulated by a variety of accessory protein complexes. A subset of the proteasome subunits, and one of the accessory complexes, PA28, are upregulated by interferon-γ and affect the generation of peptides to promote more efficient antigen recognition. The peptides are translocated into the ER by the transporter associated with antigen processing (TAP). A transient complex containing a class I heavy chain–$ß_2$ microglobulin ($ß_2$m) dimer is assembled onto the TAP molecule by successive interactions with the ER chaperones calnexin and calreticulin and a specialized molecule, tapasin. Peptide binding releases the class I–$ß_2$m dimer for transport to the cell surface, while lack of binding results in proteasome-mediated degradation (94). Finally, those peptides are presented to CD8+ T cells and elicit a CTL response.

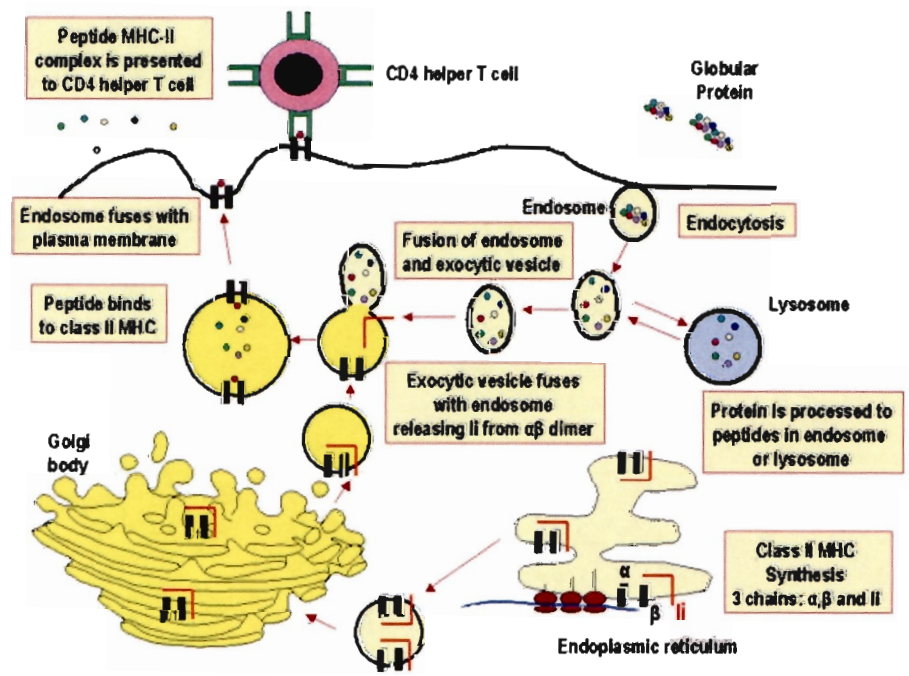
Figure 4- *Antigen processing pathways*. A) Endogenous antigen processing for presentation by Class I MHC molecules B) Exogenous antigen processing for presentation by Class II MHC molecules. See text for details.

From http://www.med.sc.edu:85/bowers/ant-pres.htm with permission of Dr Richard C. Hunt, webmaster

A


Peptide is presented by MHC-I to CD8 cytotoxic T cell
Plasma membrane
Viral protein is made on cytoplasmic ribosomes
Globular viral protein - intact
Proteasome degrades protein to peptides
rER
Peptide passes with MHC from Golgi to surface
Peptide associates with MHC-I complex
Peptide Transporter Protein moves peptide into ER
Golgi body
Peptide with MHC goes to Golgi
MHC class I alpha and beta + proteins are made on the rER

B


Peptide MHC-II complex is presented to CD4 helper T cell
CD4 helper T cell
Globular Protein
Endosome
Endocytosis
Endosome fuses with plasma membrane
Fusion of endosome and exocytic vesicle
Peptide binds to class II MHC
Lysosome
Exocytic vesicle fuses with endosome releasing Ii from αβ dimer
Protein is processed to peptides in endosome or lysosome
Golgi body
Class II MHC Synthesis 3 chains: α,β and Ii
α
β
Ii
Endoplasmic reticulum

1.2.1.3 DC migration

There are immature, resident DCs in the periphery that constitutively migrate to lymph nodes without being activated (95). These DCs do not elicit an effector response for the following reasons: a) the Ag density on the DCs is too low to elicit a strong T cell response, b) the low level of costimulatory molecules in the absence of DC activation, and c) the production by DCs of inhibitory cytokines, such as IL-10 (95). In presence of an activation stimulus, DCs migrate following chemokine signals. Immature DCs express chemokine receptor (CCR) 2 and CCR6 which promote their migration towards pathogens (96). Maturing DCs then upregulate the expression of CCR7, which is essential for their migration to the lymphatic and then to T cell areas (95). By the time they reach the lymph nodes, mature DCs produce a different set of chemokines (eg CCR7 and CCR4 ligands) which are involved in the regulation of their interaction with T cells (97, 98).

1.2.1.4 Antigen presentation and T cell activation

For T cell activation to take place, APC (in this case the DC) must meet with T cell. This encounter has been named the "immunological synapse" by analogy to the nervous system (95). The recently discovered DC-SIGN molecule plays a very important role in the formation and maintenance of the synapse (99). It appears that DC-SIGN acts as an adhesion molecule that binds ICAM-3 with high affinity. DC-SIGN would be involved as the first interaction of the immunological synapse and this adhesion would be necessary for the TCR to scan for its ligand. Once the ligand is identified, binding becomes tighter and the resting T cell becomes activated (99). DCs, pulsed with soluble Ags and injected into mice are able to elicit a very potent Ag-specific T-helper (Th) response (100). Furthermore, activated Th cells can secrete cytokines to induce activation of either antibody producing B cells or CTLs. However, DCs are equally important to prime naïve CD8+ T cells directly and without the need of Th cells (101). Strong CTL response can be elicited with DCs,

whether they are peptide-pulsed, protein-loaded, DNA-transfected or RNA pulsed (72). Although DCs can activate CD8+ T cells on their own, it has been shown that the activation occurs mainly via Th upregulation of CD40 expression on DCs (72). In other words, activated DCs are the bridge between the CD4+ and the CD8+ T cells. DCs also play a very important role in maintaining the naïve CD4+ cells alive (102) and in establishing immune T cell memory (103).

One observation could explain the capacity of DCs to prime naïve T cells: DCs have 10- to 100-fold more MHC products and MHC-peptide complexes on their surface than on other APCs (86). However, the MHC-TCR interaction is only the first signal of T cell activation. A second signal is needed to sustain T cell activation and that signal is mediated by costimulatory molecules, such as CD80 and CD86, expressed on the DC surface (Figure 1) (104). Inversely, T cells can activate DCs via the CD40L-CD40 signalling pathway and upregulate the expression of costimulatory molecules and increase the release of cytokines such as IL-1, tumor necrosis factor (TNF) and IL-12 (105).

DC subtypes exist and can elicit different types of T cell responses. Indeed, one type of DCs induces naïve CD4+ T cells to produce Th1 cytokines (consequently they are called DC1) whereas the second type of DCs induce the production of Th2 cytokines by CD4+ T cells (they are the DC2) (72). IL-12, a Th1 cytokine that is extremely important in cytotoxicity, is made only by lymphoid DCs. In addition, the costimulatory molecules CD80 and CD86 can also skew the Th1/Th2 response. A confirmation of this information is needed, but CD80 seems to promote a Th1 response, and CD86 a Th2 response (106). Overall, the direction of the immune response (Th1/Th2) induced by DCs depends on multiple factors, mostly due to the environment in which the cells are.

Although we know that distinct DC subsets have different functions, those functions can be altered or changed by the cytokine environment. Indeed, DCs normally inducing a Th1 profile can be converted to Th2-inducing cells upon treatment with an anti-inflammatory Th2 cytokine such as IL-10 or with steroids (72). It is not surprising to observe that DCs originating from different organs, and thus in a

different environment, skew the immune response differently: for example, Peyer's patches DCs elicit a Th2 response whereas splenic DCs induce a Th1 response (107).

Once DCs have interacted with T cells, they rapidly disappear from the lymph nodes. In fact, mature mouse DCs have a lifespan of about 1-2 days (108, 109). The fate of DCs after that is not known: do they undergo apotosis, are they killed by CTLs? *In vivo* trafficking experiments are still needed to solve this question.

1.2.2 Dendritic cells and B lymphocytes

DCs also have the capacity of activating naïve and memory B cells. By secreting different cytokines, DCs can induce B cell differentiation or antibody secretion (72). It is even suggested that DCs mediate naïve B cells activation during the initiation of the immune response and are involved in the development of the mucosal/humoral immune response (72). On the other hand, some cytokines, such as IL-16 secreted by B cells, play a major role in the recruitment of DCs and Th cells to the location where they are most needed (110). Therefore, B cell activation is also essential for appropriate DC function.

1.2.3 Dendritic cells and innate immunity

DCs can regulate effectors of innate immunity such as NK or natural killer T (NKT) cells either via direct cell-cell interactions or via indirect cytokine-mediated interactions. It has been shown that DCs can activate NK cells through the release of IFN-γ or by direct cell contact, leading to antiviral and antitumor activity of NK cells (111). They also act on NK/NKT cells through the release of IL-12, IL-15 and IL-18 (72). As mentioned earlier, the microenvironment in which DCs reside influences the type of immune response they will induce. Since the NKT cells have the unique capacity of producing both Th1 and Th2 cytokines depending on the signals they receive, they will have a great influence on the outcome (112). The signal to determine NKT cell fate given by DCs can also affect the NKT cell response. NKT cells are strongly activated by CD1-presented glycolipids and react by secreting IFN-

γ. Since glycolipids are mostly present on bacteria or tumor cells, NKT cells constitute a major effector mechanism of innate immunity (112).

There are also very important interactions between NK cells and DCs. When DCs are activated *in vivo* with Flt3-L (another activator of DCs), they activate NK cells and this results in the rejection of class I negative tumors. Thus, it is thought that *in vivo* expansion of both NK cells and DCs may account for the potent antitumor activity of Flt3-L (113, 114). Conversely, NK cells may elicit positive regulatory signals towards immature DCs to promote DC maturation in the spleen (114).

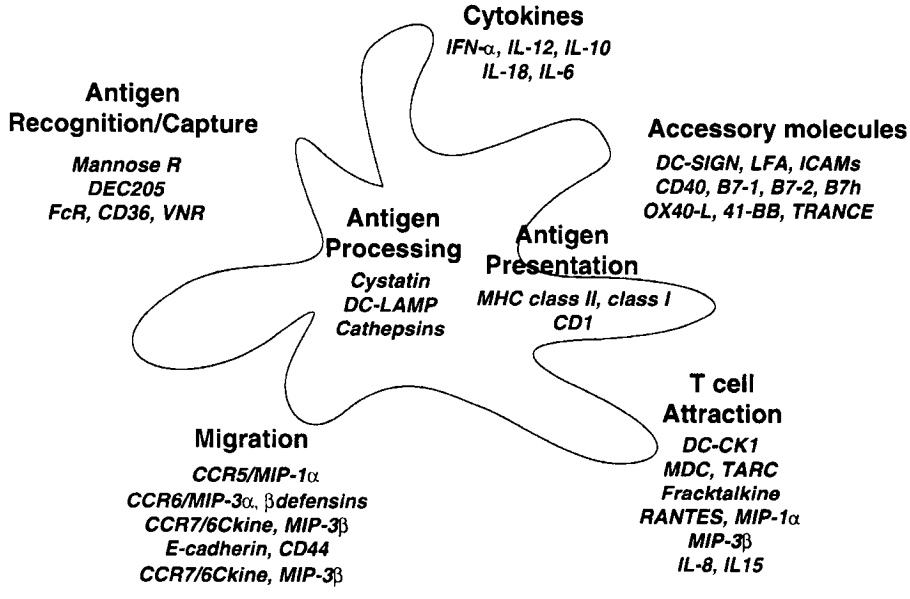
1.2.4 Potency of DCs and markers

Until recently, the potency of DCs was observed in different systems but could not be fully explained. The term "potency" indicates that a small number of DCs, and relatively small amounts of an Ag are sufficient to initiate rapid and strong responses, such as T cell proliferation and cytokine production (115). Potency also reflect on more efficient MHC class II-peptide complex formation or effectiveness even in the presence of a small number of TCRs (115). Potency is mostly attributed to accessory molecules found on DCs (figure 5), although these molecules are also shared by other APCs such as macrophages that do not exhibit such high potency (115).

The most abundant Ag receptors on DCs are C-type lectins, including DEC 205, and the mannose receptor. They both recognize glycosylated Ags (79). As mentioned earlier, DCs also express DC-SIGN, the first DC-restricted product to help stimulate resting T cells (99). Moreover, DCs express Fc receptors, receptors specific for heat shock proteins, DC91 (the α-2 macroglobulin receptor), and adhesion molecules such as CD36 and αvβ5 (79). Unfortunately, those molecules are also expressed by other cell types, contributing to the difficulty to find a specific marker for DCs (79). Figure 5 summarises the different molecules expressed by DCs.

Figure 5- *DC markers*. DCs express a unique set of molecules, which are differentially expressed at various maturation stages, and account for the ability of DC to sample antigens, process them and present them to naive T cells (see text for detail). They also express chemokines (useful for migration) and cytokines which help mount an efficient immune response.

From Nouri-Shirazi *et al.*, 2000.


Cytokines
IFN-α, IL-12, IL-10
IL-18, IL-6
Antigen Recognition/Capture
Mannose R
DEC205
FcR, CD36, VNR
Accessory molecules
DC-SIGN, LFA, ICAMs
CD40, B7-1, B7-2, B7h
OX40-L, 41-BB, TRANCE
Antigen Processing
Cystatin
DC-LAMP
Cathepsins
Antigen Presentation
MHC class II, class I
CD1
T cell Attraction
DC-CK1
MDC, TARC
Fracktalkine
RANTES, MIP-1α
MIP-3β
IL-8, IL15
Migration
CCR5/MIP-1α
CCR6/MIP-3α, βdefensins
CCR7/6Ckine, MIP-3β
E-cadherin, CD44
CCR7/6Ckine, MIP-3β

1.2.5 Maturation stimuli

As previously mentioned, DC maturation is a key event in the induction of the immune response (76). DC maturation occurs in response to several signals such as LPS, CpG-oligodeoxynucleotides (ODNs), TNF-α or CD40L. These signals represent pathogens, endogenous inflammatory signals and T cell feedback signals respectively (77, 116, 117).

The first class of maturation stimuli is induced by pathogens. DCs are activated by the LPS contained in the bacteria's cell wall following the binding to toll-like receptor (TLR) 4 (95). Moreover, it has been shown that DNA vaccination can be quite effective at inducing immunity. It is believed that this response occurs via DCs taking up DNA, making the protein and presenting peptides to induce the observed immune response (84). Some evidence suggests that the unmethylated CpG DNA motifs present in bacterial DNA are responsible for DCs activation. Some reports mentioned that CpG ODNs can induce bone marrow derived DCs, both immature and mature, to secrete high amounts of IL-12, TNF-α and IL-6 (118). Contrary to the original belief that CpG-ODNs were endocytosed non-specifically by DCs, it is now well known that signalling occurs through a receptor (TLR-9) specific for bacterial DNA located inside the cell and that the signalling cascade leads to NF-κB activation (119). Basically, CpG-ODNs skew the DC-induced immune response towards a Th1 response, thus recruiting inflammatory T cells at the sites of infection and activating macrophages to secrete chemokines that attract neutrophils to the infected area. CpG-ODNs and their receptor will be discussed further in section 3.3 as they were chosen as DC activators for the work presented in this thesis.

It has recently been suggested that adenovirus can trigger maturation. Adenovirus is often used as a vector to genetically transform DCs. Surprisingly, a recent publication suggests that adenovirus infection itself triggers not only DC maturation, but also NK activity and T cell-mediated tumor protection (120).

The second class of maturation stimuli is endogenous inflammatory signals. DCs express receptors for inflammatory cytokines such as TNF-α, type I IFNs and IL-1. The triggering of these receptors leads to DC activation. One must not forget that

DCs also express CD91, which binds to HSPs. The HSPs are recognized as a new type of endogenous danger signal since they are released from necrotic cells (121-123).

The third class of maturation stimuli is T cell feedback signals. A Th1 response involving IL-12 can be strongly induced by CD40L, which is expressed on memory T cells (124). But CD40L is only effective if the presence of CD40 on DCs has been upregulated by pathogens (125).

1.2.6 Antigen delivery strategies

In an attempt to boost the DC immune response, many strategies have been developed to deliver Ag: defined peptides of known sequence (126), undefined acid eluted class I peptide from tumor (127), whole tumor lysates (128), retroviral (129) and adenoviral vectors (130), tumor RNA (131, 132) and tumor-DC fusion (133). Some groups have also tried MHC-specific peptides derived from tumor Ags such as MART-1 or prostate-specific membrane antigen (PSMA) (134, 135). Those groups have relied on predictive algorithms to identify peptides with high binding affinities to the HLA molecule, most commonly HLA*A0201. Those peptides can be very efficient at generating cytotoxic response in HLA*A0201 patients but are totally ineffective if the patient does not bear that particular HLA. Consequently, this type of therapy is restricted to the 30-45% of the population bearing the HLA*A0201 molecule (136). Similarly, when class I restricted peptides are used, the role of MHC class II-restricted Th cells in initiating and sustaining an immune response is ignored (136).

A very interesting approach is to use full-length recombinant proteins. The DCs engulf the protein and use their antigen processing machinery to process, load and present it. Using this strategy, the treatment would not be limited to just one type of class II HLA. The DCs would "choose" the immunodominant epitopes and that would lead to an activation of the CD4+ T cells. Furthermore, because of the cross priming explained earlier, some peptides might be presented through the MHC class I pathway and elicit a CD8+ cytotoxic response. However, this probably represents a minor pathway for Ag presentation, and since most tumor cells do not express MHC

class II determinants, the generation of a CD8+ cytotoxic response is probably required to eliminate the tumor. This would necessitate the Ag to be presented through the MHC class I pathway (136). It has recently been shown that DCs take up apoptotic bodies, present proteins to T cells and can induce their proliferation (137)

However there are still some hurdles to be overcome. The major problem with the Ag stimulation strategies remains Ag delivery. One has to find a way to deliver Ag in such a way to generate both MHC class I and class II restricted response. The CD8+ cytotoxic response is necessary to kill and eliminate the tumor whereas the CD4+ helper response is needed to initiate and sustain a strong immune response.

Care must also be taken when choosing the route of injection. A pilot clinical trial for prostate cancer using a DC vaccine compared IV, intradermal and intralymphatic routes of administration. Whereas all patients developed a T cell immunity, production of IFN-γ was not seen in the IV group. In contrast, Ag-specific antibodies were seen in the IV group but to a much lesser extend in the other groups. In conclusion, the quality of the immune response and the induction of Ag-specific antibodies may be affected by the route of administration (138).

The immune system can be very powerful at eliminating cancerous cells, as evidenced by occasional spontaneous remissions in metastatic RCC (139). Tumor immunity can be initiated by the effectors of innate immunity and further developed by cells of adaptive immunity, with the DCs playing a central regulatory role. The development of tumor immunity requires multiple steps: (1) recognition of tumor Ags by DC precursors, (2) direct and cytokine-mediated killing of tumor cells by DC-activated NK/NKT cells, (3) capture and cross-presentation of tumor associated Ags (TAAs) by immature DCs, (4) selection of TAA-specific T cells as well as non-specific effectors, (5) homing of TAA-specific T cells to the site of the tumor and destruction of the tumor by the T cells (72).

Unfortunately, tumors often succeed at escaping immunosurveillance. They can alter each of the above-mentioned steps. Indeed, tumors can secrete cytokines, such as IL-6 and IL-10, and thus inhibit DC maturation and/or function (72). In fact, it has been shown that tumor-associated DCs have a low allostimulatory function. Furthermore, IL-10 is able to convert DC-APC function to the induction of Ag-

specific anergy, thus leading to tolerance against tumor tissue (140). In addition, it was recently demonstrated that tumors can also induce tumor infiltrating DC paralysis, thus preventing the generation of an efficient immune response (141)

1.2.6.1 DCs and cancer immunotherapy

Several factors have impaired the success of immunotherapy in humans. Among them, the limited availability of TAAs and the inability to deliver them so that they are rendered immunogenic (136). DCs might be a very useful tool to circumvent the above-mentioned limitations. As described earlier, the immune system is able to recognize and eliminate tumors. While both humoral and cellular effectors have been shown to play a role in this, it is felt that cytotoxic CD8+ T cells are the most important player, since their ability to recognize and kill various cancer cells was demonstrated (126, 142). It has also been demonstrated in various tumor models that DCs can prime T cells and make them capable of eliciting cytotoxicity in an Ag-specific manner (136). Furthermore, DCs are capable of inducing immunological memory, protecting the host against subsequent tumor challenge (136).

DCs have a unique ability to induce and sustain primary immune response and that striking characteristic makes them optimal candidates for vaccination protocols (139). Many animal models have been used to show that DCs loaded with the appropriate TAA can induce either protection against tumor growth or rejection of established tumor (136). Preliminary results using DC vaccination are extremely encouraging in humans (143). Many strategies are currently being developed to deliver Ag to DCs. However, induction of immunity with DCs represents a big challenge and several parameters need to be optimized before DC vaccination can be used routinely. These parameters include the source of TAA, the method for TAA preparation and loading, and the diversity of TAA subsets. Still, the observations made in animal models have established the rationale for evaluating tumor Ag-bearing DCs as therapeutic vaccines in humans.

1.2.6.2 DC vaccines in animal models

It was demonstrated that mice injected with Ag-loaded DCs could be protected against subsequent tumor challenge (144). It was also shown that tumor Ag-loaded DCs was the best strategy to induce tumor regression when compared to other types of vaccination such as naked DNA or peptides with adjuvant (145). The efficacy of peptide-loaded DC vaccination in terms of CTL induction and antitumor activity depends on additional critical factors, such as the route of DC administration and the origin of the DCs. Recent results with tumor Ag-loaded murine bone marrow-derived DCs indicated that subcutaneously injected DCs had greater antitumor activity than IV-injected DCs. Furthermore, subcutaneously injected DCs home to T-cell areas of the draining lymph nodes, whereas IV-injected DCs home to the spleen (146).

There have been experiments where DCs were engineered to produce a cytokine (eg IL-12). Upon intratumoral injection, the engineered DCs were shown to induce complete regression of established tumors, in this case CT-26 murine colon carcinoma (147). Another interesting approach led to successful prevention of tumor implantation in animals vaccinated with DC fused with tumor cells (148). Furthermore, mice with brain gliomas had improved survival when vaccinated with DC-glioma fusions, concomitantly with rIL-12 (149).

1.2.6.3 Candidate target antigens in human

For tumor immunotherapy to be efficient, DCs have to present an Ag to the immune system. There are many known Ags that have been shown to be associated with specific malignancies. They have been subdivided in five categories: tumor-specific Ags (ex: mutant p53; lung cancer), developmental Ags (ex: MART-1/melanA; melanoma), viral Ags (ex: Epstein-Barr virus; Burkitt's lymphoma), tissue-specific self-Ags (ex: prostate-specific membrane Ag (PSMA); prostate cancer) and overexpressed self-Ags (ex: carcinoembryogenic Ag (CEA); breast cancer) (136).

For most cancers, no common immunogenic Ags have been characterized. Therefore, strategies must involve tumor-derived protein extracts (128) or tumor RNA (131) as the source of Ag. In parrllel experiments must be conducted in order to identify common tumor Ags (142). Obviously, the availability of a specific tumor Ag has the potential benefit to limit non specific immune response and therefore decrease the risk of collateral damage to normal tissue (136). On the other hand, tumor Ags might vary from a tumor to another and this phenomenon may limit the efficacy of common tumor Ag (136).

Animal models can be very useful to examine immunogenicity of tumor Ag, but caution must be used when extrapolating the data to humans. First, many transgenic animals have been used to address this issue, although the differences in T cell receptor (TCR) repertoire between mice and human might provide information that is not applicable to human (136). Also, many tumor animal models are extremely immunogenic whereas most human cancers, including RCC, are not (136).

Caution must be taken when Ags chosen (e.g. CEA) are also expressed by normal tissue, as there is a theoretical risk of inducing autoimmune disease affecting vital organs. Of course, this would not matter if the Ag came from a non-essential organ (prostate) or if the organ had been removed in the course of the treatment (prostatectomy) (136).

1.2.6.4 Clinical trials for DC immunotherapy

There are many clinical trials being performed in several institutions, and very encouraging results have been reported. Although the proof of benefit using this therapy remains to be established, these trials have usually involved patients at a terminal stage of the disease, whose cancer have failed to respond to conventional treatment such as radiation therapy or chemotherapy. This could, in part, explain the mixed results obtained (136).

In the first reported clinical trial, malignant B cell lymphoma patients were treated with autologous DCs pulsed *ex vivo* with tumor-specific Ag. This represented

an ideal context because all the neoplastic cells in these patients express surface immunoglobulin receptors, and because lymphoma is monoclonal, all the cells of a given tumor express identical surface immunoglobulins. Moreover, immunoglobulins are intrinsically immunogenic, so they represent an ideal target Ag (150). The Ag was purified from a hybridoma supernatant. Patients received three IV injections of tumor-specific protein pulsed DCs at monthly intervals. DCs were obtained from precursors circulating in the blood. Two weeks after each DC injection, patients received a booster injection of purified idiotype. All patients (ten in the initial study) tolerated their infusions well and none experienced severe toxicity (150). In addition, the majority of patients developed a specific T cell proliferative response that was not present before treatment, whereas no humoral response was observed. This is consistent with the observation that DCs induces a Th1 rather than a Th2 response. Very exciting results were obtained: two patients had complete tumor regression including one who stayed in complete remission for more than three years. A third patient had a partial response while three remained stable and three had disease progression. The authors have not determined whether these patients mounted a CD8+ cytotoxic response (150).

Because of the promising results obtained in this study, several other phase I clinical trials were undertaken, including the one reported by Murphy in prostate cancer (151). In this case, DCs were obtained from peripheral blood monocytes cultured with IL-4 and GM-CSF and pulsed with a HLA*A2-specific PSMA peptide. Of the fifty-one patients enrolled in the phase I study, some were HLA*A2 negative and as expected, did not mount a T cell response. The results were less exciting than those obtain in B cell lymphoma. Of the 51 patients, only 7 had a partial response, based on the decline in PSA, a serological marker for disease burden. Nonetheless, a phase II clinical trial was undertaken with thirty-three of the fifty-one patients, and nine patients obtained a partial response, including four who had a partial response in the initial study (135).

Two trials have been reported for melanoma in which the efficacy of vaccination with DCs pulsed with a panel of HLA-restricted melanoma-derived peptides was assessed (134, 152). Both studies used monocyte-derived cells, cultured

in IL-4 and GM-CSF. The fifteen patients in Nestle's study (134) were either HLA*A1 or *A2, and five developed clinical responses, including two who had durable response. As for Lotze's study (152), one of 6 patients had a complete response.

Another interesting approach consists in transfecting DCs with RNA. It was shown that RNA-transfected DCs were capable of eliciting a strong CTL response against both prostate cancer and RCC tumors (153, 154). In this case, the challenge resides in finding the appropriate antigen and the corresponding RNA to pulse the DCs.

A very interesting, yet controversial, study has recently been reported in patients with metastatic RCC (155). The electrofusion of autologous tumor cells and allogeneic DCs was used to vaccinate seventeen patients. Vaccination was administered every three months. Of the seventeen patients enrolled in the study, four completely rejected all metastatic tumor lesions, one had a mixed response and two had a tumor mass reduction greater than 50%. None of the patients presented side effects to the vaccine and CD8+ T cells were shown to migrate to the tumor site (155). This strategy might offer an interesting approach for tumors where no common tumor Ags are known. Unfortunately, recent reports have shed doubts on the validity of these results, since the first author of the paper has been accused of scientific misconduct (156). Although no official retraction of the paper has been emitted up to now, the results must be taken with care. Several other studies involving fusion of DCs with tumor cells have been undertaken (157-160). All these studies have shown an *in vitro* activation of CTLs but *in vivo* results are awaited (157-160).

1.3 CpG oligodeoxynucleotides

1.3.1 History

As far as the 1700s, it was noted that certain infectious processes could exert beneficial therapeutic effect upon malignancy (161). Most prominent among the numerous deliberate efforts made to take advantage of these observations was the work of a pioneering New York surgeon, William B. Coley. Using a bacterial vaccine to treat primarily inoperable sarcoma, Coley accomplished a cure rate greater than 10% (162). Following this observation, the attenuated mycobacteria bacillus Calmette Guerin (BCG) has become the standard therapy for human superficial bladder cancer (163). Further studies have also demonstrated that, surprisingly, the active component in BCG was the DNA and that DNA could induce NK cell activity as well as production of type 1 and type 2 interferons *in vitro* (164). By cloning genes and synthesizing ODNs, investigators concluded that certain self complementary palindromic sequences were responsible for this effect and that the palindromes contained at least one CpG dinucleotide (117). More research led to the discovery that these CpG-ODNs had to be unmethylated to induce immune stimulation (117). Concomitantly, antisense technology was being developed and it was found that some antisense ODNs had immune stimulatory properties (117). When the two observations were put together, it was found that the CpG motifs contained in the antisense ODNs were responsible for the antitumor effects (117). Consequently, all further studies were performed with ODNs containing CpG motifs.

1.3.2 Types of CpG-ODNs

It is important to realize that different animal species will respond to different CpG-ODNs (117). Of course the CpG motifs will remain the same but the context (ie the flanking nucleotides) will be different (117). It is also important to take into account the DNA backbone of the ODNs. The ODNs formed of a phosphodiester

(PO) backbone are named CpG-A ODNs (117). They are particularly effective at initiating a NK cell response and inducing IFN-α production by DCs (117). In order for the ODN to be resistant to nucleases, the backbone has to be formed by phosphorothiorates (PS) (117) and these ODNs are called CpG-B ODNs (117). They have the capacity of highly stimulating B cells, murine DCs and human plasmacytoid DCs (117).

1.3.3 Mechanisms of action

Efforts have been devoted to understand how CpG can induce an immune response. The first question was to establish if the CpG-ODNs would bind to cell surface or not. The data acquired thus far suggest that although cellular uptake of CpG-ODNs may involve binding to cell surface proteins, the immune stimulatory effect of CpG-ODNs requires binding to an intracellular receptor (117). The phagocytic properties of DCs are very useful to internalize the CpG-ODNs (117). By using different pharmacological agents able to block endosomal acidification, suppression of the immune stimulatory effect of CpG-ODNs was observed, and they do so in a very early step in the CpG-induced signalling pathway (165, 166) Consequently, it is believed that the receptor is intracellular and probably located on the surface of endosomes (119). Recently, it has been shown that the link between CpG-ODNs and the activation of cell signaling pathways is the Toll-like receptor 9 (TLR 9), a member of the TLR family (119).

1.3.4 Toll-like receptors

TLRs are membrane receptors characterized by the presence of a leucine-rich extracellular domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain similar to the intracellular domain of the IL-1 receptor family (167). So far, ten members of this family have been identified in mammals. They play an essential role in the innate recognition of pathogen-associated molecular patterns by triggering immunity in

higher organisms (167). Individual TLRs recognize different pathogens: TLR4 recognizes LPS, TLRs 2, 1 and 6 are specific for lipoproteins, peptidoglycans and zymosan, TLR3 for double-stranded RNA, TLR5 for flagellin, TLR7 for anti viral compounds, and the one that interest us, TLR9 for CPG-ODNs (167).

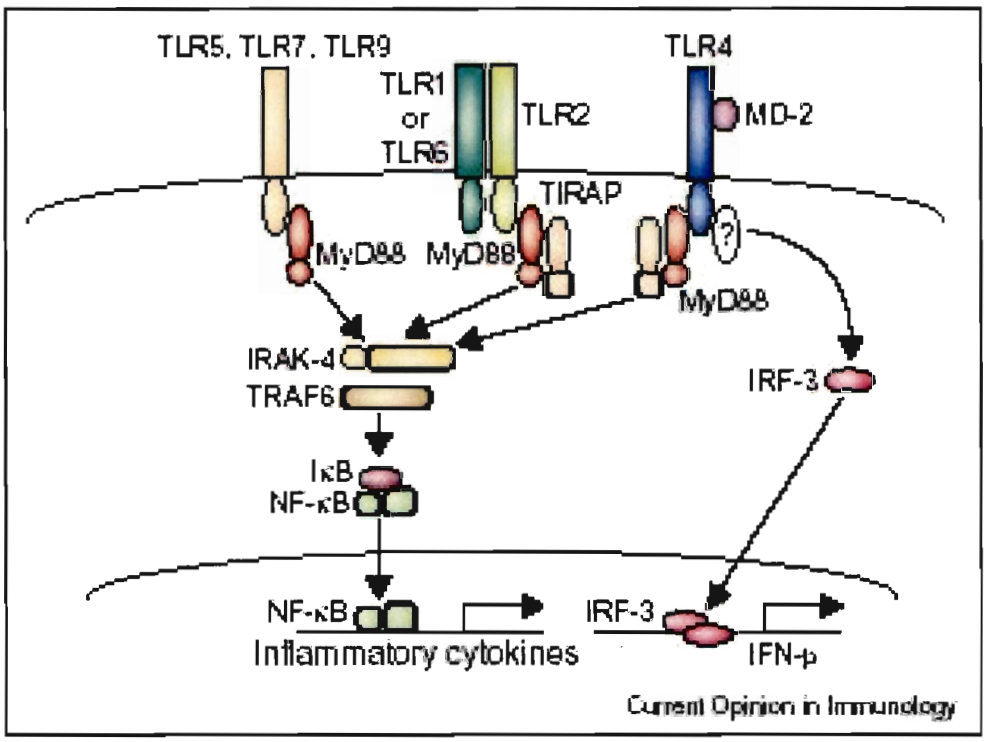
1.3.4.1 Toll-like receptor 9

TLR-9 has a transmembrane domain and is considered to be a member of the type I membrane proteins family (168). By genetically engineering cells to express TLR-9, they become fully activated by CpG-ODNs whereas they were not normally (169). This experiment indicates that TLR-9 is both necessary and sufficient for the activation of CpG-induced signaling pathways. TLR-9 is present on DCs, B cells and macrophages (117). CpG species specificity is due to the different amino acid sequence in the binding region of TLR-9 (169). Circumstantial evidences suggest that TLR-9 could be present in the endosome and it is where the CpG-ODN-TLR-9 interaction would occur and trigger signaling pathways (119, 170).

1.3.4.2 Signalling pathway

The expression of genes varies according to the TLR activated (167). The signalling cascade involving different TLRs is presented in figure 5. The adaptor proteins MyD88 and TIRAP play a very important role. MyD88 recruits the kinase IRAK-4 to the TLR after stimulation (167). It has been demonstrated that MyD88-deficient mice do not respond to CpG-ODN stimulation (171). The exact mechanism is not known but the end result is the activation of different kinases and translocation of NF-κB to the nucleus (167). It is noticeable that LPS activation of MAP kinases and NF-κB remains intact in MyD88-deficient mice, although there is a delay in the activation (172). Thus, there is a MyD88-independent pathway for activation by TLR-4 and it is thought that this pathway is mediated by IFN regulatory factor 3 (IRF-3), another adapter protein important in signalling (173).

Figure 6- *Signalling pathways triggered by TLRs*. TLR4 ligands such as LPS induce inflammatory cytokines as well as IFN-β. The induction of inflammatory cytokines is dependent on the adaptor molecules MyD88 and TIRAP, whereas the induction of IFN-β is independent of these molecules and is regulated through the phosphorylation and nuclear translocation of IFN regulatory factor 3 (IRF-3). TLR2 ligands such as mycoplasma lipoprotein and peptidoglycan induce inflammatory cytokines through the MyD88/TIRAP-dependent pathway, but do not induce IFN-β as they do not activate the MyD88-independent pathway. Cytokine induction through TLR5, TLR7 or TLR9 depends on MyD88 but not on TIRAP. TRAF is the tumour necrosis factor receptor-associated factor and IRAK is interleukin-1 (IL-1)-receptor-associated kinase, two important signalling molecules.
From Akira, 2003


TLR5, TLR7, TLR9
TLR1
or
TLR6
TLR2
TLR4
MD-2
TIRAP
MyD88
MyD88
MyD88
?
IRAK-4
TRAF6
IRF-3
IκB
NF-κB
NF-κB
Inflammatory cytokines
IRF-3
IFN-β
Current Opinion in Immunology

1.3.5 Treatment of animal tumors with CpG-ODNs

One problem encountered when vaccinating with tumor associated Ag (TAA) is the lack of strong CTL response. It has been found that repeated intraperitoneal (IP) administration of CpG-ODN as an adjuvant for the peptide increases the number of CTL precursors and DCs in the spleen. Furthermore, this treatment can prevent tumor implantation (174). When CpG-ODNs are used as a monotherapy, they need to be injected encapsulated in liposomes, and the role of NK cells and IFN-γ are primordial. If the mice are NK or IFN-γ deficient, the induced immunity is completely abrogated (175). In the C26 mouse colon carcinoma tumor model, weekly injections of CpG-ODN peritumorally could drastically help reduce tumor growth, and this effect was dependant on the presence of CD8+ T cells (176). In the same colon tumor model, but in absence of CpG ODN treatment, tumor infiltrating DCs were observed but were inactivated by the tumor. This could be reversed by *in vitro* treatment of those DCs with CpG-ODNs and anti-IL-10R antibodies, the two having a synergistic effect (177). Monoclonal antibody therapy is also enhanced by the co-administration of CpG-ODN. Specific antitumor antibodies cause antibody dependant cellular cytotoxicity. In this experiment, when mice are pre-treated with CpG-ODNs, their survival rate increases to 70-80% compared to 10% with the antibody alone (178).

1.3.6 Treatment of humans with CpG-ODNs

Many clinical trials have been undertaken with CpG-ODNs, hundreds of patients have already received them. There has been no report of association with autoimmunity and no evidence of anti DNA antibody formation (179). Furthermore, CpG-ODNs do not abrogate B or T cell tolerance or induce autoantibody production or autoimmune disease, even in genetically predisposed individuals (117), although limited data from clinical trials are available yet (180).

As mentioned earlier, the combination of CpG with monoclonal antibodies has a positive effect on tumor regression. Consequently, clinical trials combining CpG-

ODNs with Herceptin® for beast cancer and Rituxan® for non-Hodgkin's lymphoma are ongoing (180) Herceptin® is a monoclonal antibody that targets HER-2, the human epidermal growth factor receptor 2. This protein is over expressed in breast cancer which leads to abnormal cell proliferation (181). Rituxan® binds specifically to the CD20 antigen expressed on the surfaces of normal and malignant pre-B and mature B lymphocytes and induces their lysis (181). There is an ongoing clinical trial for melanoma and basal cell carcinoma where CpG-ODNs are injected weekly and where a significant clinical response is observed (180). Although final results are not yet available, the side effects are limited, consisting of local inflammation and flu-like symptoms (180). Therefore, the results of many clinical trials are impatiently awaited in order to determine the efficacy of these vaccine-based approaches.

OBJECTIVES OF STUDY

Given that RCC does not respond to conventional treatments, and that spontaneous regressions have been observed in RCC patients, major interests have focused on the role of the immune system in the pathology and treatment of this disease.

In order to establish the basis of a new treatment for human RCC, it was important to validate our hypothesis on a mouse RCC model. RENCA was chosen because of the similarities it shares with the human disease. Thus the goal of this research was to obtain results that would permit us to establish a new treatment for human RCC based on a DC vaccine, potentiated by the treatment of DCs by CpG-ODNs.

More specifically, the objectives were:

- Establish culture conditions for the growth of murine DCs
- Validate RENCA as a good murine model for the development of a DC vaccine
- Characterize the immune response in naïve, RENCA-bearing and RENCA-bearing/DC-vaccinated animals
- Characterize the impact of CpG-ODNs on DC activation and maturation and how this affects tumor growth and tumor challenge
- Evaluate lymphocytic infiltration in the tumor of vaccinated vs non-vaccinated mice
- Evaluate the capacity of CpG-treated DCs (RENCA-pulsed or not) to confer long-term memory to the animals (as seen by a T cell transfer and further resistance to tumor growth), as well as to induce a specific immune response.

CHAPTER 2

Chagnon, F., Thompson-Snipes, L., Elhilali, M.M., Tanguay, S. Murine renal cell carcinoma: evaluation of a dendritic cell tumour vaccine. BJU International (2001) 88: 418-424.

In collaboration with Dr. Thompson-Snipes, I have established the basis of this project. The first thing that had to be done was to establish the conditions for DC culture. After following the protocols reported in the literature, we came across the technology established by StemCell (Vancouver, BC, Canada), which enabled us to do negative selection of the hematopoietic progenitors. The protocols for antigen extraction also had to be validated in our hands. With these tools developed, we went on to validate the RENCA model *in vivo*. This paves the road for future studies using a DC vaccine to treat RENCA tumors.

For contribution of authors, see p. xii

MURINE RENAL CELL CARCINOMA: EVALUATION OF A DENDRITIC CELL TUMOR VACCINE

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SUMMARY

Objective To use a murine model of renal cell carcinoma (RCC), RENCA, to aid in developing a dendritic cell (DC)-mediated tumour vaccine for RCC; as conventional therapy has been unsuccessful for RCC and therapy using immune modulators has had limited success, novel therapies enhancing further the immune system must be developed.

Materials and methods DCs were obtained from mouse bone marrow enriched for the haematopoietic progenitors, and cultured in the presence of interleukin-4 (IL-4) and granulocyte macrophage-colony stimulating factor (GM-CSF). *In vivo* vaccines and *in vitro* proliferation assays were used to assess ability of the DCs to present tumour antigen.

Results The presence of DCs was confirmed in the cultures by fluorescent-activated cell sorting analysis. *In vivo*, tumour-bearing animals receiving tumour extract-pulsed DCs as a vaccine showed a 2–3-fold reduction in tumour growth at day 12 and day 16 but no significant difference at day 28. *In vitro*, tumour extract-pulsed DCs stimulated significant proliferation of splenocytes from naive animals but not tumour-bearing animals. In addition, splenocytes from tumour-bearing animals had an attenuated immune response *in vitro*.

Conclusion These results show that it is possible to use the DC vaccine to modulate the immune response to achieve an antitumour effect, but further manipulation of the DC vaccine may be needed to overcome the tumour-induced immune suppression.

Keywords dendritic cell, tumour immunity, renal cell carcinoma, RENCA, tumour vaccine, immunotherapy, immune inhibition

RCC accounts for ≈2% of cancers or 150 000 new cases per year worldwide and is a major clinical problem with nearly one third of the patients having metastatic disease and unfavourable prognosis [1]. RCC is a particularly difficult tumour to treat as it presents relatively late in the course of the disease and responds poorly to conventional treatments such as chemotherapy or radiation therapy [2]. However, as some renal cell metastases show spontaneous regression it appears that this disease can be modulated by the immune system. Accordingly, the strategy of most recent treatment protocols for RCC is to stimulate the patient's immune system to generate an antitumour response, using immunostimulatory cytokines such as interleukin-2 (IL-2) and/or interferon-α as therapeutic agents [3]. Treatment with high-dose IL-2 produces response rates of ≈20% that are durable for 3 years [3]. To increase the response rate, improved therapies using lymphokine-activated killer cells (LAKs) and tumour-infiltrating lymphocytes (TILs) in combination with IL-2, have been attempted. In one phase III clinical trial using TILs or LAKs in combination with IL-2 there was better survival than in patients receiving IL-2 alone [4]. One of the major difficulties in the successful treatment of advanced RCC seems to be the ability of the tumour cells to suppress the patient's immune response against the tumour [5].

As an alternative approach to RCC treatment, we propose using dendritic cells (DCs) in a tumour vaccine. DCs are capable of presenting tumour antigens to immune effector cells and stimulating an effective antitumour response [6]. Indeed, DC therapy has already shown some efficacy in the treatment of human prostate cancer, B-cell lymphoma and melanoma [7–10] as well as in many murine tumour models, including B-cell lymphoma and sarcoma [6]. More recently, a DC vaccination strategy for RCC using tumour cell-DC hybrids as antigen [11] has shown therapeutic potential. In this trial, four patients of 17 completely rejected the metastatic tumour lesions, one had a mixed response, and in two the tumour mass reduced by more than half. Although this trial was promising, several variables can be optimized to generate a more effective antitumour response. A good animal model for developing DC strategies to vaccinate against RCC would be helpful in optimizing and overcoming

the many technical challenges posed by immunotherapeutic approaches to RCC.

We have begun to evaluate the efficacy of DC vaccination for the treatment of RCC in the murine form of RCC known as RENCA. The RENCA model of RCC fulfils many of the requirements for a useful animal tumour model; the tumour cells are of spontaneous origin, syngeneic to BALB/c mice, confirmed to be an adenocarcinoma by histology, have a predictable growth rate both *in vitro* and *in vivo*, and form a primary tumour mass within the mouse. In addition, RENCA shows progressive disease stages similar to human RCC. For example, transplanted RENCA forms solid tumours that develop spontaneous metastases to distant organs such as lungs and liver [12]. Furthermore, RENCA induces many of the immunosuppressive effects of human RCC such as defective T-cell signalling [13,14]. Taken together, all these features make RENCA a challenging but relevant model of RCC.

There have been many attempts to treat RENCA, e.g. many groups have tried with success to induce tumour regression with cytokines, such as IL-12, IL-2 and IL-4 [15–17]. Gene transfection of tumour cells with apoptosis-related molecules such as Fas have also been attempted with success [18]. These methods are very useful to understand more about RENCA but they are not applicable in humans, mostly because of toxicity or lack of effectiveness [4,19]. It is self-evident to find a way to reduce tumour growth in RENCA that would also be applicable to human; the DC vaccine fulfils that criterion.

Using the RENCA model, we tried to determine whether the DC vaccine could be a strategy of choice to treat RCC and overcome the immune suppression it induces. In this report we provide evidence that RENCA shows immune suppression similar to that from human RCC. In addition, we show that the use of DCs to stimulate the immune response to tumour is promising for developing a vaccine against the tumour.

MATERIALS AND METHODS

BALB/c mice (4–6 weeks old) were generously provided by the Montreal General Hospital Research Institute breeding facility, or were purchased from Charles River (Saint-Constant, Quebec). The BALB/c RCC cell line, RENCA, was obtained from Dr

I. J. Fidler (Houston, TX), and the BALB/c fibroblast cell line, CL-7, was obtained from the ATCC (Rockville, MD). The cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin G, streptomycin and amphotericin B (Gibco BRL, Life Technologies, Grand Island, NY).

To purify T cells, spleens were collected from normal or tumour-bearing animals. The red blood cells were lysed and the remaining cells submitted to the StemSep negative-selection system (StemCell Inc., Vancouver, BC). The T cells were then used for the ELISPOT assay. The T cells were cultured in RPMI containing 5% FBS, 2 mM L-glutamine, penicillin G, streptomycin and amphotericin B (Gibco).

To measure interferon-γ (IFN-γ) the ELISPOT assay was used; all the antibodies were purchased from Pharmingen (Mississauga, ON). Briefly, ELISA plates were coated with the capture primary antibody (purified rat antimouse IFN-γ, 5 μg/ml and incubated overnight at 4 °C. The plates were then blocked with PBS containing 1% BSA and 0.5% Tween-20. After washing the plates three times with PBS/0.5% Tween-20 (wash buffer), the cells were added in complete medium and incubated overnight at 37 °C. The plates were washed three times and the second biotinylated antibody added (biotin rat antimouse IFN-γ, 0.5 μg/ml). The plates were incubated for 1 h at room temperature, washed three times, and avidin-horseradish peroxidase added (5 μg/ml). After incubating the plates for 1 h at room temperature they were washed five times and 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB, Vector Laboratories, Burlingame, CA) was added, followed by incubation in the dark for 30 min. Finally, TMB was removed, the plated rinsed with water, and fluid removed. The number of spots present were counted under a dissecting microscope and expressed as mean of triplicate cultures.

DC cultures were established as described by Inaba et al. [20]; briefly, bone marrow cells were submitted to a Lympholite density gradient (Cedarlane, Hornby, ON, Canada) and enriched for haematopoietic progenitors using the StemSep negative selection system (StemCell Inc.). The cells were cultured at a concentration of 10^6 cells/ml in a 24-well plate in OPTI-MEM supplemented with 5% heat inactivated FBS (Gibco), and 1000 U/ml each of GM-CSF and IL-4 (generously provided by Schering-

Plough, Madison, NJ). The cultures were fed every other day by removing half of the medium and replacing it with fresh medium containing GM-CSF and IL-4. The cells were harvested after 6–9 days of culture.

For immunocytometry, the cell samples containing 10^6 DCs were incubated with 2.4G2, an antibody directed against the FcRIIγ receptor, and then stained with different directly fluorescence-conjugated antibodies at 4 °C for 40 min. The antibodies used were anti-CD11c, anti-B7.1, and the appropriate isotype controls (Pharmingen). Propidium iodide (Roche Diagnostics, Laval, Qc, Canada) was used to exclude dead cells from the analysis. The cells were analysed on a fluorescent-activated cell sorting (FACS) Scanner using the CellQuest software (Becton Dickinson, Mississauga, ON).

To extract tumour antigen, RENCA and BALB/c CL-7 cell lines were cultured in 150 cm^2 Petri dishes (Sarstedt, St-Leonard, QC) using phenol red-free DMEM supplemented with 5% FBS, 2 mM L-glutamine, penicillin G, streptomycin and amphotericin B (Gibco). Cell-surface antigens were extracted from semi-confluent cultures with citrate-phosphate buffer (0.131 M citric acid/ 0.066 M Na_2HPO_4, pH 3) as previously described [21]. The cell extracts were subsequently concentrated on SepPak C_{18} cartridge (Millipore, Bedford MA), lyophilized (Virtis, Gardiner, NY) and stored at – 80°C. The protein concentration was determined by BCA analysis (Pierce, Rockford, IL). The cell extracts were reconstituted in serum-free OPTI-MEM before loading onto DCs.

For the splenocyte proliferation assay, mice were injected intraperitoneally with 10^6 RENCA cells, and their spleens harvested 7–14 days later. Then 10^6 DCs were pulsed for 3 h with either 10 μg of RENCA, or CL-7 cell extracts, or incubated with serum-free OPTI-MEM as a negative control. DCs were irradiated (3000 rads) before being extensively washed. Irradiated DCs (5×10^4) were incubated, in triplicates, with 2.5×10^5 splenocytes in round-bottom 96-well plates. Spleens cells alone were used as the background control. As a positive control, spleen cells were incubated with 2.5 μg/ml of concanavalin A (Roche Diagnostics); 2 days later, the cells were incubated overnight with 37 Bq/well of ^{3}H-thymidine (248 GBq/mmol). The cells were then harvested and counted using a MicroBeta Instrument (Wallac Oy, Turku,

Finland).

For the therapeutic experiments, 40 BALB/c mice were injected with 10^5 RENCA cells subcutaneously on day 0. The vaccine was prepared by pulsing DCs as described above. The mice were treated by intravenous injections of antigen-pulsed DCs twice a week, for 4 weeks, starting on day 4 after tumour cell injection. Groups containing 10 animals each received 5×10^5 DCs/mouse, pulsed with either RENCA extracts, BALB/c CL-7 extracts, medium, or 200 µl of Hank's balanced salt solution alone. The tumours were measured using a Vernier caliper (Scienceware, Pequannock, NJ) in two dimensions twice a week and the tumour volume determined using the formula $(A \times B^2)/2$, where A is the longest measurement and B the shortest. All data were assessed statistically using ANOVA.

RESULTS

We have tested whether the course of the growth of an implantable model of RCC in mice can be modulated by RENCA antigen pulsed DC vaccination. We generated DCs by incubating murine bone marrow progenitors for 6-9 days with GM-CSF and IL-4. Analysis by FACS confirmed that most cells within the DC cultures showed typical DC markers such as CD11c and B7–1 [22], as shown in Fig. 1. For the DC vaccination, we pulsed the DCs with tumor cell extracts obtained from RENCA cells as the antigen or from the BALB/c fibroblast cell line CL-7 as a control antigen. The results of DC vaccination on the growth of RENCA tumours in mice are shown in Table 1; only vaccination using DCs pulsed with RENCA cell extracts significantly slowed the initial growth of RENCA tumours during the first 2 weeks. On day 12 the mean tumour volume in animals receiving RENCA-pulsed DCs was less than that in animals receiving unpulsed DCs ($P < 0.05$). By day 16 the tumours in control animals had grown significantly (mean 152 mm^3) while the tumours in animals receiving RENCA-pulsed DCs were only 54 mm^3 ($P < 0.05$). Thus, DC vaccination reproducibly shows antitumour effects in an antigen-specific manner in the early stages of tumour growth.

However, continued treatment with RENCA antigen-pulsed DCs was unable to prevent the rapid increase in tumour growth that normally occurs at 2–4 weeks in this

model; by day 28, the tumour volume was similar in all experimental groups, irrespective of DC vaccination (Table 1). Thus, the antigen-specific, antitumour effects of the DC vaccination at the early times were only transient in this murine model of RCC, suggesting that either the DC vaccine is an ineffective long-term treatment for RCC, or that additional immunosuppressive factors need to be addressed before DC vaccination for RCC is to be effective.

Immunosuppression is a condition that might limit the effectiveness of the DC vaccine. To verify that mice injected with RENCA tumour cells have a suppressed immune response, whole spleen cells were tested for their *in vitro* response to mitogen stimulation. Table 2 shows that splenocytes from RENCA tumour-bearing mice exhibit a marked decrease in proliferation in response to stimulation with the mitogen concanavalin A compared to normal mice.

DCs are known to be potent APCs, as they have the unique ability to stimulate naive T cells *in vitro* to proliferate in response to antigenic stimulation [6]. DCs might assist in reactivating the proliferative response of the RENCA growth-suppressed splenocytes. To test this, we investigated whether DCs loaded with tumor cell derived antigens could stimulate lymphocyte proliferation *in vitro*. As shown in Table 2, DCs loaded with either RENCA or CL-7 cell extracts were able to stimulate *in vitro* twice the proliferation in normal mouse splenic T cells than in control cultures using unpulsed DCs ($P < 0.05$). This reaffirmed the ability of DCs to stimulate splenic T cells *in vitro* to any antigen. However, lymphocytes from tumour-bearing animals had a significantly lower proliferative capacity in response to DC stimulation than splenocytes from normal animals. Tumour-bearing animals appear to have immunosuppressed splenoctyes.

To test whether some immune functions are preserved in tumour-bearing animals, we determined whether T cells from tumour-bearing animals could be induced to produce IFN-γ an activator of natural killer cells and cytotoxic T cells, both important effector cells for tumour killing. The production of IFN-γ induced by phorbol myristate acetate and ionomycin was no less in T cells from tumour-bearing mice than in T cells from normal age-matched controls, with respective mean (SEM) values of 9.3 (1.1) and 10.9 (2.9) spots/10^4 cells, respectively. Thus, although the proliferative

capacity of the lymphocytes to mitogens and DC stimulation is markedly reduced in tumour-bearing animals, at least some of the immune effector functions, as exemplified by the preservation of IFN-γ inducibility, remained intact.

DISCUSSION

RCC has been known to suppress the immune response in humans and in animal models [13,14,23]. DCs have been used successfully to stimulate significant immune responses to a variety of tumours [6–10]. The aim of this study was to determine if tumour antigen-pulsed DCs were able to generate a specific immune response leading to tumour regression and overcoming immune suppression in an animal model of RCC. We successfully generated DCs from cultures of mouse bone marrow progenitor cells in the presence of IL-4 and GM-CSF. These DC preparations had high levels of expression of surface markers CD11c and B7–1, typical of the DC phenotype [22]. We intend to use these cells in the present animal model of RCC to develop a successful vaccine against the tumour.

The RENCA tumour cells suppressed the immune response when administered *in vivo*, consistent with what others have shown [13,14]. Spleen cells from animals exposed to the RENCA tumour *in vivo* have a lower mitogenic response *in vitro* than do normal animal splenocytes. In addition, we showed, for the first time in the RENCA model system using DCs as antigen presenters, that RENCA-bearing animals have a reduced response to stimulation by DCs. In a normal *in vitro* challenge with antigen, as others have shown [24], the antigen-pulsed DCs would have been expected to stimulate the proliferation of splenocytes that had already been exposed to antigen *in vivo.* The absence of such a response and the presence of inhibition indicates the presence of strong inhibitory influences by the RENCA tumour. This is consistent with and reinforces the previous observation by Gregorian et al. [13,14] of a suppression in immune effector cells in RENCA-bearing mice.

Previous studies and more recent evidence suggest that the immune suppression is induced by the release of factors such as prostaglandins and gangliosides by tumour cells [13,14,23]. These factors could be responsible for inactivating the transcription

factor NF-κB, recently observed in patients with renal cancer [23,25]. The NF-κB malfunction was also detected in RENCA-bearing mice [26]. Despite the complexity of the immune system, there are only a few signalling pathways involved; one of the most important is the NF-κB pathway, which is involved in the transcription of almost all cytokines and some of the cytokine receptors. One example of a cytokine using the NF-κB pathway is IL-2, a key factor for T cell growth [27]. Consequently, a NF-κB malfunction results in inadequate T cell proliferation and expansion. Overcoming or inhibiting the inactivation of NF-κB by renal tumours will be necessary to achieve optimal immune response to tumour. In the present study, spleen cells taken from tumour-bearing animals had a decreased mitogenic response *in vitro* to stimuli, but there was no decrease in the ability of T cells to produce IFN-γ. Thus, the ability of the cells to divide and expand in response to signals may be inhibited, but some of the effector functions are not compromised.

The results from the present *in vivo* vaccination experiment are consistent with a deleterious effect of tumour burden on the immune response to RENCA tumour. There was an adequate antitumour response at the beginning of the treatment while the tumour was small, but the response declined and by day 28 the tumour size increased dramatically, despite continuous vaccine treatment. No significant difference was detected between the tumour size in mice receiving RENCA-pulsed DCs and the control groups after 4 weeks. These results show that DCs pulsed with tumour antigen can affect the growth of RENCA tumour in the animals while tumour burden is low, but also reinforces the hypothesis that the RENCA cells produce an inhibitory factor acting on the immune system when the tumour burden is high. The present results suggest that there was a major inhibition of the immune response when a high tumour burden was present in the host; this might explain the prolonged survival in patients undergoing surgical resection of metastasis or nephrectomy in the presence of metastatic disease [28].

The response to RENCA tumour *in vivo* was tumour-specific; by using an irrelevant antigen (CL-7) to pulse the DCs, the specificity of the vaccine was assessed. Indeed, while DCs pulsed with the CL-7 antigen stimulated the proliferation of splenic T cells *in vitro*, vaccination with DCs pulsed with CL-7 extracts did not inhibit tumour

growth, similar to un-pulsed DCs *in vivo*. Preliminary data on a human DC vaccine for RCC are very promising, with the generation of a strong specific antitumour immune response [11,29].

In conclusion, the present results indicate that the DC vaccine is a promising strategy to elicit a tumour-specific antitumour response. However, to improve the immune response against RCC, the inhibitory components generated by the tumours will have to be eliminated or overcome. We propose that the use of the RENCA model for vaccine development is relevant to aid in the design of a DC-based tumour vaccine for RCC in humans. Tempering the immune-suppression caused by RENCA will be a major challenge for future work.

ACKNOWLEDGEMENTS

The authors thank Drs Mary Stevenson and George J. Snipes for helpful suggestions and critical review of the manuscript. This work was supported by a grant to S.T. from F.R.S.Q. F. Chagnon was supported by a scholarship from F.R.S.Q.-F.C.A.R. Sante. L.T-S. was supported by a scholarship from the Fast Foundation of the M.G.H. Research Institute.

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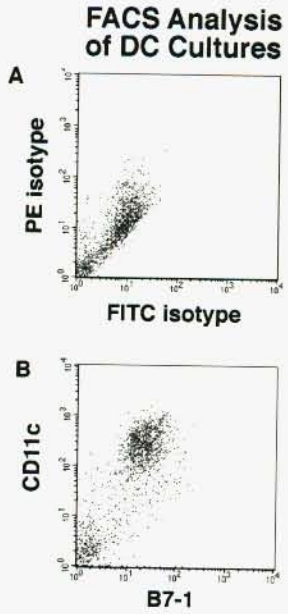
Abbreviations: RCC, renal cell carcinoma; IL, interleukin; IFN, interferon; LAKs, lymphokine-activated killer cells; TILs, tumour-infiltrating lymphocytes; DC, dendritic cell; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; TMB, 3,3',5,5'-tetramethylbenzidine; GM-CSF granulocyte macrophage-colony stimulating factor; FACS, fluorescent-activated cell sorting;

Fig. 1. a, The cell surface phenotype of bone marrow-derived DCs as determined by FACS analysis. On day 7 of culture, 10^6 cells were double-stained with fluorescent-labelled antibodies and propidium iodide used to exclude dead cells. Upper panel (A) isotype controls; lower panel (B) B7 coupled to FITC (fluorescein isothiocyanate) and CD11c coupled to PE (phycoerythrin). b, Left panel (A), DCs in culture fixed onto glass slides and viewed by phase-contrast microscopy. Right panel (B), haematoxylin-eosin stained cells. Both X250.

I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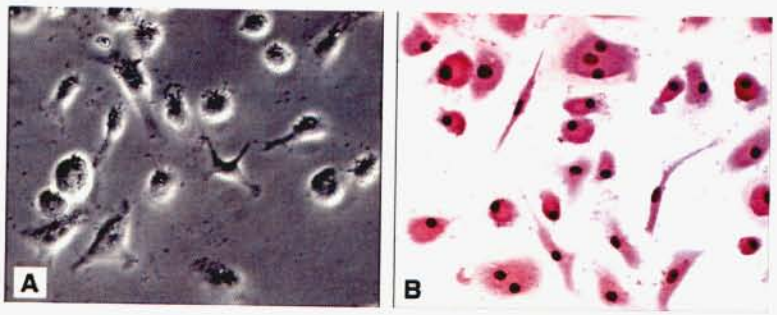
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Table 1 The effect of DC vaccination on subcutaneous RENCA tumour growth

Mean (SEM) variable	DC alone	DC + CL-7	DC + RENCA	HBSS
Tumour volume (mm^3) at day				
12	76 (7)	71 (14)	12 (6)*	61 (20)
16	152 (39)	164 (50)	54 (14)*	154 (24)
28	494 (129)	844 (237)	809 (210)	683 (33)

* significantly smaller tumour volume ($P < 0.05$) between RENCA-pulsed DC and the other groups. There was no significant difference between the groups at day 28. Representative data from three different experiments.

Table 2 DC- and ConA-mediated stimulation of splenocytes and splenic T cell proliferation

Mean (SEM) variable	normal	tumour-bearing
Splenic T-cell proliferation, d.p.m.		
DC + RENCA	6470 (120)	443 (120)
DC + CL-7	6120 (912)	1643 (656)
DC alone	3317 (727)†	1556 (438)
Spleen-cell proliferation, d.p.m.		
ConA (2,5 μg/ml)	234,200 (9100)	127,000 (5000)†

†$P < 0.05$, values are mean of triplicates.

CHAPTER 3

Fanny Chagnon, Simon Tanguay, O. Levent Ozdal, Meng Guan, Mario Chevrette, Mostafa M. Elhilali and LuAnn Thompson-Snipes. Potentiation of a Dendritic Cell Vaccine for Murine Renal Cell Carcinoma by CpG Oligonucleotides. (2003) Submitted to Cell. Immunol.

The mechanism and timing of the suspected RENCA-induced immunosuppression had to be better understood in order to circumvent the immunosuppressive effects. Based on the immunosurveillance hypothesis, it is believed that the immune system is activated by the tumor and then as the tumor burden become overwhelming, inactivated by it. Thus, administration of the vaccine had to be timed correctlys to take advantage of this enhancement of the immune response. Secondly, it was thought that the DC vaccine was not powerful enough to overcome the RENCA-induced immunosuppression. A strategy had to be developed to potentiate our DC vaccine. Since it was demonstrated in the literature that success was obtained with CpG-ODNs, we chose to use that compound. If a DC vaccine was to be attempted in a clinical setting, it was important to establish a strong protocol in the RENCA mouse model.

For contribution of authors, see p. xii

Potentiation of a Dendritic Cell Vaccine for Murine Renal Cell Carcinoma by CpG Oligonucleotides

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Running title: Vaccine for renal carcinoma

Key words: rodent, dendritic cells, tumor immunity, vaccination

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SUMMARY

An inadequate immunity to tumors could be related to a deficiency in the professional antigen presenting cell, the dendritic cell (DC). Here, we investigated the impact of CpG oligodeoxynucleotides (CpG-ODN) on tumor antigen-pulsed bone marrow derived DCs in the treatment of the murine Renal Cell Carcinoma RENCA. Tumor growth was shown to negatively impact on spleen cells and T cell proliferation, interferon gamma production, NK cell activity and decrease the translocation of the transcription factor NF-κB to the nucleus. We have demonstrated that RENCA-pulsed CpG-ODN treated DCs were able not only to significantly reduce tumor growth, but to also prevent tumor implantation in 60% of animals. This data demonstrates that RENCA impacts negatively on the animal's immunity and that the DC therapy, in conjunction with CpG-ODN, can overcome this problem. These experiments also emphasize the importance of an optimal host immune response for the positive outcome of a therapy for cancer.

INTRODUCTION

Renal cell carcinoma (RCC) is a relatively uncommon cancer (31,000 new cases diagnosed in the US in 2001; American Cancer Society, 2001) yet it remains a significant health problem because of its severe morbidity and mortality, despite treatment by conventional radiation and chemotherapies (1). Occasionally, however, RCC may spontaneously regress (2) suggesting the possibility that augmenting the host's natural tumor defense mechanisms might provide a basis for treatment. Accordingly, clinical trials have demonstrated that cytokine stimulation of the immune response with IL-2 and/or IFN-α could induce tumor regression in a subset of patients with metastatic RCC (3), but is associated with high treatment toxicity (2, 3). Additional T-cell modulating treatment strategies, developed for the treatment or prevention of RCC, largely have failed to elicit a durable immune T cell response (4-review, 5).

The use of dendritic cells (DCs) in immune therapy could help sustain a more durable T cell response, as DCs constitute the most potent antigen presenting cells, and could function as important initiators and modulators of an immune response against tumor antigens as reviewed in reference (4). Indeed, the early signals given by DCs during the formation of the T-cell response can determine the scope and nature of the immune response. DCs produce IL-12 and other cytokines that activate a cytotoxic response by T cells or NK cells against tumors, in part, by stimulating T-helper type 1 (Th1) cells to produce cytokines such as IFN-γ (6, 7). Although initial clinical trials evaluating the efficacy of DC vaccination in patients with metastatic RCC were disappointing (8, 9), Kugler et al developed a promising DC vaccination strategy for

RCC, using tumor-DC cell hybrids as a source of antigen, and obtained a partial or complete rejection of metastatic tumor lesions in 7 out of 17 patients (10). These results although still controversial, confirm the ability of DC vaccination, under favorable conditions, to eradicate metastatic disease. However, more effective strategies for DC activation are needed.

We have used the murine renal cell carcinoma (RENCA) model to study tumor targeted DC vaccination (11). We demonstrated that DCs pulsed with RENCA tumor extracts reduce tumor growth in animals that carry a limited tumor burden. However, once tumor burden reaches a critical volume, the beneficial anti-tumor effects generated by DC vaccination are overcome leading to rapid and uncontrolled tumor growth. These results would argue that RENCA induces an immunosuppression similar to that observed with human RCC (12, 13). However, the nature and kinetics of the murine immune response generated against RENCA, and the immunosuppression caused by RENCA remain unknown. This could be important for the development of vaccine strategies.

Treatment of DCs with unmethylated CpG oligodeoxynucleotides (CpG-ODN), promotes the production of cytokines such as IL-12, IL-18, IFN-α and TNF-α creating a Th1-like milieu as reviewed in reference (14). In addition, CpG-ODN can enhance the immune response in vaccine models for infectious agents and tumors (14). CpG motifs have been proposed to stimulate the maturation of DCs as an explanation for the adjuvant effects of CpG-ODN; however, the exact mechanism by which CpG-ODN improves the effectiveness of DCs is unknown. However, it is established that CpG-ODN binds to the Toll-like receptor 9 (TLR9), which is present in DCs and signals to the nucleus via the activation of the transcription factor NF-κB (15, 16). Irrespective of the exact mechanism

of action of CpG-ODN and based on a number of studies, we propose that CpG-ODN will enhance our DC vaccine by enabling DCs to overcome the immunosuppressive effects of RENCA.

In this study, we observe RENCA-induced immunosuppression between 3 to 6 days after tumor injection. When we induce DC maturation with CpG-ODN, we see a dramatic increase in the effectiveness of the tumor vaccine to the point where tumor growth is virtually eliminated after 21 days. We suggest that CpG-activated DCs can override the immunosuppressive effects of RCC in a mouse model and may be useful against human cancer.

MATERIALS AND METHODS

Mice and cells. BALB/c mice, 4-6 weeks old, were obtained from Charles River (Saint-Constant, QC, Canada) or Taconic (Germantown, NY, USA). The BALB/c renal cell carcinoma cell line, RENCA, was a generous gift from Dr I. J. Fidler (M.D. Anderson Cancer Center, Houston. TX, USA). RENCA cells were cultured in DMEM supplemented with 5% FBS, 2 mM L-glutamine, penicillin G, streptomycin and amphotericin B (Invitrogen, Burlington, ON, Canada). The RENCA cells used for this study tested negative for murine viral pathogens. YAC-1 cells were kindly provided by Dr. Mary M. Stevenson (McGill University, Montreal, QC, Canada) and were grown in RPMI 1640 (Invitrogen, Burlington, ON, Canada) supplemented with 5% FBS, 2 mM L-glutamine, 10 mM HEPES, penicillin G, streptomycin and amphotericin B.

Time course. Mice were injected intra-peritoneally with 1 x 10^6 RENCA cells and

spleens collected on days 3, 5, 7, 10 and 14. Splenocytes were then isolated on a Lympholyte M density gradient (Cedarlane, Hornby, ON, Canada). Splenocytes (2.5 x 10^5) from each time point were incubated in triplicate with 2.5 μg/ml Concanavalin A (ConA) (Roche Diagnostics, Laval, QC, Canada) and T-cells were isolated from the remaining splenocytes by StemSep negative selection system (StemCell Inc., Vancouver, BC, Canada). The T cells obtained were assayed by ELISPOT for IFN-γ production and for their proliferative response to 1000 U/ml recombinant human IL-2 (Pharmingen, Mississauga, ON, Canada). The splenocytes and T cells were cultured in RPMI containing 5% FBS, 2 mM L-glutamine, penicillin G, streptomycin, and amphotericin B. In the proliferation assays, 1 μCi/well of ^{3}H-thymidine (6.7 Ci/mM) (ICN, Costa Mesa, CA, USA) was added overnight after 48 hours of culture. The cells were then harvested and the radioactivity was counted using a MicroBeta Instrument (Wallac Oy, Turku, Finland).

NK cell assay. NK (Natural killer) cell cytotoxic activity in total spleen cells was measured as previously described (17). Mice were injected with 1 x 10^6 RENCA cells, intraperitoneally at 3, 7, and 14 days prior to spleen collection. Two mice from each group were injected intraperitoneally with 150 μg poly I:C (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada) 24 hours before harvest. Spleens were collected and mononuclear cells were enriched using Lympholyte M density gradients. Cells were cultured at a concentration of 2 x 10^6 cells/ml along with 2 x 10^4 YAC-1 cells previously labeled with $Na_2{}^{51}CrO_4$ in a classical ^{51}Cr release-cytotoxic assay. Following 4 h incubation at 37°C, supernatants were collected and assessed as cpm of sample. Cells were treated with 1% SDS to determine maximum ^{51}Cr release. Supernatants from

labeled YAC-1 cells without spleen cells present were evaluated for spontaneous ^{51}Cr release. All experiments were done in quadruplicates. Radioactivity was measured using a MicroBeta Instrument. The specific lysis of target cells was calculated as follow:

$$\text{Specific lysis of target cells} = \frac{\text{cpm of sample} - \text{cpm spontaneous}}{\text{cpm max} - \text{cpm spontaneous}} \times 100$$

Electrophoretic mobility shift assay (EMSA). Mice were injected intra-peritoneally with 1 x 10^6 RENCA cells and spleens from 7 days RENCA-injected mice and naïve mice were collected. Splenocytes were then isolated on a Lympholite M density gradient and T-cells isolated from the splenocytes by StemSep negative selection system. Cells were cultured for 30 minutes in RPMI containing 5% FBS, 2 mM L-glutamine, penicillin G, streptomycin, and amphotericin B with or without phorbol myristate acetate (PMA) (Sigma-Aldrich, Oakville, ON, Canada) (10 ng/ml) and ionomycin (Calbiochem, La Jolla, CA, USA) (0.75 μg/ml). The nuclear proteins were extracted using the Cell Lytic Nuclear extraction kit (Sigma-Aldrich, Oakville, ON, Canada) and the lysates were analyzed for total protein content by BCA assay (Pierce, Rockford, IL, USA). EMSAs were performed as previously described (18). The NF-κB probe used is from HIV-LTR, is designated N and its sequence is 5`-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3. A control probe containing a mutant binding site for NF-κB is designated M and its sequence is 5'-TTGTAACAACTCACTTTCCGCTGCTCACTTTCCAGGGAGGCGTGG-3'. Gels were analyzed using a phophorimager Storm 860 and Image Quant software (Amersham, Sunnyvale, CA, USA).

Dendritic cell cultures. DC cultures were established as previously described (11), in

the presence of IL-4 (1000 U/ml) (R&D Systems, Minneapolis, MN) and GM-CSF (20 ng/ml) (generously provided by Schering-Plough, Madison, NJ, USA).

Treatment of DCs with CpG-ODN. After 6 days of culture, DCs were incubated overnight with 4 μg/ml of active phosphorothiorate-modified oligodeoxynucleotide (CpG-ODN), designated "1826" (5'-TTC ATG ACG TTC CTG ACG TT-3') or inactive control CpG-ODN, designated "1982" (5'- TCC AGG ACT TCT CTC AGG TT-3') described by Yi et al. (19) (generously provided by Coley Pharmaceutical Group, Ottawa, ON, Canada).

Cytokine measurement and IFN-γ ELISPOT. Supernatants from the DC cultures were collected after 18 h of incubation with or without CpG-ODN and assayed for IL-12p40/IL-12p70 with an ELISA kit (Biosource International, Camarillo, CA, USA). All antibodies for the ELISPOT were obtained from Pharmingen (Mississauga, ON, Canada) and the IFN-γ ELISPOT assay was performed as previously described (20). The number of spots present in each well were counted under a dissecting microscope and expressed as the mean of triplicates.

RNase protection assay. RNA of DCs treated with the active CpG-ODN 1826, the control CpG-ODN 1982, or untreated was extracted with Trizol (Invitrogen, Burlington, ON, Canada) following the manufacturer's instructions. The RNA was purified from the same cells which supernatant was assayed in the IL-12 ELISA assay. Cytokine mRNA levels were determined by RNase protection assay using the Riboquant multiprobe RNase Protection Assay System and the mCK-2b template set (PharMingen, San Diego, CA, USA) following the instructions of the supplier. For quantification, cytokine values were expressed as a percentage of the mean values of the housekeeping gene (fixed at 100%),

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each condition/gel lane. The bands were quantified using a Storm 860 Phosphorimager and the Image Quant software.

Tumor antigen extraction. Tumor antigens were extracted from confluent RENCA cells using citrate-phosphate buffer as described (11). The extracted polypeptides were subsequently concentrated on SepPak C_{18} cartridge (Millipore, Bedford MA, USA), lyophilized (Virtis, Gardiner, NY, USA) and stored at –80 °C.

Therapeutic experiments. BALB/c mice (6 groups of 10 animals per group) were injected with 1 x 10^5 RENCA cells subcutaneously on day 0. Group 6 received HBSS along with the RENCA cells and group 5 received 10 μg of active CpG-ODN 1826 concomitantly with RENCA cells but did not receive DCs. The mice from the remaining 4 groups were treated by subcutaneous injections of DCs on day –4 relative to tumor injection and on day +6 following tumor injection ipsilateral to the tumor cells. Groups received 2.55 x 10^5 unstimulated DCs/mouse (group 4), DCs treated with 4μg/ml of active CpG-ODN 1826 (group 3), DCs pulsed with RENCA peptides (DC-RENCA) (10 μg/ml/10^6cells; group 2) or DCs pulsed with RENCA peptides and CpG-ODN (group 1). The tumors were measured using a Vernier caliper (Scienceware, Pequannock, NJ, USA) in two dimensions perpendicular to each other twice a week. The tumor measures were collected by a person who did not know what type of injections the mice had received (blinded). Tumor volume was calculated using the formula $(AxB^2)/2$, where A is the largest of the two measurements, and B the shortest.

Statistical analysis. All statistics were performed with the multivariance analysis test ANOVA.

RESULTS

Time course of immune response in RENCA-bearing animals. As we previously described (11), DC vaccine reduces RENCA growth only in animals with small tumor burden. To define the steps leading to the immunosuppression induced by the RENCA tumor, we have monitored a number of measures of the immune response over several days following tumor injection. We thus examined splenocyte proliferation in response to Con A (Fig. 1A), T cell proliferation in response to IL-2 (Fig. 1B), T cell production of IFN-γ (Fig. 1C) and NK cell activity (Fig. 1D) as a function of time following administration of tumor cells. For all parameters examined, we observed an enhanced immune response in the splenocytes or T-cells from tumor bearing mice as compared to naive animals for the immediate 3 days following tumor injection. Since tumor burden is small during this initial period, these results are consistent with our previous observations that DC vaccine is effective in animals with a limited tumor burden (11). However, as the tumor grows, the observed initial heightened immune response diminishes to near control levels for most parameters at days 6-9 following tumor injection, but with an even lower response in NK cell activity (Fig. 1D). This experiment was repeated three times with similar results.

NF-κB activation is modulated by the presence of RENCA tumor. The rise and fall of the immune response correlated well with the activation and inactivation of NF-κB in the splenic T-cells of tumor bearing mice (Fig. 2). At the lowest point of the immune activity

(day 6 figure 1), the ability to activate NF-κB using PMA and ionomycin stimulation of splenic T-cells was significantly reduced (in naïve mice, the intensity of the band is 87% over background while it is only 39% in tumor-bearing animals). This reduction of NF-κB seven days after tumor injection was reproducible. This binding was also specific since a probe containing a mutated binding site for NF-κB did not exhibit any binding to the transcription factor. Since CpG motifs have been shown to activate DCs and increase NF-κB activation as reviewed in reference (14), we chose to examine the effects of CpG-ODN on our DC preparations.

Effect of CpG-ODN on DC phenotype. Bacterial DNA containing CpG motifs are known to stimulate DCs (14-review) and upregulate the expression of cell surface markers including MHC class I and II molecules, as well as CD80 and CD86 (21). In conjunction with these phenotypic changes, CpG-treatment impacts on cytokine expression by DCs (22). To test whether CpG-ODN treatment of our bone marrow derived DC cultures would upregulate gene expression of Th1 cytokines and thus facilitate tumor regression, we used an RNAse protection assay to simultaneously assess the mRNA expression levels of a panel of cytokines including Th1, Th2 and some inflammatory cytokines. The results of the RNase protection assay performed with RNA from DCs treated with CpG-ODN 1826, control CpG-ODN 1982 or untreated DCs are displayed in Figures 3A and 3B. When compared to their GAPDH level of expression, DCs treated with active CpG-ODN 1826 show an increased level of IL-12 p40, IL-1 α and β, IL-1Rα, IL–6 and IL-18 mRNA as compared with DCs treated with control inactive CpG-ODN 1982, or with untreated DCs. For example, the DCs treated with

active CpG-ODN 1826 showed a 12-fold increase in IL-12 p40 mRNA expression over the media control (Figure 3B). This correlates with the production of IL-12 p40 plus IL-12 p70 protein as assessed using ELISA assay of DC cell supernatants, 18 hours after stimulation with CpG-ODN (see Figure 3C). The IL-12 measurement in the supernatant was performed on the same cultures from which the RNA had been extracted and on which the RNAse protection assay was performed. The control CpG-ODN 1982 treated DCs did not significantly differ from the media control treated samples. Consistently we see a significant induction of IL-6 and TNF-α protein levels as well in our bone marrow derived DCs cultures when stimulated with CpG-ODN 1826 (data not shown).

Animal vaccination with CpG-ODN 1826 treated DCs. As we have seen, CpG-ODN 1826 treatment promotes the maturation of DCs (14-review, 21), and the secretion of Th1 type cytokines. The impact of these changes on the efficacy of DC vaccination was tested in an anti-tumor assay *in vivo*. Since the control CpG-ODN 1982 did not induce any expression of Th1 cytokines in our *in vitro* assay (see Fig 3), we chose to decrease the number of mice needed in our vaccine experiments and exclude its use. Groups of 10 mice were injected with 1 x 10^5 RENCA cells subcutaneously on day 0. Animals were treated with DC vaccine on day –4 (before tumor injection) and +6 days after tumor injection. Although the immunosuppression caused by the tumor may have been affected by the previous DC administration, we choose to boost at day 6 corresponding to the time at which the immune response begins to fall following tumor injection as shown in Figure 1. Animals were treated with either HBSS (group 6), CpG-ODN concomitantly with tumor (group 5), unstimulated DCs (group 4), CpG-ODN-treated DCs (group 3), RENCA

antigen-pulsed DCs (group 2), or DCs treated with CpG-ODN and pulsed with RENCA antigen (group 1). As shown in Figure 4, the effect of CpG-ODN-treated DCs on tumor growth was striking. By day 19 post tumor injection, control animals (group 5 and 6) had developed significant tumors, with a size: of 343 +/- 106 mm^3 (group 6), and 236 +/- 175mm^3 (group 5). These results were not statistically different from one another ($p=0.36$). Animals vaccinated with unstimulated DC (group 4) or DC RENCA (group 2) presented similar average tumor sizes, and although a tendency to have smaller tumors size than controls could be observed, results were not statistically different ($p=0.06$). However, when these DCs (RENCA pulsed or not) were also treated with CPG-ODN (groups 3 and 1, respectively), a strikingly smaller tumor size was observed. At day 19 , the tumor size for group 3 was 33 +/- 80mm^3, and for group 1 it was 3 +/- 4mm^3, which is statistically different from control groups ($p<0.01$ for both groups). It must also be noted that in the CpG-ODN treated DC groups only one mouse demonstrated a small palpable tumor at 19 days. Over the duplicate set of experiment, 6/10 mice from group 3 and 11/18 mice from group 1 were tumor free at that time (data not shown). Furthermore, we have correlated an increase in splenic T-cell IFN-γ production in mice without tumor growth from group 3 (DC+RENCA+CpG) indicating a replenished T-cell activity (ELISpot data not shown).

Lymphocyte infiltration in tumors of animals vaccinated with CpG-ODN 1826 treated DCs. Figure 5 shows the hematoxylin and eosin staining of tissue sections from formalin fixed tumors obtained 19 days after tumor injection. Only one tumor was palpable and recovered from the animals receiving CpG-ODN treated DCs (group 3). This tumor

revealed significant lymphocytic infiltration within the small residual tumor, suggesting that lymphocytes were activating a cellular response to the tumor (Figure 5, B and D). No significant inflammatory cell infiltrate was observed in control groups (groups 5 and 6), including the animals receiving RENCA treated DCs or unstimulated DCs (groups 2 and 4: data not shown).

DISCUSSION

This study demonstrates that RENCA suppresses immune function; however, the immune function can be restored successfully by treatment with autologous DC activated with proper CpG-ODN. In earlier work, we demonstrated that DCs pulsed with tumor-specific antigens can decrease RENCA tumor growth in animals, but only during the early phases of tumor growth (11). The inability of tumor antigen pulsed DCs to inhibit tumor growth at later time points correlated with loss of specific immune functions, suggesting that the RENCA cells themselves suppressed anti-tumor immune surveillance. The concept of RCC induced immune suppression has been recognized for over a decade (12, 13, 23). One aim of the present study is to identify the components of the immunosuppressive effects of RENCA and delineate the time course of the immunosuppression. This has important implications for therapy as it could identify the appropriate time for intervention. Finally, we evaluate whether anti-tumor immune function could be restored by directly activating DCs with CpG-ODN *in vitro* prior to injection into animals. As will be discussed more fully below, the ability of synthetic CpG-ODN to activate NF-κB, restore DC function, and stimulate T cell function may

explain how they promote an effective immune response for the treatment of RCC.

In the present study, we expanded our previous observations by analyzing the time-course of alterations in several measures of the host's immune response to the RENCA tumor. We found that RENCA tumors initially increased a) the proliferative response of splenocytes to IL-2 and ConA, b) IFN-γ production by T- cells, and c) NK cell activity, but only in the 3 days following tumor administration. However, after 6 days post-tumor injection, all of these measures of increased immune activity declined to either baseline or below compared to naïve animals. The rise and fall of the immune response in these animals suggests that the animals' immune mechanisms are initially being activated by the tumor, but that prolonged exposure to tumor dampens the immune response. This observation of decrease in the immune response in RENCA-bearing animals supports the results of Gregorian et al. (12, 13), who demonstrated that spleen cells taken from RENCA bearing mice do not develop lymphokine-activated killer cells *in vitro* compared to spleen cells from normal animals.

Human RCC also suppresses the immune system. In human RCC, a tumor associated immune suppression is affiliated with a decrease in the activation of the transcription factor NF-κB and increased T cell apoptosis (24). NF-κB is a highly conserved inducible transcription factor that is important in both innate and acquired immunity as it regulates gene expression of many cytokines and chemokines (25). In the mouse RENCA model, we observe a significant decline in NF-κB activation seven days following RENCA cell injection into the mice. This decrease is associated with a significant decrease in the mitogenic response of the murine spleen T-cells to ConA, and IL-2, and to produce IFN-γ in response to PMA/ionomycin (Figure 1). The NK cell inactivation in these mice

parallels the NF-κB inactivation. By analogy, we speculate that T-cell, NK cell and DC deficiencies in tumor-bearing mice may be secondary to NF-κB inactivation induced by tumor cells, leading to a dampening of the multiple cytokine signaling pathways dependent on NF-κB activation (26-review, 27).

Cells in the innate immune system, such as macrophages and DCs, express TLR9 that is activated upon binding to CpG motifs (28). TLR9 activates a signaling cascade that eventually leads to NF-κB translocation to the nucleus (28) and activation of a family of mitogen-activated protein kinases (MAPKs) found in monocytes, DCs (19), macrophages and B cells (16), thus, triggering an optimal adaptive immune response.. In our study, CpG motifs may override the tumor associated inhibition of NF-κB activation in the DCs themselves, through one of the alternate pathways of NF-κB regulation such a post transcriptional regulation (25). It is possible that the initial immune response to the tumor leads to activation and nuclear localization of NF-κB but the presence of tumor decreases the transcriptional activation of NF-κB leading to a dampened immune response. These CpG motifs may antagonize the tumor inhibitory effect on NF-kB activity by directly stimulating transcription of NF-κB in DCs. Future studies will be necessary to analyze the transcriptional activation of NF-κB by CpG-ODN and its effects on other critical components of this pathway, which includes the IκB kinase complex. Such studies are relevant since it is likely that NF-κB is central to the function of DCs but also to the tumor specific T-cells that are activated by DCs, cytokines and co-stimulatory cell surface molecules such as CD40, B7-1 and B7-2.

The RENCA tumor may directly inhibit the function of DCs (29). There is evidence that DC function is suppressed by tumors (30). Indeed, histopathological studies indicate

that most human RCCs are poorly infiltrated by DCs (31). One mechanism by which tumors could affect DC function is by down-regulating chemokine receptors on DCs thereby preventing them from being recruited to the tumor, or from homing to lymphoid tissues to present antigen to T-cells. In addition, some tumors down-regulate the expression of MHC class II molecules on DCs (32). This combination of decreased DC functions, impaired trafficking and antigen presentation, may be a major impediment to an effective immune response in advanced RCC (33, 34). Interestingly, the presence of RENCA tumor upregulates the expression of the co-stimulatory molecules CD80, CD40 and MHC class II on the surface of a CD11c+/CD11b+ population of spleen DCs 5-14 fold compared to DCs from naïve mice (data not shown). The expression of these cell surface molecules is usually correlated with increased DC function and T-cell activation as reviewed in reference (4). However, despite the activation of DCs in tumor-bearing animals, the tumors are not rejected unless the animals receive the DC/CpG-ODN vaccine treatment. In addition, DCs from the spleens of unvaccinated RENCA bearing mice are normal in their ability to stimulate a classical mixed lymphocyte reaction (data not shown). It would thus appear that the function of these DCs *in vitro* is normal. However, CpG-ODN treated DCs are capable of inducing a tumor response *in vivo*, while untreated DCs do not, implying that some critical function of the tumor-bearing animals DCs is being supplemented by CpG-ODN activated DCs.

One of our rationales for using CpG-ODN derives from recent studies demonstrating the efficacy of CpG-ODN as DC activators and inducers of Th-1 responses (35). One important characteristic of CpG-ODN remains their ability to activate and induce DC maturation (21). Our results are consistent with a role for CpG-ODN in influencing DCs, perhaps via activation of NF-κB, to create a Th-1 environment, thus activating a strong

CTL response. We found that active CpG-ODN 1826 induces DCs to produce more IL-12 p40 and IL-18 mRNAs, whereas control CpG-ODN did not (Figure 3A and 3B). Our cytokine analysis also revealed the production of active IL-12 in the form of IL-12 p70 in response to CpG-ODN 1826 (Figure 3C). These results are consistent with DC's ability to promote a more efficient Th1 environment in the context of a specific pathogenic or tumor antigen presentation leading to a cytotoxic response to tumor.

Irrespective of the mechanism of action of the CpG-ODN, it is clear that CpG-ODN treated DCs strongly activate the *in vivo* response to RENCA tumor in our vaccine paradigm. In this study, we carefully chose the times for injection of the DC vaccine based on the time-course of the immune responses. Thus, the first vaccination was administered 4 days prior to tumor injection in order to best model the status of a patient following removal of a tumor (i.e. low tumor presence) and concomitant with injection of a DC vaccine. The second vaccination was administered 6 days after tumor injection in order to stimulate an immune response to the RENCA antigens during a period just prior to peak immune suppression. By day 19 post tumor injection, there was a highly significant difference ($p<0.01$) in tumor size between the control groups (#6, HBSS and #5 ,CpG-ODN) and the groups that received CpG-ODN treated DCs, (#3 and #1), whether they were pulsed with RENCA antigen or not. One possible explanation for the activity of CpG-ODN treated DCs in the absence of tumor antigen stimulation *in vitro* could be that DCs *in vivo* are activated by antigens originating from apoptotic tumor cells at the draining peripheral lymph node. Indeed, there is evidence that DCs can acquire antigens from apoptotic cells (36). Thus, it may be possible to boost a patients' immune response by activating autologous DCs with CpG-ODN without the requirement for the

isolation of specific tumor antigens. However, IV injection or contralateral injection of DC vaccine may not give the same response. This may have important implications for the clinical efficacy of this vaccine regime, provided the DCs are administered near the tumor draining lymph nodes.

Since CpG-ODN strongly affect DC maturation (14), they should find utility in improving DC vaccines. Two recent reports support our results. One group (37) used *in situ* stimulation of DCs by injecting CpG-ODN concomitantly with tumor cells coupled with intraperitoneal injection of Flt3 ligand (FL). The latter is known to expand the number of DCs in lymph nodes, spleens and other tissues (38). Although this treatment is convenient in that it does not require *ex vivo* manipulation of DCs and was effective against the tumor, FL may not be tolerated by some patients. In the other report, tumor-infiltrating DCs (TIDCs) were found to have a functional defect that can be reversed by incubation with CpG-ODN along with anti-IL-10 receptor antibodies (30). CpG-ODN alone could not overcome the DC impairment in the C26 colon carcinoma tumor model used. Vicari et al attributed the refractory state of the DC to IL-10 produced by the tumors. They speculated that by blocking the effects of IL-10 with anti-IL10 receptor antibodies, the threshold for activation with CpG motifs would be lowered thereby allowing TIDC activation leading to an increase in animal survival. However, they injected anti-IL-10R antibody intraperitoneally, a method that may not be tolerated by a human patient. Furthermore, they injected CpG-ODN directly into the tumor unlike our experiments that used CpG-ODN to stimulate DCs *in vitro* followed by removal of excess CpG-ODN prior to injection, thus, eliminating the risk of an inflammatory response generated by the CpG-ODN. In our DC vaccine we were able to inhibit tumor growth without the apparent need to block IL-10 activity. This may be an advantage of our

strategy since ablating IL-10 may have other effects on a patient's general immune responses and may not be tolerated.

Several clinical trials have been undertaken in humans utilizing DCs as a cancer vaccine. However, it is difficult to compare their efficacies since there is no standardized protocol. Nevertheless, some success of DC vaccines has been reported in RCC, prostate cancer, and melanoma. In the RCC trials by Kugler et al, only 4 of 17 patients showed a complete response to a vaccine of fused DCs and tumor cells (10). However, this could reflect a low efficiency of cell fusion that could be overcome using our protocol of direct antigen stimulation followed by CpG-ODN stimulation. For prostate cancer, Murphy's group obtained an overall response rate of 25-30% (39) by vaccinating with DCs pulsed with two HLA-A2-restricted prostate-specific membrane antigen peptides. Unfortunately, this strategy requires the use HLA-A2-restricted peptides and must be tailored to individual patients. In melanoma, the most successful results were obtained in patients vaccinated with DCs pulsed with a cocktail of melanoma antigens (40). In the melanoma trial, three out of sixteen patients had a complete response. These results encourage the use of DCs as anti-cancer vaccines, but overall the results are not as successful as we would like. Improving the efficacy of the DC vaccines may require a method of activating the antigen presenting cells to produce a stronger stimulus to the effector T-cells. The CpG-ODN have been shown to have an effect on tumor growth when injected directly in the tumor, but complete tumor regression has not been observed (14).

In this report we demonstrate the use of CpG-ODN to activate DCs in a murine tumor vaccine model. We postulate that CpG-ODNs are activating the DCs in a way that overcomes the immunosuppressive effects of the RENCA tumor. Experiments are underway to assess the tumor specificity and longevity of this immune response to

RENCA. Further analysis of the long term effects of this vaccine will be pursued. We anticipate that the use of CpG-ODN-treated DCs for the treatment of human RCC will become an extremely attractive treatment strategy.

ACKNOWLEDGEMENTS

We wish to thank Dr. G. Jackson Snipes for the pathology and critical reading of the manuscript, Dr. Pierre Allard for critical reading of the manuscript, as well as Martha Zewdie, Bettye Cox and Jean-Sébastien Ripeau for technical assistance.

This research was funded by the Kidney Foundation of Canada. ST received funding as a Chercheur-Clinicien and MC received funding as a Chercheur-Boursier Senior from the FRSQ. FC received a scholarship from the FRSQ-FCAR Santé. L.T-S. was supported by a scholarship from the Fast Foundation of the M.G.H. Research Institute.

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FOOTNOTES

1. This research was funded by the Kidney Foundation of Canada.

2. Abbreviations used in this paper: DC: dendritic cell, RCC: Renal cell carcinoma, CpG- ODN: CpG-oligodeoxynucleotides, TIDC: tumor-infiltrating DCs, TLR: toll-like receptor

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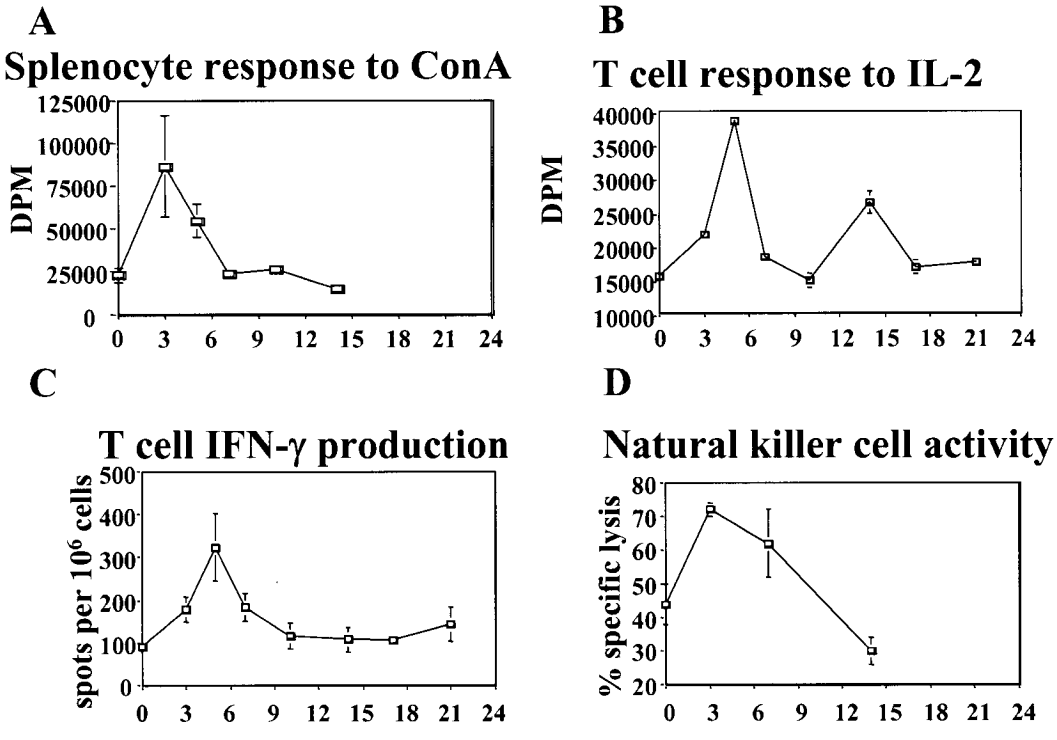
Figure 1. The immune response is decreased over time in the presence of tumor. Mice received 10^6 RENCA tumor cells by intraperitoneal injection and T-cells were purified from the spleens of normal and tumor bearing animals at intervals during tumor growth. The following *in vitro* measurements were performed: A) proliferative response of spleen cells to ConA . B) proliferative response of T-cells to IL-2. C) PMA/ionomycin induction of IFN-γ by T-cells using ELISPOT assay. D) Natural killer cell activity of splenocytes against ^{51}Cr-labeled YAC-1 cells.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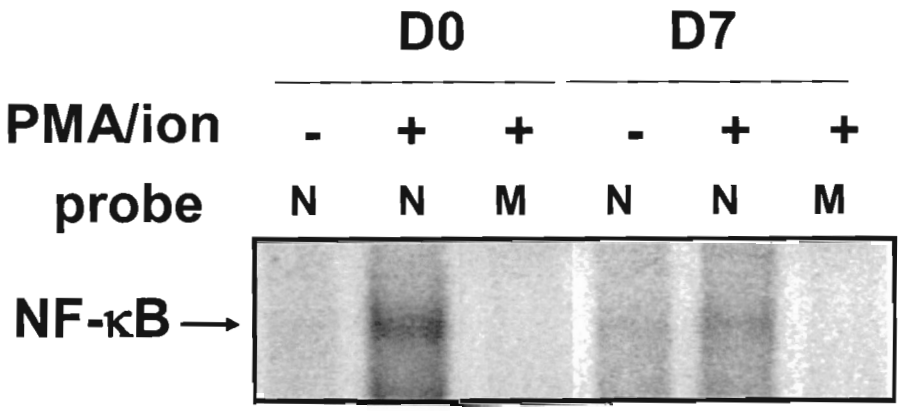
25000
20000
15000
10000
0 3 6 9 12 15 18 21 24
C
T cell IFN-γ production
spots per 10^6 cells
500
400
300
200
100
0
0 3 6 9 12 15 18 21 24
D
Natural killer cell activity
% specific lysis
80
70
60
50
40
30
20
0 3 6 9 12 15 18 21 24

Figure 2. NF-κB activation is decreased in tumor bearing mice. The activation of NF-κB was evaluated in isolated T-cells from spleen, just before (D0) and 7 days after injection of 1 x 10^6 RENCA cells (D7) in mice. Isolated T-cells were then stimulated (+) or not (-) with PMA/ionomycin, and NF-κB activity was revealed using an EMSA assay detecting the nuclear binding of NF-κB with the HIV-LTR probe (N). A probe containing a mutated binding site for NF-κB (M) was used to assess specificity.


D0
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NF-κB

Figure 3. CpG-ODN 1826 stimulated cytokine production in DCs. Cytokine mRNA expression and IL-12p70 + p40 ELISA from the same DC culture were assessed. A) RNA was extracted from DCs treated with CpG-ODN 1826 (3), with control CpG-ODN 1982 (2) or non-treated (1). The RNA was then submitted to an RNase protection assay using the mCK-2b multi probe template set. B) The bands were quantified and expressed as % of GADPH intensity. This is representative of two replicate experiments. C) IL-12 p70 + p40 ELISA. Supernatants of DC cultures treated with CpG-ODN 1826, with control CpG-ODN 1982 or non-treated were assayed for IL-12 p70 + p40 presence by ELISA.

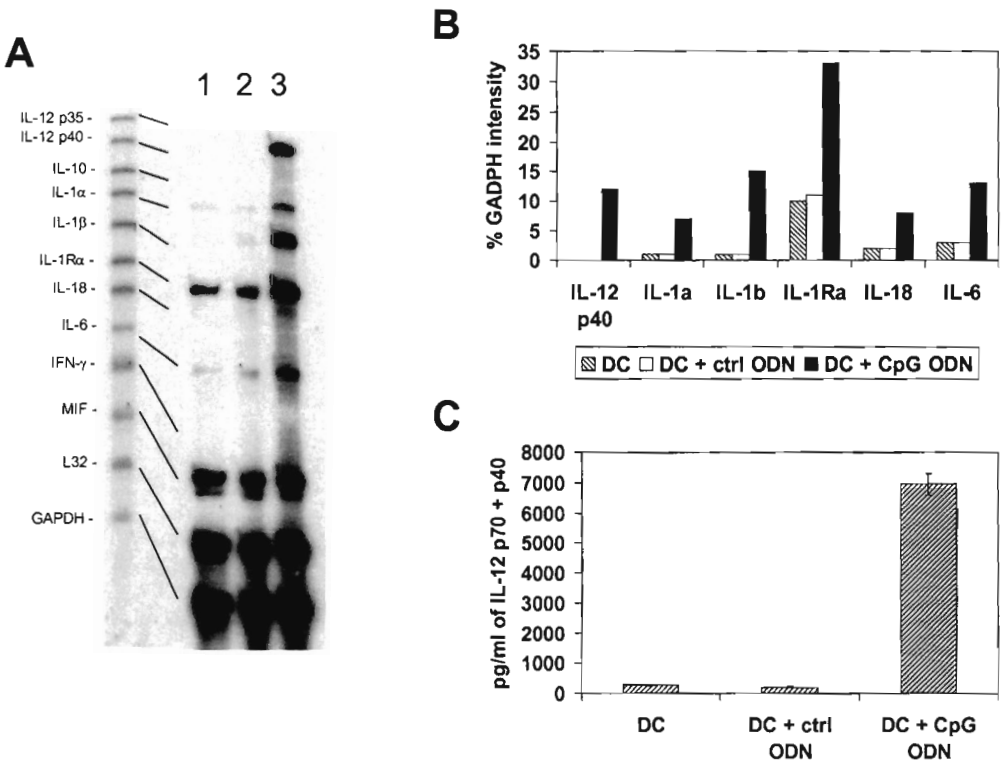

A
1 2 3
IL-12 p35 -
IL-12 p40 -
IL-10 -
IL-1α -
IL-1β -
IL-1Rα -
IL-18 -
IL-6 -
IFN-γ -
MIF -
L32 -
GAPDH -
B
% GADPH intensity
35
30
25
20
15
10
5
0
IL-12 p40
IL-1a
IL-1b
IL-1Ra
IL-18
IL-6
DC
DC + ctrl ODN
DC + CpG ODN
C
pg/ml of IL-12 p70 + p40
8000
7000
6000
5000
4000
3000
2000
1000
0
DC
DC + ctrl ODN
DC + CpG ODN

Figure 4. CpG-ODN 1826 treated DCs elicit a strong anti-tumor response to RENCA. Groups of 10 mice were injected with 1 x 10^5 RENCA cells subcutaneously on day 0. Animals were treated with DC vaccine on day –4 (before tumor injection) and +6 days after tumor injection. Animals were treated with either HBSS (group 6), CpG-ODN (group 5), unstimulated DC (group 4), CpG-ODN-treated DC (group 3), RENCA antigen-pulsed DC (group 2), or DC treated with CpG-ODN and pulsed with RENCA antigen (group 1). The * and ** indicates a statistically significant smaller tumor volume ($P<0.05$ and $P<0.01$ respectively) when comparing DC-CpG-ODN (group 3) and DC-RENCA-CpG-ODN (group 1) to HBSS (group 6). Values represent mean +/- SEM. This *is* representative of two replicate experiments.

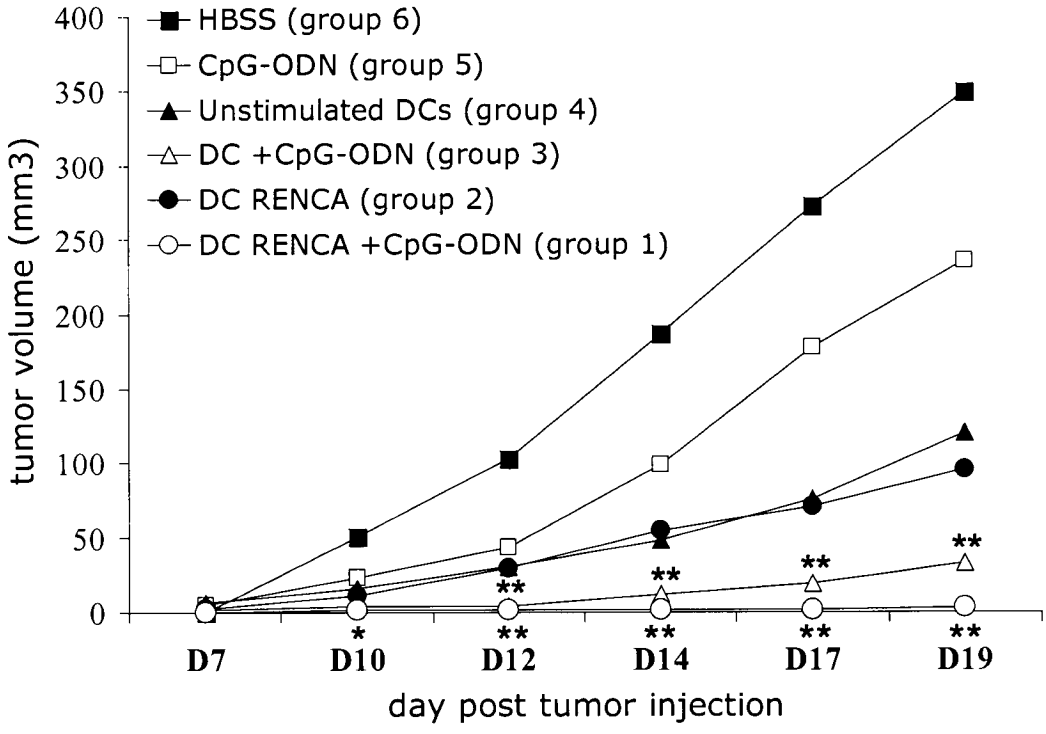
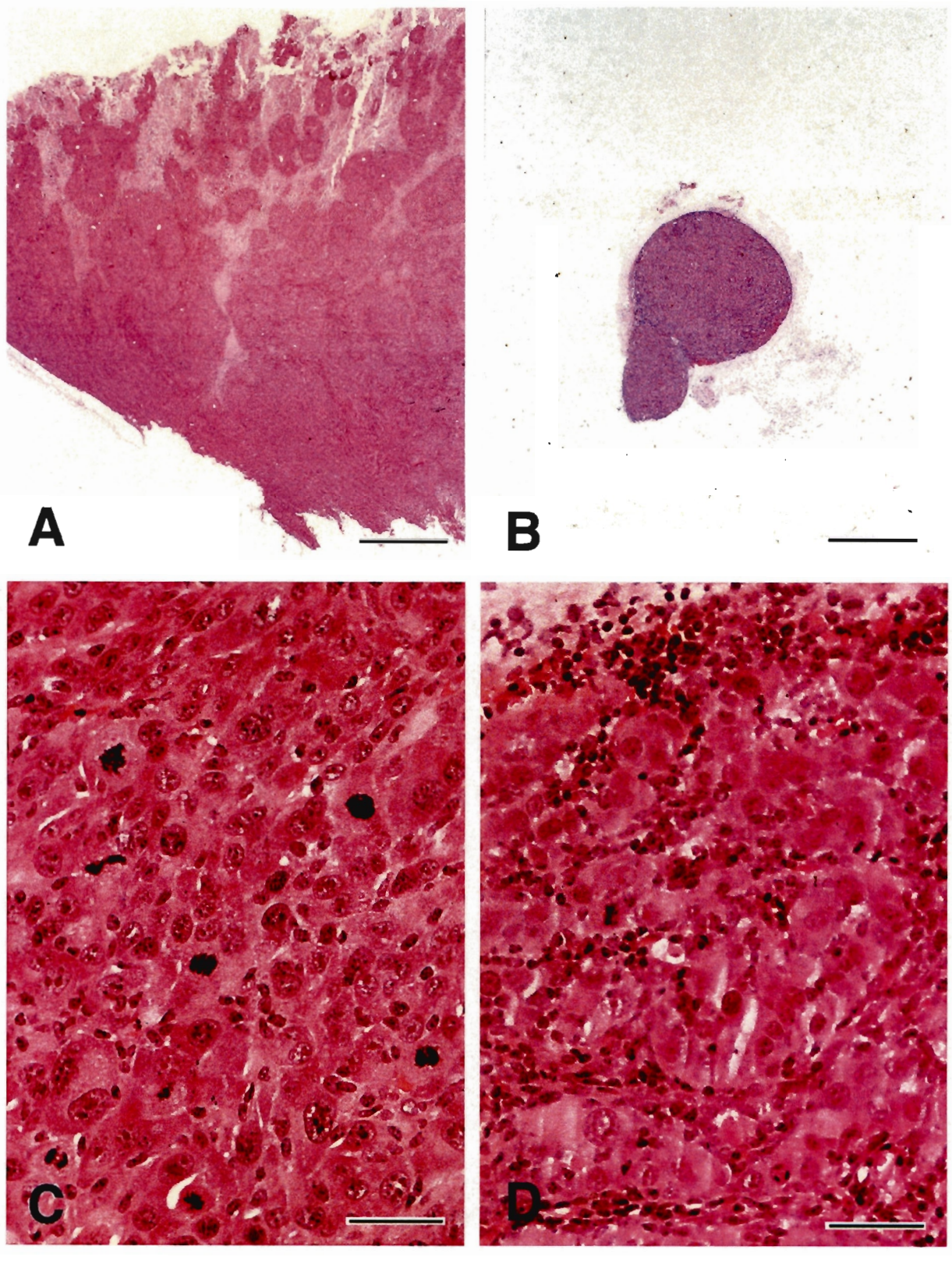

HBSS (group 6)
CpG-ODN (group 5)
Unstimulated DCs (group 4)
DC +CpG-ODN (group 3)
DC RENCA (group 2)
DC RENCA +CpG-ODN (group 1)
tumor volume (mm3)
400
350
300
250
200
150
100
50
0
D7
D10
D12
D14
D17
D19
day post tumor injection
*
**

Figure 5. CpG-ODN 1826 DC tumor vaccine demonstrates a strong lymphocyte response within the tumors. Histopathology of the RENCA tumors isolated from a control-HBSS treated animal (panels A and C) and DC-CpG-ODN treated animal (panels B and D) 19 days after injection. Note the small size of the tumor in the DC-CpG treated animal (B compared to A) and the large number of lymphocytes infiltrating the periphery of the DC-treated tumor compared to the HBSS treated tumors (D compared to C). Panels A and B, bar = 1mm. Panels C and D, bar = 50 μm.


A
B
C
D

CHAPTER 4

Fanny Chagnon, Mario Chevrette, O. Levent Ozdal, Jean-Sébastien Ripeau, Mostafa M. Elhilali and Simon Tanguay. Long term memory and immunity conferred by a CpG-ODN-treated DC vaccine for murine renal cell carcinoma. (2003) Will be submitted to BJU International.

1.

Having shown that vaccination with CpG-ODN-treated DCs could efficiently prevent tumor growth, likely via a T-cell mediated mechanism, the characteristics of the conferred protection had to be assessed. It was important to verify if resistant animals could sustain a tumor challenge, as this mimics cancer relapses. Furthermore, we had seen that small growing tumors in treated animals were infiltrated with lymphocytes. Thus, it was important to verify if the protection could be transferred by splenic T cells to naïve animals. Lastly, since CpG-ODN-DCs could confer protection in the absence of antigen, the specificity of the protection had to be assessed by implanting a different tumor. All this would give us valuable information about the long-term memory and the specificity of the conferred protection of our CpG-ODN DC vaccine.

For contribution of authors, see p. xii

Long term memory and immunity conferred by a CpG-ODN-treated DC vaccine for murine renal cell carcinoma

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SUMMARY

Objective Conventional therapy has limited success in metastatic renal cell carcinoma (RCC). Novel therapies enhancing the immune response should however be very powerful treatment tools. It is in this last line of research that we studied the long-term effect of a CpG-ODN-treated DC vaccine for the treatment of the murine model of RCC, RENCA.

Materials and methods DCs were obtained from mouse bone marrow, cultured in the presence of interleukin-4 (IL-4) and granulocyte macrophage-colony stimulating factor (GM-CSF). These cells were further treated with CpG-ODNs alone (CpG-ODN), or CpG-ODNs and RENCA peptide extracts (RENCA-CpG-ODN), and then used to vaccinate animals. Vaccinated mice protected from RENCA implantation were subjected to tumour challenges and used in immune transfers to assess long-term protection and memory.

Results A significant proportion of animals that remained tumour free after the RENCA-CpG-ODN vaccine were also resistant to a second tumour challenge. Splenic T cells isolated from the RENCA-CpG-ODN treated group, when transferred to naïve animals, conferred specific protection against RENCA tumour growth in a good proportion of animals.

Conclusion These results argue that CpG-ODN-treated DCs are able to establish a long-term specific memory against RENCA and that the presence of antigen in the vaccine is essential in order to establish long term memory.

Keywords dendritic cell, tumour immunity, renal cell carcinoma, RENCA, tumour vaccine, immunotherapy.

INTRODUCTION

Renal cell carcinoma (RCC) is a relatively uncommon cancer with an estimated 31,900 new cases in the USA in 2003 (American Cancer Society, 2003). However, renal cancer remains a significant health problem mainly because of its potentially high morbidity and mortality (1). The occasional spontaneous regression and the observed response to biological response modifiers reinforce the assumption that augmenting the host's natural tumour defense mechanisms might provide a basis for an effective treatment. Accordingly, clinical trials have demonstrated that cytokine therapy with interleukin-2 (IL-2) and/or interferon-α (IFN-α) could induce tumour regression in a subset of patients with metastatic RCC (2). Interferon-α based therapy was also shown to impact on patient survival. However, these treatments are associated with significant toxicity and a limited response rates (2, 3). Additional T cell modulating treatment strategies, including treatment with *ex vivo* expanded tumour infiltrating lymphocytes (TILs) failed to elicit a durable immune T cell response (4, 5).

The use of dendritic cells (DCs) in immune therapy could help sustain a more durable T cell response, as DCs constitute the most potent antigen presenting cells. Moreover, DCs could function as important initiators and modulators of an immune response against specific tumour antigens (4). Indeed, the early signals given by DCs during the formation of the T cell response can determine the scope and nature of the immune response. DCs produce IL-12 and other cytokines activating anti-tumours cytotoxic response in T cells or NK cells, in part, by stimulating T-helper type 1 (Th1) cells to produce cytokines such as IFN-γ (6, 7). Although initial clinical trials evaluating

the efficacy of DC vaccination in patients with metastatic RCC were disappointing (8, 9), Kugler et al recently developed a promising DC vaccination strategy for RCC. Using tumour-DC cell hybrids as a source of antigen, they obtained a partial or complete rejection of metastatic tumour lesions in 7 out of 17 patients (10). These results although still controversial, confirm the ability of DC vaccination, under favorable conditions, to eradicate metastatic disease. However, more effective strategies for DC activation are needed.

We have used the murine renal cell carcinoma (RENCA) model to study tumour targeted DC vaccination (11). We previously demonstrated that DCs pulsed with RENCA tumour extracts reduce tumour growth in animals carrying a limited tumour burden. However, once tumour burden reaches a critical volume, the beneficial anti-tumour effects generated by DC vaccination are overcome, leading to rapid and uncontrolled tumour growth. These results would support the induction of an immunosuppression in the RENCA model similar to what is observed with human RCC (12, 13). We have recently shown that RENCA indeed induced an immunosuppression (Chagnon et al, 2003), as measured by changes in splenocyte proliferation in response to ConA, T cell proliferation in response to IL-2, T cell IFN-γ production, NK cell activity and NF-κB translocation to the nucleus. In these experiments, the peak of immune activity was observed 4 days after tumour injection and dropped to or below control levels between days 6 to 9 (Chagnon et al, 2003).

Treatment of DCs with unmethylated CpG oligodeoxynucleotides (CpG-ODNs), promotes the production of cytokines such as IL-12, IL-18, IFN-α and TNF-α creating a Th1-like milieu (14, Chagnon et al, 2003). In addition, CpG-ODNs can enhance the

immune response in vaccine models against infectious agents and tumours (14). CpG motifs have been proposed to stimulate the maturation of DCs and this can in part explain in part the adjuvant effects of CpG-ODNs. However, the exact mechanism by which CpG-ODNs improves the effectiveness of DCs remains to be elucidated. CpG-ODNs are known to bind to the Toll-like receptor 9 (TLR9), which is present on DCs and signals to the nucleus via the activation of the transcription factor NF-κB (15, 16). Irrespective of the exact mechanism of action of CpG-ODNs, we proposed that CpG-ODNs will enhance our DC vaccine by enabling DCs to overcome the immunosuppressive effects of RENCA.

We have previously shown that vaccination with tumour-Ag-treated, CpG-ODN-activated DCs could prevent tumour growth in a subset of treated animals (Chagnon et al, 2003). In this report, we demonstrate that vaccinated tumour-free mice can resist to a second tumour challenge. Furthermore, by adoptively transferring the splenic T cells of immunized animals to naïve mice, we were able to transfer the immunity to RENCA in 50% of the animals. Finally, we have confirmed the specificity of immunity to RENCA cells since the transferred animals could not sustain a challenge by CT-26, a murine colon carcinoma.

MATERIALS AND METHODS

Mice and cells. BALB/c mice, 4-6 weeks old, were obtained from Charles River (Saint-Constant, QC, Canada). The BALB/c renal cell carcinoma cell line, RENCA, was a generous gift from Dr I. J. Fidler (M.D. Anderson Cancer Center, Houston, TX, USA). RENCA cells were cultured in DMEM supplemented with 5% FBS, 2 mM L-glutamine,

penicillin G, streptomycin and amphotericin B (Invitrogen, Burlington, ON, Canada). The RENCA cells used for this study tested negative for murine viral pathogens (MAP test) murine colon carcinoma cell line was obtained from ATCC (Rockville, MD, USA) and was grown in RPMI 1640 (Invitrogen, Burlington, ON, Canada) supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, penicillin G, streptomycin and amphotericin B (all (Invitrogen, Burlington, ON, Canada).

Dendritic cell cultures. DC cultures were established as previously described (11), in the presence of IL-4 (1000 U/ml) and GM-CSF (20 ng/ml) (R&D Systems, Minneapolis, MN, USA).

Treatment of DCs with CpG-ODNs. After 6 days of culture, DCs were incubated overnight with 4 μg/ml of active phosphorothiorate-modified oligodeoxynucleotides (CpG-ODNs), designated "1826" (5'-TTC ATG ACG TTC CTG ACG TT-3') described by Yi et al. (17) (generously provided by Coley Pharmaceutical Group, Ottawa, ON, Canada).

Tumour antigen extraction. Tumour antigens were extracted from confluent RENCA cells using citrate-phosphate buffer as described (11). The extracted polypeptides were subsequently concentrated on SepPak C_{18} cartridge (Millipore, Bedford MA, USA), lyophilized (Virtis, Gardiner, NY, USA) and stored at –80 °C until use.

Therapeutic experiments. The vaccination experiments were previously described (Chagnon et al, 2003). Briefly, BALB/c mice were injected with 1 x 10^5 RENCA cells subcutaneously on the right flank on day 0. The treatment groups consisted of one group receiving HBSS as a control and one group receiving 10 μg of active CpG-ODN 1826 concomitantly with RENCA cells but without DCs. Mice from the remaining 3 groups

were treated by subcutaneous injections of DCs on day –4 and +6 relative to tumour injection. Groups received either 2.55 x 10^5 unstimulated DCs/mouse, DCs treated with 4μg/ml of active CpG-ODNs, or DCs pulsed with RENCA antigen (10 μg/ml/10^6cells) and CpG-ODNs. Tumours were measured using a Vernier caliper (Scienceware, Pequannock, NJ, USA) in two dimensions twice a week. Tumour volume was calculated using the formula (AxB^2)/2, where A is the largest of the two measurements, and B the shortest.

Challenge. All tumour-free animals were challenged on day 34 post RENCA-injection with a second injection of 1 x 10^5 RENCA cells subcutaneously in the left flank.

Adoptive transfer. Spleens of tumour free mice were harvested on D22 post-challenge. Splenocytes were then isolated on a Lympholite M density gradient and T cells further isolated by StemSep negative selection system (StemCell Inc., Vancouver, BC, Canada). Naïve mice received 5 to 7 x 10^6 T cells IV, whereas control animals received HBSS. The animals were injected with 1 x 10^5 RENCA cells subcutaneously on the right flank four days after T cells infusion.

CT-26 challenge. Tumour-free transferred animals were challenged with 1 x 10^6 murine CT-26 colon carcinoma cells subcutaneously on the left flank (18).

RESULTS

Challenge of tumour-free animals. We previously described how vaccination with CpG-ODN-treated DCs could prevent RENCA tumour growth in a subset of treated animals (Chagnon et al, 2003). To evaluate if animals remaining tumour free 34 days

following RENCA injection could sustain a second tumour challenge, RENCA cells were injected in the flank opposite to the initial tumor injection. In three consecutive experiments, 10 to 30% (between 1 and 3 animals out of 10) of animals resisted to the initial tumor inoculation. These animals were subsequently used for the second tumor challenge. Table 1 provides a summary of the results. Previous experiments with RENCA confirmed that all animals will develop tumor growth following s/c injection within 9 to 11 days. In the first set of experiments, two out of three vaccinated mice remained tumour-free and were sacrificed 56 days after the first tumour injection and 22 days after the challenge. Repeated experiments confirmed that tumor rarely ever grows after 20 days. Therefore, 22 days following tumor challenge was chosen to sacrifice animals and harvest their splenic T cells for transfer to naïve animals. In subsequent experiments, a group vaccinated with CpG-ODN-treated DCs was included. In those two experiments, all animals vaccinated with DC-RENCA-CpG ODN resisted tumour challenge.

Immune transfer. Splenic T cells of animals remaining tumour-free following tumor challenge were isolated and injected IV to naïve animals. Control animals wew injectted IV with HBSS. The number of animals receiving T cell transfer varied according to the yield of splenic T cell recovery in order to inject 5-10 x 10^6 T cells per animals. In both transfer experiments, all control animals developed tumour whereas 50% (3/6) of animals receiving T cells from DC-RENCA-CpG ODN vaccinated animals remained tumour free (Table 2). Results shown in Table 2 also demonstrate the importance of antigen to obtain long-term memory. In fact, animals receiving T cells from DC-CpG ODN vaccinated animals developed tumours as the control animals did.

Specificity of immunity. CT-26, a colon carcinoma syngeneic to BALB/c mice was used to test the specificity of the immunity conferred by T cell transfer. Although preliminary, an animal immune to RENCA growth following T cell transfer from DC-RENCA-CpG ODN vaccinated animals was unable to prevent CT26 tumor growth (1/1 animal). All control animals (naïve animals) also developed tumors (2/2 animals).

DISCUSSION

In the field of cancer immunotherapy, a variety of tumour antigens have been identified, and different strategies are being developed to mimic the presence of a bacterial infection to initiate a tumour Ag-specific T cell response. This study demonstrates that DC vaccination against RENCA can provide long lasting, transferable and specific immunity, when DCs are treated with CpG-ODNs in conjunction with RENCA antigens. In earlier studies, we demonstrated that DCs pulsed with tumour-specific antigens could decrease RENCA tumour growth as long as tumour burden remained limited (11). Our results suggested that RENCA cells were inducing an immunosuppression that eventually overcame the host immune response as the tumor burden was increasing. At this later stage, DC vaccination had lost its efficacy. This phenomenon shares great similarity with the immune suppression observed in human RCC (19). We have subsequently shown that the immune function could be restored successfully by treating DCs with CpG-ODNs (Chagnon et al, 2003). These very interesting findings could have important implications if we plan to apply this treatment strategy to human.

Tumour cells secrete factors such as gangliosides known to inactivate T cells. The exact mechanism by which this suppression is induced is not fully understood but results in part, in inactivation of the transcription factor NF-κB (19). This transcription factor positively regulates the expression of genes involved in the immune response (such as cytokine genes) and in cell survival (such as anti-apoptotic molecules like Bcl-2) (20).

CpG-ODNs are known to bind the Toll-like receptor 9 (TLR 9) present in DCs (21). The signaling pathway triggered by this binding leads to the translocation of NF-κB to the nucleus. CpG-ODNs were also shown to be powerful activators of DCs (14). CpG-ODNs-induced DC maturation lead to strong expression of co-stimulatory molecules and secretion of Th1 cytokine (including IL-12). These changes can explain in part the ability of DC-RENCA CpG-ODNs to overcome the RENCA induced immunosuppression (Chagnon et al, 2003).

In the present study, we have expanded those results by showing that a subset of CpG-ODN-DC-vaccinated animals is resistant to a subsequent tumour challenge. This observation can be very important in the context of relapses seen in patients with RCC. It is attractive to extrapolate that humans treated with CpG-ODNs-DCs could have a decreased relapse rate. Furthermore, we have shown that transfer of splenic T cells from DC-RENCA-CpG vaccinated mice to naïve animals was able to elicit protective immunity against a subsequent RENCA tumour challenge. This shows that DCs are able to trigger long term immunity, confirming their role as initiators of the immune response (4). It is important to note that only T cells harvested from animals vaccinated with DC-RENCA CpG-ODN elicited protection whereas T cells from DC-CpG-ODN vaccinated mice did not result in tumor protection. This emphasizes the importance of vaccinating

with DCs exposed to tumor antigens in order to elicit specific long-term memory. In the C26 colon carcinoma model, Heckelsmiller et al. have shown that peritumoural injections of CpG-ODNs were able to elicit an anti-tumour response in animals with established tumours (22). They also demonstrated the ability of their treatment strategy to protect animals against a subsequent tumour challenge. Furthermore, spleen cells of animals protected against a second challenge displayed a CD8 T cell-dependent lytic activity against tumour cells (22). Kawarada et al. have shown that, depending on the model used, either NK cells or CD8 T cells were involved in the antitumour activity generated by CpG-ODNs (23). This could argue that the splenic T cell population responsible for the observed protection is probably in part the population of CD8 cytotoxic T cells, although CD4 T cells could have a role in the memory (24). In our experiments, animals resistant to RENCA challenge following T cell transfer had developed an antigen-specific immunity. This was further reinforced by the observation that these animals were not resistant to the CT-26 colon carcinoma tumour challenge.

2. The CpG-ODNs have distinct effects and specificities in human and mouse. This raises some caution in predicting the anti-tumour activity of CpG-ODN-DC treatment in cancer patients. Several points have to be considered if this treatment is to be applied in a clinical setting. First, the appropriate CpG-ODNs have to be selected, since the CpG motif that is optimal to activate human immune cells is different from the CpG motifs needed for the murine immune system. These differences are likely due to the species-specificity conferred by the TLR9 (15, 25). Second, major differences exist between species regarding the existence of different DC subsets and their susceptibility to stimulation by CpG-ODNs (26-28). Furthermore, as previously reported by

Heckelsmiller et al. and Vicari et al, injecting CpG-ODNs directly in the tumour might elicit an inflammatory response. In our experiments, CpG-ODNs were used to stimulate DCs *in vitro* and the excess of CpG-ODNs was carefully removed prior to injection in animals. It is thus our belief that a carefully selected CpG-ODNs, used in a similar strategy could be a successful treatment option in patients with metastatic RCC.

3. There have been few successful clinical trials for cancer patients using a DC-based strategy. However, their efficacy are difficult to compare since there are no established standardized protocols. The most successful trials took place in melanoma and prostate cancer patients. In melanoma, the most encouraging results were obtained by vaccinating patients with DCs pulsed with a cocktail of melanoma antigens (29). In this trial, three out of sixteen patients had a complete response (29). In prostate cancer, an overall response rate of 25-30% was obtained by vaccinating with DCs pulsed with two HLA-A2-restricted prostate-specific membrane antigen peptides (30). Although quite successful, this strategy requires the use of HLA-A2-restricted peptides and must be specifically made for individual patients. It thus eliminates patients that do not carry these specific HLA-A2 antigens. By using CpG-ODN-activated DCs and a pool of tumour antigens, we believe that our strategy will help increase the vaccine efficacy.

4. In this report, we demonstrated the impact of a CpG-ODN-DC vaccine on the immune response. The vaccine helps to mount such a strong immune response that not only a subset of animals are resistant to tumour development, but they can also be resistant to a tumour challenge. Furthermore, this vaccination strategy proved helpful in allowing the immune system to develop long-term memory since splenic T cells were able to transfer immunity to naïve animals. Finally, we confirmed the tumor specificity of

the immunity conferred by the vaccine. These results suggest that the use of CpG-ODN treated DCs may be a good strategy for the treatment of advanced RCC and one can speculate that the strategy should also be effective in other cancers.

5.

6. ACKNOWLEDGEMENTS

We wish to thank Dr. Pierre Allard for critical reading of the manuscript.

This research was funded by the Kidney Foundation of Canada. ST and MC received funding from the FRSQ. FC received a scholarship from the FRSQ-FCAR Santé.

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Abbreviations: RCC, renal cell carcinoma; IL, interleukin; IFN, interferon; TILs, tumour-infiltrating lymphocytes; DC, dendritic cell; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; GM-CSF granulocyte macrophage-colony stimulating factor; CpG- ODN, CpG-oligodeoxynucleotides; TLR, toll-like receptor; Th, T helper.

Table 1- Resistance to a tumor challenge by vaccinated mice. Tumor-free animals following injection of RENCA cells and tumor vaccine, were challenged with a second injection of RENCA cells. The animals were considered tumor free 55 days after the first tumor injection and 22 days after the second tumor challenge.

Group (vaccine)	tumor free mice		
	exp. 1	exp. 2	exp. 3
DC-CpG-ODN	n/a	1/1	1/2
DC-RENCA-CpG-ODN	2/3	1/1	2/2

7.

Table 2 – Immune transfer by splenic T cells and resistance to tumor growth. T cells harvested from tumor free animals following challenge with RENCA cells were injected into naïve animals prior to s/c RENCA injection. Animals were considered tumor free in the absence of tumor growth 29 days following tumor injection.

group	tumor free mice	
	exp. 1	exp. 2
T cells from Control	0/2	0/5
T cells from DC-CpG-ODN	n/a	0/2
T cells from DC-RENCA-CpG-ODN	2/4	1/2

CHAPTER 5: GENERAL DISCUSSION

Thesis summary

The results presented in this thesis can be summarized by the following points:

- We have established a method that works in our hands for murine DC cultures. Using the StemSep negative selection system, the hematopoietic progenitors were isolated from the bone marrow of femurs and tibias of BALB/c mice. The progenitors were then cultured for 6 to 9 days with mGM-CSF and mIL-4. Differentiation of progenitors to DCs was verified by FACS analysis on the population obtained with the characteristic of DCs markers B7-1 and CD11c.

- RENCA Ag-pulsed DCs were able to prevent tumor growth, as long as tumor burden remained low. However despite continued treatment, RENCA Ag-pulsed DCs were unable to prevent the rapid increase in tumor growth that normally occurs at 2-4 weeks in RENCA.

- In earlier studies, it was suspected that RENCA induced an immunosuppression. The indication of this effect was first seen when comparing splenocyte proliferation to ConA in normal animals, compared to RENCA-bearing animals for 7 days. Indeed, splenocytes from RENCA-bearing animals showed much less proliferation than splenocytes from normal mice.

- DCs are potent APCs known to stimulate the proliferation of naïve T cells *in vitro* in response to antigenic stimulation. Indeed, Ag pulsed-DCs stimulated the proliferation of splenocytes isolated from normal animals twice as much as unpulsed DCs. However, DCs were unable to counteract the RENCA-induced immune suppression. In fact, RENCA-pulsed DCs induced less proliferation than unpulsed DCs in splenocytes isolated from RENCA-bearing animals.

- Splenic T cells from tumor-bearing animals maintained their IFN-γ production when compared to splenic T cells from normal animals. IFN-γ is a key cytokine to assess cytotoxic activity. In this case, the animals had received RENCA 7 days prior to the assay. Further studies have demonstrated that IFN-γ production was affected before day 7 and that it came back to normal levels thereafter (see next point).

- We realized at this point that the time after tumor injection at which the assays were performed was critical. A time course was performed in order to target the moment at which immune suppression was induced. Compared to normal animals, all parameters measured (splenocyte proliferation in response to ConA, T cell proliferation in response to IL-2, T cells IFN-γ production or NK cell activity) lead to an increased 3 to 5 days following tumor injection followed by a decreased to or below normal levels between days 6 to 9.

- As NF-κB is a very important transcription factor for the immune system, and because previous studies had demonstrated that its DNA-binding capacity was affected in human RCC, an EMSA was performed. It showed that NF-κB DNA binding activity was decreased in splenic T cells from tumor bearing animals compared to splenic T cells of normal animals.

- CpG-ODNs are known to induce DC maturation and Th1 cytokine production. DCs were treated with CpG-ODNs, control ODNs containing no CpG motifs or left untreated. RNA from DCs was extracted and submitted to an RNase protection assay. As expected, CpG-ODNs induced the expression of Th1 cytokines compared to control ODNs. To further confirm those results, IL-12 was detected by ELISA in the supernatant of CpG-ODN-treated DCs, whereas IL-12 was undetectable in supernantants of control CpG-ODN-treated- or untreated DCs.

- Having found the right timing for intervention and having in hand mature, Th1 cytokines-producing DCs, we tested our new strategy *in vivo*. We found that CpG-ODNs treated-DCs could virtually prevent tumor growth. This effect was potentiated when DCs were also pulsed with RENCA Ag.

- When tumors were detected in the CpG-ODNs-treated-DCs vaccinated animals group, they were very small and contained extensive lymphocyte infiltration compared to tumors from control animals.

- Animals from the CpG-ODNs and CpG-ODNs-RENCA pulsed- DCs groups that remained tumor free were able to sustain challenge from a second tumor dose.

- T cells isolated from tumor free challenged animals, and transferred to naive animals, conferred protection against tumor growth only when these T cells were isolated from CpG-ODN-treated-RENCA pulsed DC-vaccinated animals. In other words, only T cells from this group developed immune memory that was transferable to a naïve animal.

- Immunity conferred by transferred T cells was tumor specific, as the animals could not sustain tumor challenge from another tumor (specifically CT-26, a BALB/c colon carcinoma tumor).

Discussion

The main goal of this work was to study alternative therapies for RCC. As explained in the introduction, RCC does not respond to conventional therapies such as chemotherapy, radiation therapy or hormonal therapies (27, 32). The main factor responsible for this resistance (especially for chemotherapy and radiation therapy) is believed to be the expression of the *MDR-1* multidrug resistance gene (31). This is a logical explanation since the main function of the protein encoded by *MDR-1* is to prevent entry of drugs into the cell, or to promote their expulsion from the cell if they enter (182). Since spontaneous regressions of metastasis were observed in patients with RCC (1), immune-based therapies were attempted. However, only limited success has been obtained so far (6).

The critical question to answer remains why do the immune-based therapies have such limited success. There are two main categories (that can be combined) of immune-based therapies: the use of biological response modifiers (such as cytokines), the use of cellular therapy (such as TILs) or a combination of both. The major limitation of cytokine therapy remains its significant clinical toxicity (6, 30). The use of TILs involves extracting lymphocytes directly from the tumor, activating and expanding them *ex vivo* and re-infusing them to the patient (42). A randomized controlled trial using nephrectomy, blinded TIL/placebo infusion and IV IL-2 therapy did not demonstrate a benefit in the use of TILs over IL-2 alone (43). Although CTL clones, thought to be at the core of an effective antitumor immunity, constitute part of the TILs, this expansion and reinfusion technique failed to demonstrate a clinical advantage. One can postulate that the tumor itself may cause the TILs to be neutralized. This is very likely since RCC has been found to shed gangliosides (183) responsible in part with the inactivation of the transcription factor NF-κB and induction of T cell apoptosis (50). In consideration of these results, the development of an immune-based strategy to overcome this RCC-induced immunosuppression should allow a greater chance of success.

The theory of immunosurveillance was first elaborated early in the 20th century by Paul Ehrlich (184). He postulated that the immune system could repress a potentially "overwhelming frequency" of carcinomas. This idea was quite controversial at the time

and was not further explored until the 1950's when significant improvement in understanding the immune system occured. After many challenges to the theory, the official concept of immunosurvellance was defined by Burnet as follows: "In large, long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character." (185). This supports the concept that lymphocytes are sentinels able to eliminate nascent cancer cells.

As all hypotheses in science, this concept was later challenged. The study of tumors in nude mice triggered the opposition. Following the concept of immunosurveillance, it was logical to think that an immunocompromised host would be prone to develop spontaneous tumors and also be more susceptible to chemically induced tumors. However, it was observed that nude mice did not develop more tumors than their parental strain (186). Since the immunological defects of the nude mouse were not well characterized at the time, this and other data were highly convincing to reject the immnosurveillance hypothesis. Of course, we now know that nude mice are not completely immunoincompetent, due to NK cells and γδ T cells which are not thymic-dependent for their development (187). Consequent to the limited knowledge at the time, little interest was paid to the concept of immunosurveillance in the following years.

Enthusiasm for the hypothesis resurged in the mid 1990's when the role for different cytokines in tumor control was elucidated. Many studies were focused on IFN-γ. Whether IFN-γ signal transcription was disrupted, its receptor or gene knocked out, the results were the same: animals lacking IFN-γ activity were unable to control tumor growth (188). Similar results were obtained with perforin knock-out mice (189). Perforin is a key element in cell lysis by cytotoxic T cells and NK cells (190). The availability of RAG-1 and RAG-2 knock out mice lacking NKT, T and B cells (191) confirmed the immunosurveillance hypothesis. RAG (recombination activated gene) is involved in the repair of double-stranded DNA breaks, exclusively in the lymphoid

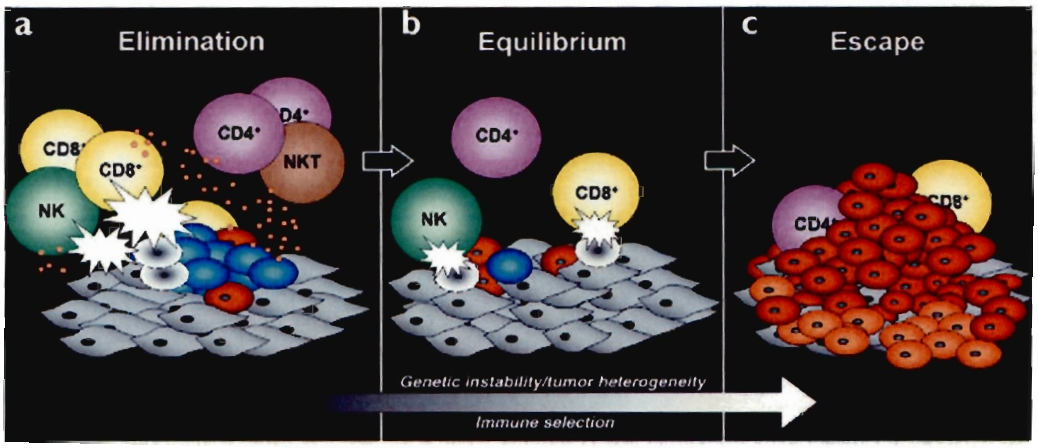
compartment. Consequently, the immune system in those animals is completely disrupted allowing the development of spontaneous tumors (192).

The question then arose as to whether immunosurveillance also occurs in humans. It was first demonstrated that immunosuppressed individuals, such as AIDS patients, were more susceptible to virally-induced tumors caused by Epstein-Barr virus, human herpesvirus 8 or human papillomavirus (193). It was also observed that patients who received immunosuppressive drugs following transplantation were more susceptible to develop spontaneous tumors (194). This supports the hypothesis of immunosurveillance in human.

Dunn et al. recently observed that tumors formed in immunoincompetent animals were rejected when transferred to an immunocompetent host (184). These results suggest that tumors are imprinted by the immunologic environment in which they form. This process can often result in the generation of tumors that are able to better resist to the immune system by eliminating highly immunogenic tumor cells. However, this process could also leave behind tumor variants of reduced immunogenicity (or that have acquired other mechanisms to evade or suppress immune attack) that have a better chance of surviving in the immunocompetent host. The hypothesis is that immunologic sculpting of tumors occurs continuously, but the sculpting is most prominent early when the tumor is very small and not clinically detectable. Consequently, the immunogenicity of most tumors that are clinically apparent has already been modified to some degree by their interactions with the immune system. Because the immune system exerts both host-protecting and tumor-sculpting effects on developing tumors, the term cancer immunosurveillance may no longer be appropriate to accurately describe this process. In fact, Dunn et al. have proposed the use of the broader term "cancer immunoediting" to describe more appropriately the dual host-protecting and tumor-sculpting actions of the immune system that not only prevent but also shape developing tumors. Figure 1 illustrates this theory.

Figure 1. *The three Es of cancer immunoediting.* Cancer immunoediting encompasses three processes. (a) Elimination corresponds to immunosurveillance. (b) Equilibrium represents the process by which the immune system iteratively selects and/or promotes the generation of tumor cell variants with increasing capacities to survive immune attack. (c) Escape is the process wherein the immunologically sculpted tumor expands in an uncontrolled manner in the immunocompetent host. In a and b, developing tumor cells (blue), tumor cell variants (red) and underlying stroma and nontransformed cells (gray) are shown; in c, additional tumor variants (orange) that have formed as a result of the equilibrium process are shown. Different lymphocyte populations are as marked. The small orange circles represent cytokines and the white flashes represent cytotoxic activity of lymphocytes against tumor cells.

From Dunn et al., 2002


a
Elimination
CD8+
CD8+
CD4+
NKT
NK
b
Equilibrium
CD4+
CD8+
NK
c
Escape
CD8+
Genetic instability/tumor heterogeneity
Immune selection

The data presented here are very convincing as to the existence of immunosurveillance in RENCA and further emphasize the special relationship that RENCA and RCC have with the immune system. Firstly, we have shown that a DC vaccine was not able to prevent tumor growth in an animal with a large tumor burden (Chapter 2, Table 1). Furthermore, RENCA has a detrimental effect on splenocyte proliferation (Chapter 2, Table 2). We then characterized how RENCA affects the immune response over time. We showed that splenic T cell IFN-γ production was affected by RENCA: levels of IFN-γ increased shortly after tumor implantation in the peritoneal cavity and decreased thereafter (Chapter 3, Figure 1C). It has been shown that IFN-γ is extremely important in immunosurvellance (188). Consequently, it is not surprising to observe an increase in tumor growth when immunosuppression occurs. Obviously, DCs pulsed with tumor antigens were not powerful enough to overcome the immune suppression induced by these cells.

Similar conclusions can be drawn from the loss of NK cell activity observed following tumor implantation. NK cells are part of the innate immune response and contribute to the elimination of foreign cells via perforin-mediated lysis (190). Perforin was shown to be a key element in immunosurveillance (189). Since the NK cell activity and perforin mechanism are closely linked, it is not surprising to observe a reduction in control of tumor growth that correlates with loss of NK cell activity (Chapter 3, Figure 1D).

Figure 3 in Chapter 3 confirms the ability of CpG-ODN treated DCs to allow activation and maturation of DCs. Although it has been thoroughly shown in the literature that CpG-ODNs activate DCs (117, 118, 195, 196), the use of the RNAse protection assay is novel. This technique is very powerful as it enables one to simultaneously visualize the increase in RNA transcripts of many different molecules, in this case, cytokines. This experiment clearly showed the increase of RNA transcripts of Th1 cytokines in CpG-ODN-treated DCs. It is important to realize that care must be taken when interpreting those results, as an increase in RNA levels does not necessarily reflect an increase in protein levels. Consequently, we chose to determine IL-12 concentration by ELISA, since this assay enables to measure protein concentration. IL-12 is a very important cytokine

for the orchestration of a Th1 response; it was chosen for this reason (Chapter 3, Figure 3C). Its concentration was indeed increased 26-fold when DCs were treated with CpG-ODNs as compared to DCs alone or DCs treated with a control ODN. This demonstrates the ability of CpG-ODNs to induce the production of Th1 cytokines by DCs and help orchestrate a significant cytotoxic immune response, which is essential for an anti-tumor response.

The core of the thesis is Figure 4 of Chapter 3 where we demonstrate that tumor growth can be prevented by vaccinating with CpG-ODN-treated DCs. It is very interesting to note that similar results were obtained with vaccination by CpG-ODN-treated DCs, whether these cells are RENCA antigen-pulsed or not. It is thus possible that the DCs recognize and process the tumor antigen *in vivo*. It has already been demonstrated, although in another tumor model, that DC can uptake, process and present antigens from apoptotic bodies (137) and also from live cells (197). The small difference in treatment efficacy that we observed between the RENCA pulsed and unpulsed groups, could be due to a difference in antigen uptake. Indeed, in the DC RENCA-CpG-ODN group, DCs are pulsed with antigens for four hours and then treated with CpG-ODN, inducing activation and maturation. In the case of the DC CpG-ODN group, their encounter with antigen happens only after maturation. It has been demonstrated that antigen uptake is much less efficient following DCs maturation (72). The data presented in chapter 4 suggest that although antigens may not be essential to establish tumor protection, their presence is required to establish a strong and durable long-term immune response. One could also argue that the response generated is not tumor specific since the presence of the antigen does not seem to be required. This hypothesis is refuted by the results reported in chapters 2 and 4. Indeed, we could clearly demonstrate that animals initially protected against RENCA tumor growth could sustain a second challenge with RENCA, but were not protected against another tumor challenge, in this case, the BALB/c syngeneic colon carcinoma CT-26.

Another group has obtained data similar to ours concerning tumor challenge (177). In both cases, a high percentage of animals were resistant to tumor challenge (Chagnon et al. 2003; 50%, Vicari et al. 2002, 75%). Of course, the fact that CpG-ODN-

treated DCs build a strong immune response plays a major role in this. But it is possible that the tumor cell population is not homogenous. This possibility is highly plausible. Could it be that the tumor cells are in fact composed of two or more populations? In the case of RENCA, this tumor has emerged spontaneously in a BALB/c mouse and has been passaged both *in vivo* and *in vitro*. To obtain a sufficient number of cells, the tumor has been passaged in many mice and pooled together (65). Thus, it is possible that, according to the immunoediting theory, the immune system has "sculpted" the cells differently. It is even plausible that the effect of the immune system has "sculpted" a certain population of cells to express non-immunogenic antigens. If the cells are not a homogenous population and express somewhat different antigens, the response to the vaccine would be different. This could explain why the anti-tumor response of the vaccine never reached 100%.

One of the perspectives (see below for more details) emerging from this work is the possibility to use the CpG-ODN-DC vaccine to treat humans affected with RCC. Some vaccination strategies have been attempted for RCC with limited success. I will discuss four strategies that present the most interest. The first strategy involves vaccination with a VHL peptide containing a specific mutation with an adjuvant (30). Although promising, this strategy has a major drawback: vaccine peptides have not been shown to be efficient to elicit a systemic immunity, especially the CD8+ cytotoxic arm, which is crucial for an anticancer response (198). The second strategy used in clinical trials is an HSP-based vaccine. The HSP is isolated from the tumor and carries tumor-specific peptides (44). The same drawback can be raised again, that these peptides are not efficient to help mount a cytotoxic response (198), since peptides are usually presented via MHC class II molecules to CD4+ T cells. An additional concern is the need for the vaccine to be custom-made for each patient, which can prove to be quite costly. However, most vaccine strategies chosen will need to be in part custom-made. The third strategy is to genetically engineer tumor cells to express co-stimulatory molecules (such as B7-1) to enhance the antitumor immunological response. So far, no benefit from this strategy has been demonstrated in RCC (45). There are probably technical reasons to explain these results such as the difficulty to achieve high expression levels of a specific molecule. Finally, a very promising approach was published by a German group in 2000 (155). It involved fusion of DCs to tumor cells, using these fused cells as a vaccine.

Promising results were obtained: four out of nineteen patients had a regression of the tumor. They explained their relatively low response rate by a technical problem associated with the fusion by electroporation (155). Unfortunately, a recent article put a shadow on the study (156). It seems that the author was found guilty of negligence for forging some of the results. Although no retraction has been issued as of now, this puts a serious doubt on the reported results. Therefore, none of these four strategies are today satisfactory enough to think about using them on a larger scale for cancer treatment. The data presented in this thesis are convincing enough to suggest that our strategy could be one that is likely to give positive results in humans.

Indeed, Hartmann and collaborators have clearly shown that human DCs responded well to CpG-ODNs (84). Tumors have been treated in many mouse models by injecting CpG-ODNs directly in the tumor (176, 177). These groups have suggested that this strategy can lead to *in vivo* DC activation. However, if we directly apply this strategy to humans, the dose of CpG-ODNs required for administration would be unrealistic. Furthermore, such an amount of CpG-ODNs might cause an adverse inflammatory response. So far, no CpG-ODN-DC vaccines have been attempted in human, but many clinical trials confirmed that the clinical use of DCs has little or no side effects (134, 135, 199). In clinical trials for prostate cancer, DCs were pulsed with a specific PSMA peptide that was HLA-A2-restricted (199). Although the response rate was significant, patients not bearing the HLA-A2 molecules were excluded from this protocol. The vast majority of Caucasians bear the HLA-A2 antigen, but it is rarely found in African-Americans for example (199). As there is no specific antigen identified yet for RCC, the DCs will have to be pulsed with an antigen extract made from the patient's own tumor. This targeted strategy will permit the inclusion of all patients. Ideally, DCs would be pulsed with one or more RCC antigens (when they are identified) and this would greatly decrease the labor involved in making a vaccine.

The choice to treat or not with this vaccine will have to be a choice of society. In an ideal world, cost would not be an issue and science would predominate. Unfortunately this is not the case and it is important for scientists to realize whether their project is feasible or not. The DC vaccine will be costly; on the other hand, there are no effective treatments for RCC at this time. One has also to consider that treatment with cytokines,

such as IFN-γ and IL-2, are also very costly. The side effects from these treatments prevent patients from working and often require patients to be hospitalized; there are thus indirect costs associated with such treatments. Such hidden costs should probably also be taken into account. Finally, RCC often affects people in the productive part of their life leading to a great loss, both on the human level and for the society at large. Although DC vaccine could be costly at first, it could also be adopted for the treatment of many cancers. In the long term, if demand increases, costs of production should go down and the DC vaccine should become accessible to a larger population. All sides considered, this approach has great potential.

Perspectives

This project opens the door to many possibilities, both to better understand the mechanism involved in tumor protection in animals, but also to the possibility of pursuing human clinical trials as previously discussed. Before proceeding to human clinical trials however, it will first be essential to evaluate the activity of DCs in RENCA bearing animals. Although our results show that our immune-based stategy can prevent the initial growth and development of tumor cells, we need to show that it can also have a significant impact on animals with established tumors. We have shown in chapter 2 that the DC vaccine is not powerful enough to overcome RENCA-induced immunosuppression, presumably caused by a loss of the expression of co-activation molecules over time. However, we have also shown that much better results were obtained when DCs were also stimulated with CpG-ODNs. Therefore, although it is nearly certain that the DCs pulsed with RENCA antigens alone will have no or only very minor impact on tumor growth of tumor-bearing animals, effects of CpG-ODN-RENCA pulsed DCs would be very interesting to study.

Another way to answer this essential question would be to do *in vivo* trafficking experiments by comparing DCs alone and CpG-ODN-treated DCs in naïve and tumor bearing animals. The lack of efficacy of the DC vaccine could be explained by a shorter half-life of the DCs when they are not treated with CpG-ODNs. We showed in Figure 2 of Chapter 3 that the translocation of NF-κB to the nucleus of splenic T cells is defective. Consequently, it would also be interesting to demonstrate that the activity is reconstituted in animals vaccinated with RENCA pulsed-CpG-ODN-treated DCs. The activity of NF-κB is expected to be restored and this would explain the effectiveness of the vaccine.

The results from these animal studies would be easier to project to a human setting if regression of established tumor is achieved. The data presented here showed that prevention of tumor growth is an important first step in this process. But thinking in terms of human treatment, a preventive vaccine would not be feasible. First, the entire population could not be vaccinated against every cancer and second, the costs of such an operation would be enormous and not acceptable.

In order to better understand the mechanism of the RENCA-pulsed-CpG-ODN-treated-DC vaccine-induced long term immunity, further analysis of the immune transfer experiments are needed. In the experiments performed, whole splenic T cells were transferred. To understand how this protective effect mediated by T cells happens, transfer of CD4+ and CD8+ T cells separately would be indicated. It is expected that this protection will be mediated by CD8+ cytotoxic T cells since it has already been shown that CD8+ depleted animals are not protected against tumor by CpG-ODNs (177).

The ultimate goal of this work remains to apply this approach to treatment of human RCC. The protocol for establishing human DCs from peripheral blood is well established since clinical trials are being performed (135). Nevertheless, our team will have to establish that the technique works in their hands and the entire protocol will have to be performed in an adequate facility. The protocol for tumor antigen extraction will also have to be validated. We have shown the effectiveness of the technique for RENCA (Chapter 2), it has to be demonstrated to be the same for RCC. Finally, it has been demonstrated the TLR9 has different specificities in different species (161). Consequently, care will have to be taken when the CpG-ODN is chosen.

Ultimately, the understanding of the precise mechanism underlying the protection induced by antigen-pulsed-CpG-ODN-treated-DC vaccination could help to cure not only patients with RCC, but also patients with many different types of cancers, thus representing a more than welcome alternative for many people.

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APPENDICES

Reprint

Chagnon, F., Thompson-Snipes, L., Elhilali, M.M., Tanguay, S. Murine renal cell carcinoma: evaluation of a dendritic cell tumour vaccine. BJU International (2001) 88: 418-424.

BJU International (2001), **88**, 418–424

Murine renal cell carcinoma: evaluation of a dendritic-cell tumour vaccine

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Objective To use a murine model of renal cell carcinoma (RCC), Renca, to aid in developing a dendritic cell (DC)-mediated tumour vaccine for RCC; as conventional therapy has been unsuccessful for RCC and therapy using immune modulators has had limited success, novel therapies enhancing further the immune system must be developed.

Materials and methods DCs were obtained from mouse bone marrow enriched for the haematopoietic progenitors, and cultured in the presence of interleukin-4 and granulocyte macrophage-colony stimulating factor. *In vivo* vaccines and *in vitro* proliferation assays were used to assess ability of the DCs to present tumour antigen.

Results The presence of DCs was confirmed in the cultures by fluorescent-activated cell sorting analysis. *In vivo*, tumour-bearing animals receiving tumour extract-pulsed DCs as a vaccine showed a two to threefold reduction in tumour growth at day 12 and day 16 but no significant difference at day 28. *In vitro*, tumour extract-pulsed DCs stimulated significant proliferation of splenocytes from naive animals but not tumour-bearing animals. In addition, splenocytes from tumour-bearing animals had an attenuated immune response *in vitro*.

Conclusion These results show that it is possible to use the DC vaccine to modulate the immune response to achieve an antitumour effect, but further manipulation of the DC vaccine may be needed to overcome the tumour-induced immune suppression.

Keywords dendritic cell, tumour immunity, renal cell carcinoma, Renca, tumour vaccine, immunotherapy, immune inhibition

Introduction

RCC accounts for ≈ 2% of cancers or 150 000 new cases per year worldwide and is a major clinical problem with nearly one third of the patients having metastatic disease and unfavourable prognosis [1]. RCC is a particularly difficult tumour to treat as it presents relatively late in the course of the disease and responds poorly to conventional treatments such as chemotherapy or radiation therapy [2]. However, as some renal cell metastases show spontaneous regression it appears that this disease can be modulated by the immune system. Accordingly, the strategy of most recent treatment protocols for RCC is to stimulate the patient's immune system to generate an antitumour response, using immunostimulatory cytokines such as interleukin-2 and/or interferon-α as therapeutic agents [3]. Treatment with high-dose interleukin-2 produces response rates of ≈ 20% that are durable for 3 years [3]. To increase the response rate, improved therapies using lymphokine-activated killer cells (LAKs) and tumour-infiltrating lymphocytes (TILs) in combination with interleukin-2, have been attempted. In one phase III clinical trial using TILs or LAKs in combination with interleukin-2 there was better survival than in patients receiving interleukin-2 alone [4]. One of the major difficulties in the successful treatment of advanced RCC seems to be the ability of the tumour cells to suppress the patient's immune response against the tumour [5].

As an alternative approach to RCC treatment, we propose using dendritic cells (DCs) in a tumour vaccine. DCs are capable of presenting tumour antigens to immune effector cells and stimulating an effective antitumour response [6]. Indeed, DC therapy has already shown some efficacy in the treatment of human prostate cancer, B-cell lymphoma and melanoma [7–10] as well as in many murine tumour models, including B-cell lymphoma and sarcoma [6]. More recently, a DC vaccination strategy for RCC using tumour cell-DC hybrids as antigen [11] has shown therapeutic potential. In this trial, four patients of 17 completely rejected the metastatic tumour lesions, one had a mixed response,

Accepted for publication 5 April 2001

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and in two the tumour mass reduced by more than half. Although this trial was promising, several variables can be optimized to generate a more effective antitumour response. A good animal model for developing DC strategies to vaccinate against RCC would be helpful in optimizing and overcoming the many technical challenges posed by immunotherapeutic approaches to RCC.

We have begun to evaluate the efficacy of DC vaccination for the treatment of RCC in the murine form of RCC known as Renca. The Renca model of RCC fulfils many of the requirements for a useful animal tumour model; the tumour cells are of spontaneous origin, syngeneic to Balb/c mice, confirmed to be an adenocarcinoma by histology, have a predictable growth rate both *in vitro* and *in vivo*, and form a primary tumour mass within the mouse. In addition, Renca shows progressive disease stages similar to human RCC. For example, transplanted Renca forms solid tumours that develop spontaneous metastases to distant organs such as lungs and liver [12]. Furthermore, Renca induces many of the immunosuppressive effects of human RCC such as defective T-cell signalling [13,14]. Taken together, all these features make Renca a challenging but relevant model of RCC.

There have been many attempts to treat Renca, e.g. many groups have tried with success to induce tumour regression with cytokines, such as interleukin-12, -2 and -4 [15–17]. Gene transfection of tumour cells with apoptosis-related molecules such as Fas have also been attempted with success [18]. These methods are very useful to understand more about Renca but they are not applicable in humans, mostly because of toxicity or lack of effectiveness [4,19]. It is self-evident to find a way to reduce tumour growth in Renca that would also be applicable to human; the DC vaccine fulfils that criterion.

Using the Renca model, we tried to determine whether the DC vaccine could be a strategy of choice to treat RCC and overcome the immune suppression it induces. In this report we provide evidence that Renca shows immune suppression similar to that from human RCC. In addition, we show that the use of DCs to stimulate the immune response to tumour is promising for developing a vaccine against the tumour.

Materials and methods

Balb/c mice (4–6 weeks old) were generously provided by the Montreal General Hospital Research Institute breeding facility, or were purchased from Charles River (Saint-Constant, Quebec, Canada). The Balb/c RCC cell line, Renca, was obtained from Dr I. J. Fidler (Houston, TX, WA), and the Balb/c fibroblast cell line, CL-7, was obtained from the ATCC (Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 5% fetal bovine serum (FBS), 2 mmol/L L-glutamine, penicillin G, streptomycin and amphotericin B (Gibco BRL, Life Technologies, Grand Island, NY, USA).

To purify T cells, spleens were collected from normal or tumour-bearing animals. The red blood cells were lysed and the remaining cells submitted to the StemSep negative-selection system (StemCell Inc., Vancouver, BC, Canada). The T cells were then used for the ELISPOT® assay or for measuring their proliferation in response to 1000 U of recombinant human interleukin-2 (Pharmingen, Mississauga, ON, USA). The T cells were cultured in RPMI containing 5% FBS, 2 mmol/L L-glutamine, penicillin G, streptomycin and amphotericin B (Gibco).

To measure interferon-γ the ELISPOT assay was used; all the antibodies were purchased from Pharmingen. Briefly, ELISA plates were coated with the capture primary antibody (purified rat antimouse interferon-γ, 5 μg/mL) and incubated overnight at 4°C. The plates were then blocked with PBS containing 1% BSA and 0.05% Tween-20. After washing the plates three times with PBS/0.05% Tween-20 (wash buffer), the cells were added in complete medium and incubated overnight at 37°C. The plates were washed three times and the second biotinylated antibody added (biotin rat antimouse interferon-γ, 0.5 μg/mL). The plates were incubated for 1 h at room temperature, washed three times, and avidin-horseradish peroxidase added (5 μg/mL). After incubating the plates for 1 h at room temperature they were washed five times and 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB, Vector Laboratories, Burlingame, CA, USA) was added, followed by incubation in the dark for 30 min. Finally, TMB was removed, the plated rinsed with water, and fluid removed. The number of spots present were counted under a dissecting microscope and expressed as mean of triplicate cultures.

DC cultures were established as described by Inaba *et al.* [20]; briefly, bone marrow cells were submitted to a Lympholite density gradient (Cedarlane, Hornby, ON, Canada) and enriched for haematopoietic progenitors using the StemSep negative selection system (StemCell Inc.). The cells were cultured at a concentration of 10^6 cells/mL in a 24-well plate in OPTI-MEM supplemented with 5% heat inactivated FBS (Gibco), and 1000 U/mL each of granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (generously provided by Schering-Plough, Madison, NJ, USA). The cultures were fed every other day by removing half of the medium and replacing it with fresh medium containing GM-CSF and interleukin-4. The cells were harvested after 6–9 days of culture.

For immunocytometry, the cell samples containing 10^6 DCs were incubated with 2.4G2, an antibody

directed against the FcRIIγ receptor, and then stained with different directly fluorescence-conjugated antibodies at 4°C for 40 min. The antibodies used were anti-CD11c, anti-B7.1, and the appropriate isotype controls (Pharmingen). Propidium iodide (Roche Diagnostics, Laval, QC, Canada) was used to exclude dead cells from the analysis. The cells were analysed on a fluorescent-activated cell sorting (FACS) Scanner using the CellQuest software (Becton Dickinson, Mississauga, ON, USA).

To extract tumour antigen, Renca and Balb/c CL-7 cell lines were cultured in 150 cm^2 Petri dishes (Sarstedt, St-Leonard, QC, Canada) using phenol red-free DMEM supplemented with 5% FBS, 2 mmol/L L-glutamine, penicillin G, streptomycin and amphotericin B (Gibco). Cell-surface antigens were extracted from semi-confluent cultures with citrate-phosphate buffer (0.131 mol/L citric acid/0.066 mol/L Na_2HPO_4, pH 3) as previously described [21]. The cell extracts were subsequently concentrated on SepPak C_{18} cartridge (Millipore, Bedford MA, USA), lyophilized (Virtis, Gardiner, NY, USA) and stored at −80°C. The protein concentration was determined by BCA analysis (Pierce, Rockford, IL, USA). The cell extracts were reconstituted in serum-free OPTI-MEM before loading onto DCs.

For the splenocyte proliferation assay, mice were injected intraperitoneally with 10^6 Renca cells, and their spleens harvested 7–14 days later. Then 10^6 DCs were pulsed for 3 h with either 10 μg of Renca, or CL-7 cell extracts, or incubated with serum-free OPTI-MEM as a negative control. DCs were irradiated (3000 rads) before being extensively washed. Irradiated DCs (5×10^4) were incubated, in triplicates, with 2.5×10^5 splenocytes in round-bottom 96-well plates. Spleens cells alone were used as the background control. As a positive control, spleen cells were incubated with 2.5 μg/mL of concanavalin A (Roche Diagnostics); 2 days later, the cells were incubated overnight with 37 Bq/well of ^{3}H-thymidine (248 GBq/mmol). The cells were then harvested and counted using a MicroBeta Instrument (Wallac Oy, Turku, Finland).

For the therapeutic experiments, 40 Balb/c mice were injected with 10^5 Renca cells subcutaneously on day 0. The vaccine was prepared by pulsing DCs as described above. The mice were treated by intravenous injections of antigen-pulsed DCs twice a week, for 4 weeks, starting on day 4 after tumour cell injection. Groups containing 10 animals each received 5×10^5 DCs/mouse, pulsed with either Renca extracts, Balb/c CL-7 extracts, medium, or 200 μL of Hank's balanced salt solution alone. The tumours were measured using a Vernier calliper (Scienceware, Pequannock, NJ, USA) in two dimensions twice a week and the tumour volume determined using the formula $(A \times B^2)/2$, where A is the longest measurement and B the shortest. All data were assessed statistically using ANOVA.

Results

Analysis by FACS confirmed that most cells within the DC cultures showed typical DC markers such as CD11c and B7–1 [22], as shown in Fig. 1. The results of DC vaccination on the growth of Renca tumours in mice are shown in Table 1; only vaccination using DCs pulsed with Renca cell extracts significantly slowed the initial growth of Renca tumours during the first 3 weeks. On day 12 the mean tumour volume in animals receiving Renca-pulsed DCs was less than that in animals receiving unpulsed DCs ($P<0.05$). By day 16 the tumours in control animals had grown significantly (mean 152 mm^3) while the tumours in animals receiving Renca-pulsed DCs were only 54 mm^3 ($P<0.05$). Thus, DC vaccination reproducibly shows antitumour effects in an antigen-specific manner in the early stages of tumour growth.

However, continued treatment with Renca antigen-pulsed DCs was unable to prevent the rapid increase in tumour growth that normally occurs at 2–4 weeks in this model; by day 28, the tumour volume was similar in all experimental groups, irrespective of DC vaccination (Table 1). Thus, the antigen-specific, antitumour effects of the DC vaccination at the early times were only transient in this murine model of RCC, suggesting that either the DC vaccine is an ineffective long-term treatment for RCC, or that additional immunosuppressive factors need to be addressed before DC vaccination for RCC is to be effective.

Immunosuppression is a condition that might limit the effectiveness of the DC vaccine. To verify that mice injected with Renca tumour cells have a suppressed immune response, whole spleen cells were tested for their *in vitro* response to mitogen stimulation. Table 2 shows that splenocytes from Renca tumour-bearing mice showed much less proliferation in response to stimulation with the mitogen concanavalin A than did normal mice.

DCs are known to be potent antigen-presenting cells, as they have the unique ability to stimulate naive T cells *in vitro* to proliferate in response to antigenic stimulation [6]. DCs might assist in reactivating the proliferative response of the Renca growth-suppressed splenocytes. To test this, we investigated whether DCs loaded with tumour-cell derived antigens could stimulate lymphocyte proliferation *in vitro*. As shown in Table 2, DCs loaded with either Renca or CL-7 cell extracts were able to stimulate *in vitro* twice the proliferation in normal mouse spleen cells than in control cultures using unpulsed DCs ($P<0.05$). This reaffirmed the ability of DCs to stimulate

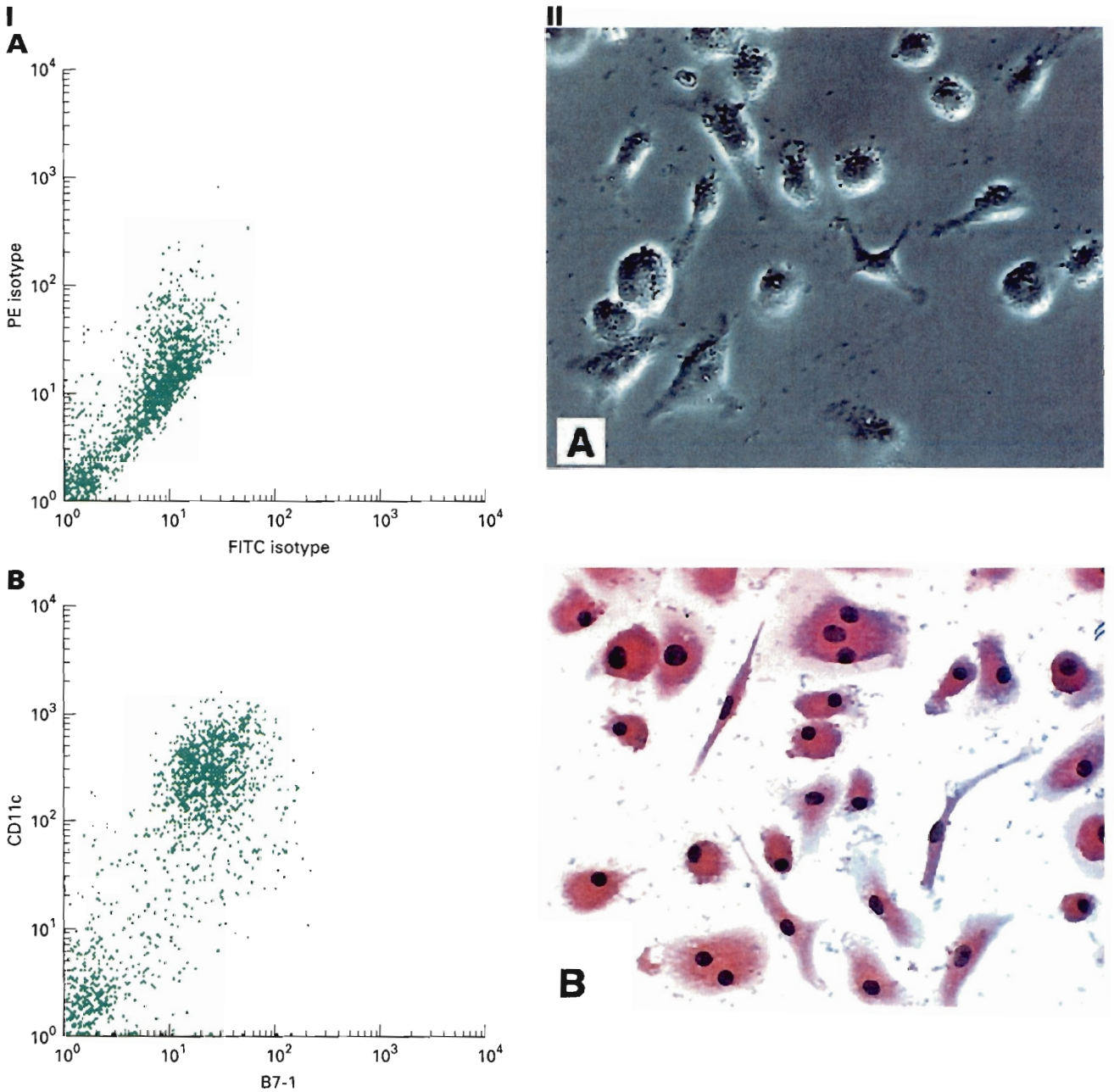

Fig. 1. I, The cell surface phenotype of bone marrow-derived DCs as determined by FACS analysis. On day 7 of culture, 10^6 cells were double-stained with fluorescent-labelled antibodies and propidium iodide used to exclude dead cells. Upper panel (A) isotype controls; lower panel (B) B7 coupled to fluorescein isothiocyanate and CD11c coupled to phycoerythrin. II, (A), DCs in culture fixed onto glass slides and viewed by phase-contrast microscopy. (B), Haematoxylin-eosin stained cells. Both ×250.

spleen cells *in vitro* to any antigen. However, splenocytes from tumour-bearing animals had a significantly lower proliferative capacity in response to DC stimulation than splenocytes from normal animals. Tumour-bearing animals appear to have immunosuppressed splenoctyes.

To test whether some immune functions are preserved in tumour-bearing animals, we determined whether T cells from tumour-bearing animals could be induced to produce interferon-γ, an activator of natural killer cells and cytotoxic T cells, both important effector cells for tumour killing. The production of interferon-γ induced by phorbol myristate acetate and ionomycin was no less in T cells from tumour-bearing mice than in T cells from normal age-matched controls, with respective mean (SEM)

Table 1 The effect of DC vaccination on subcutaneous Renca tumour growth

Mean (SEM) variable	*DC alone*	*DC+CL-7*	*DC+Renca*	*HBBS*
Tumour volume (mm^3) at day				
12	76 (7)	71 (14)	12 (6)*	61 (20)
16	152 (39)	164 (50)	54 (14)*	154 (24)
28	494 (129)	844 (237)	809 (210)	683 (33)

*Significantly smaller tumour volume ($P<0.05$) between Renca-pulsed DC and the other groups. There was no significant difference between the groups at day 28. Representative data from three different experiments.

Table 2 DC- and ConA-mediated stimulation of spleen-cell proliferation

	Mean (SEM) spleen-cell proliferation, d.p.m.	
Group	*Normal*	*Tumour-bearing*
DC+Renca	6470 (120)	443 (120)
DC+CL-7	6120 (912)	1643 (656)
DC alone	3317 (727)*	1556 (438)
ConA (2.5 μ/mL)	234200 (9100)	127000 (5000)*

*$P<0.05$, values are mean of triplicates.

values of 9.3 (1.1) and 10.9 (2.9) spots/10^4 cells, respectively. Thus, although the proliferative capacity of the lymphocytes to mitogens and DC stimulation is markedly reduced in tumour-bearing animals, at least some of the immune effector functions, as exemplified by the preservation of interferon-γ inducibility, remained intact.

Discussion

RCC has been known to suppress the immune response in humans and in animal models [13,14,23]. DCs have been used successfully to stimulate significant immune responses to a variety of tumours [6–10]. The aim of this study was to determine if tumour antigen-pulsed DCs were able to generate a specific immune response leading to tumour regression and overcoming immune suppression in an animal model of RCC. We successfully generated DCs from cultures of mouse bone marrow progenitor cells in the presence of interleukin-4 and GM-CSF. These DC preparations had high levels of expression of surface markers CD11c and B7–1, typical of the DC phenotype [22]. We intend to use these cells in the present animal model of RCC to develop a successful vaccine against the tumour.

The Renca tumour cells suppressed the immune response when administered *in vivo*, consistent with what others have shown [13,14]. Spleen cells from animals exposed to the Renca tumour *in vivo* have a lower mitogenic response *in vitro* than do normal animal splenocytes. In addition, we showed, for the first time in the Renca model system using DCs as antigen presenters, that Renca-bearing animals have a reduced response to stimulation by DCs. In a normal *in vitro* challenge with antigen, as others have shown [24], the antigen-pulsed DCs would have been expected to stimulate the proliferation of splenocytes that had already been exposed to antigen *in vivo*. The absence of such a response and the presence of inhibition indicates the presence of strong inhibitory influences by the Renca tumour. This is consistent with and reinforces the previous observation by Gregorian *et al.* [13,14] of a suppression in immune effector cells in Renca-bearing mice.

Previous studies and more recent evidence suggest that the immune suppression is induced by the release of factors such as PGs and gangliosides by tumour cells [13,14,23]. These factors could be responsible for inactivating the transcription factor NF-κB, recently observed in patients with renal cancer [23,25]. The NF-κB malfunction was also detected in Renca-bearing mice [26]. Despite the complexity of the immune system, there are only a few signalling pathways involved; one of the most important is the NF-κB pathway, which is involved in the transcription of almost all cytokines and some of the cytokine receptors. One example of a cytokine using the NF-κB pathway is interleukin-2, a key factor for T-cell growth [27]. Consequently, a NF-κB malfunction results in inadequate T-cell proliferation and expansion. Overcoming or inhibiting the inactivation of NF-κB by renal tumours will be necessary to achieve optimal immune response to tumour. In the present study, spleen cells taken from tumour-bearing animals had a decreased mitogenic response *in vitro* to stimuli, but there was no decrease in the ability of T cells to produce interferon-γ. Thus, the ability of the cells to divide and expand in response to signals may be inhibited, but some of the effector functions are not compromised.

The results from the present *in vivo* vaccination experiment are consistent with a deleterious effect of tumour burden on the immune response to Renca tumour. There was an adequate antitumour response at the beginning of the treatment while the tumour was small, but the response declined and by day 28 the tumour size increased dramatically, despite continuous vaccine treatment. No significant difference was detected between the tumour size in mice receiving Renca-pulsed DCs and the control groups after 4 weeks. These results show that DCs pulsed with tumour antigen can affect the growth of Renca tumour in the animals while tumour burden is low, but also reinforces the hypothesis that the Renca cells produce an inhibitory factor acting on the immune system when the tumour burden is high. The

present results suggest that there was a major inhibition of the immune response when a high tumour burden was present in the host; this might explain the prolonged survival in patients undergoing surgical resection of metastasis or nephrectomy in the presence of metastatic disease [28].

The response to Renca tumour *in vivo* was tumour-specific; by using an irrelevant antigen (CL-7) to pulse the DCs, the specificity of the vaccine was assessed. Indeed, while DCs pulsed with the CL-7 antigen stimulated the proliferation of splenocytes *in vitro*, vaccination with DCs pulsed with CL-7 extracts did not inhibit tumour growth, similar to un-pulsed DCs *in vivo*. Preliminary data on a human DC vaccine for RCC are very promising, with the generation of a strong specific antitumour immune response [11,29].

In conclusion, the present results indicate that the DC vaccine is a promising strategy to elicit a tumour-specific antitumour response. However, to improve the immune response against RCC, the inhibitory components generated by the tumours will have to be eliminated or overcome. We propose that the use of the Renca model for vaccine development is relevant to aid in the design of a DC-based tumour vaccine for RCC in humans. Tempering the immune-suppression caused by Renca will be a major challenge for future work.

Acknowledgements

The authors thank Drs Mary Stevenson and George J. Snipes for helpful suggestions and critical review of the manuscript. This work was supported by a grant to S.T. from F.R.S.Q. F. Chagnon was supported by a scholarship from F.R.S.Q.-F.C.A.R. Sante. L.T-S. was supported by a scholarship from the Fast Foundation of the M.G.H. Research Institute.

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Abbreviations: **LAKs**, lymphokine-activated killer cells; **TILs**, tumour-infiltrating lymphocytes; **DC**, dendritic cell; **DMEM**, Dulbecco's modified Eagle medium; **FBS**, fetal bovine serum; **TMB**, 3,3′,5,5′-tetramethylbenzidine; **GM-CSF**, granulocyte macrophage-colony stimulating factor; **FACS**, fluorescent-activated cell sorting.

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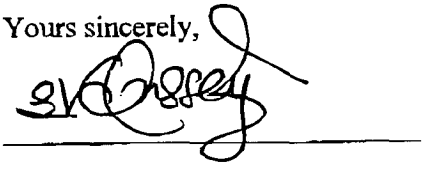

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Sincerely,

LuAnn Thompson-Snipes, Ph.D.
Department of Pathology,
Baylor College of Medicine,
Houston, TX
77030,
USA

McGill

July 29, 2003

Graduate and Postdoctoral Studies
James Administration Building, 4th floor
845 Sherbrooke Street West
Montreal, Quebec
H3A 2T5

To Whom It May Concern:

I, hereby, grant permission to Fanny Chagnon to use the manuscript entitled Potentiation of a Dendritic Cell Vaccine for Murine Renal Cell Carcinoma by CpG Oligonucleotides in her PhD thesis.

Sincerely,

Ozdem Levent Ozdal, MD
Urology Department
Montreal General Hospital
1650 Ave Cedar
Montreal, Quebec
H3G 1A4

Centre universitaire de santé McGill
McGill University Health Centre

Mostafa M. Elhilali, O.C., M.D., Ph.D., F.R.C.S. (C)

Université McGill
Titulaire de la Chaire Stephen Jarislowsky
Directeur, Division d'urologie

Hôpital Général de Montréal
Hôpital Royal Victoria
Urologue-en-chef

McGill University
Stephen Jarislowsky Chair of Urology
Director, Division of Urology

Montreal General Hospital
Royal Victoria Hospital
Urologist-in-Chief

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Mostafa M. Elhilali, MD, Ph.D.
Urology department
Montreal General Hospital
1650 Cedar
Montreal, Quebec
H3G 1A4

HÔPITAL ROYAL VICTORIA HOSPITAL, 687, av. des Pins O., S6.95, Montréal (Québec) H3A 1A1
Tél.: (514) 843-1516, Télec.: (514) 843-1552, e-mail: mostafa.elhilali@muhc.mcgill.ca

Centre universitaire de santé McGill
McGill University Health Centre

Simon Tanguay, M.D. F.R.C.S. (c)

Université McGill
Professeur Agrégé
Urologie Oncologique

McGill University
Associate Professor
Urologic Oncology

July 29, 2003

Graduate and Postdoctoral Studies
James Administration Building, 4th floor
845 Sherbrooke Street West
Montreal, Quebec
H3A 2T5

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I, hereby, grant permission to Fanny Chagnon to use the manuscript entitled: *Potentiation of a Dendritic Cell Vaccine for Murine Renal Cell Carcinoma by CpG Oligonucleotides* in her PhD thesis.

Sincerely,

Simon Tanguay, MD

HÔPITAL GÉNÉRAL DE MONTRÉAL
1650, av. Cedar, Montréal L8-309 (Québec) H3G 1A4, Tél.: (514) 934-8295 • Fax: (514) 934-8297
e-mail: stangu@po-box.mcgill.ca

Centre universitaire de santé McGill
McGill University Health Centre

Simon Tanguay, M.D. F.R.C.S. (c)

Université McGill
Professeur Agrégé
Urologie Oncologique

McGill University
Associate Professor
Urologic Oncology

July 29, 2003

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H3A 2T5

To Whom It May Concern:

I, hereby, grant permission to Fanny Chagnon to use the manuscript entitled: *Long term memory and immunity conferred by a CpG-ODN-treated DC vaccine for murine renal cell carcinoma* in her PhD thesis.

Sincerely,

Simon Tanguay, MD

HÔPITAL GÉNÉRAL DE MONTRÉAL
1650, av. Cedar, Montréal L8-309 (Québec) H3G 1A4. Tél.: (514) 934-8295 • Fax: (514) 934-8297
e-mail: stangu@po-box.mcgill.ca

Centre universitaire de santé McGill
McGill University Health Centre

Mostafa M. Elhilali, O.C., M.D., Ph.D., F.R.C.S. (C)

Université McGill
Titulaire de la Chaire Stephen Jarislowsky
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July 29, 2003

Graduate and Postdoctoral Studies
James Administration Building, 4th floor
845 Sherbrooke Street West
Montreal, Quebec
H3A 2T5

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Sincerely,

Mostafa M. Elhilali, MD, Ph.D.
Urology department
Montreal General Hospital
1650 Cedar
Montreal, Quebec
H3G 1A4

Centre universitaire de santé McGill
McGill University Health Centre

July 29, 2003

Graduate and Postdoctoral Studies
James Administration Building, 4th floor
845 Sherbrooke Street West
Montreal, Quebec
H3A 2T5

To Whom It May Concern:

I, hereby, grant permission to Fanny Chagnon to use the manuscript entitled Long term memory and immunity conferred by a CpG-ODN-treated DC vaccine for murine renal cell carcinoma in her PhD thesis.

Sincerely,

Mario Chevrette, Ph.D.
Urology department
Montreal General Hospital
1650 Cedar
Montreal, Quebec
H3G 1A4

Centre universitaire de santé McGill
McGill University Health Centre

July 29, 2003

Graduate and Postdoctoral Studies
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845 Sherbrooke Street West
Montreal, Quebec
H3A 2T5

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Sincerely,

Jean-Sébastien Ripeau

Jean-Sébastien Ripeau, M.Sc.
Urology department
Montreal General Hospital
1650 Cedar
Montreal, Quebec
H3G 1A4

July 29, 2003

Graduate and Postdoctoral Studies
James Administration Building, 4th floor
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Sincerely,

Ozdem Levent Ozdal, MD
Urology department
Montreal General Hospital
1650 Cedar
Montreal, Quebec
H3G 1A4

Compliance certificates

Centre universitaire de santé McGill
McGill University Health Centre

To whom it may concern,

A request was made by Ms. Fanny Chagnon to the McGill University Health Centre's Radiation Protection Service to provide a letter confirming that all radioisotope work was performed under a valid Internal Radioisotope Permit.

We can confirm through our records that Ms. Chagnon manipulated radioisotopes under an Internal Permit issued by the Radiation Protection Service to Dr. Simon Tanguay for the period beginning May 1999 through to May 2003. I am aware however that Ms Chagnon began using radioisotopes in September 1998. Unfortunately, due to the restructuring of the department that has occurred several times throughout the past 5 years, along with the relocation of documentation, I am unable to confirm that the proper paperwork was in order prior to May 1999. I will however state that I am quite confident that the acting Radiation Safety Officer at the time ensured that that all work was done under a valid Internal Radioisotope Permit.

Should you require any other information, please do not hesitate to contact me at (514) 842-1231 extension 36484.

Regards,

Daniel Alu
MUHC Radiation Protection Service

Centre universitaire de santé McGill
McGill University Health Centre

Internal Permit #: 6-1011-04

Permit Holder: Simon Tanguay **Office:** R1.107

Department: Urology **Telephone:** 44275

(A) Location

Room	Classification
R1.107	Basic
R1.113	Basic
R1.127	Basic
R3.107	Basic

(B) Authorized Activity

Isotope	Max Manipulated (MBq)	Max Purchase (MBq)
P32	9.25	9.25
H3	37	37

(C) Authorised Users

Last Name	First Name	P32	P33	S35	H3	C14	Fe59	I125	Ca45	Co57	Cr51	Na22	Rb86
Chagnon	Fanny	☑	☐	☐	☑	☐	☐	☐	☐	☐	☐	☐	☐
Ozdal	Levent	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Tanguay	Simon	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

(D) Conditions

The internal permit holder and the persons listed in section (C) are authorised to use the designated radioisotopes. The radioisotopes and their respective activities listed in section (B) can only be used in the laboratories listed in section (A) in accordance with the conditions listed in section (D). Importation, storage, manipulation and disposition of radioactive material must be performed in conformity with our CNSC licence, with Federal regulations and with the MUHC Radiation Safety Policies and Procedures. A copy of our CNSC licence is posted at RVH Research secretariat office and is also available at Radiation Protection Service (room S4.79, local 36133).

Approved by: Maureen McQueen
Radiation Safety Manager (36133)

Date issued: 26 June, 2002

Expiration date: June 01, 2004

Permis interne: 6-1011-04

Détenteur de permis: Simon Tanguay **Bureau:** R1.10

Département: Urology **Téléphone:** 44275

(A) Localisation

Room	Classification
R1.107	Élémentaire
R1.113	Élémentaire
R1.127	Élémentaire
R3.107	Élémentaire

(B) Activité authorisée

Isotope	Max Manipulated (MBq)	Max Purchase (MBq)
P32	9.25	9.25
H3	37	37

(C) Personnel authorisé

Last Name	First Name	P32	P33	S35	H3	C14	Fe59	I125	Ca45	Co57	Cr51	Na22	Rb86
Chagnon	Fanny	☑	☐	☐	☑	☐	☐	☐	☐	☐	☐	☐	☐
Ozdal	Levent	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Tanguay	Simon	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

(D) Conditions

Le détenteur de permis interne et les personnes inscrites dans la section (C) sont autorisés à utiliser les radioisotopes désignés. Les radioisotopes et leur activité respective listé dans la section (B) peuvent être utilisé dans les laboratoires listés dans la section (A), en conformité avec les conditions inscrites dans la section (D). L'importation, le storage, la manipulation ainsi que la disposition du matériel radioactif doit être effectué en conformité avec la license de la CCSN, les règles fédérales ainsi que les politiques et procédures de la radioprotection. La copie de la license de la CCSN est disponible au secrétariat de la recherche (HRV) et au bureau de la radioprotection (HRV salle S4.79, ext. 36133)

Approuvée par: _____
Chef du Service de radioprotection (36133)

Date d'entrée en vigueur: 26 June, 2002

Date d'expiration: 01 Juin, 2004

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 3716
Investigator #: 887
Approval End Date: Oct. 31, 2003
Facility Committee: MGH

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 3716

Title (must match the title of the funding source application): The immunomodulation of Renal Cell Carcinoma

1. Investigator Data:

Principal Investigator: Dr. Simon Tanguay **Office #:** 934-8295
Department: Urology **Fax#:** 934-8297
Address: MGH R1-107 **Email:** stangu@po-box.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Simon Tanguay	**Work #:**	934-8295	**Emergency #:**	428-8947
Name:	Fanny Chagnon	**Work #:**	934-1934 #44612	**Emergency #:**	734-0269

3. Funding Source:

External ☒
Source (s): Kidney Foundation of Canada
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: 07/2001 to 06/2003

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

ACTION	✓	DATE
	✓	Nov 28/02
DE		
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: Date: Oct 3 2002

Approval Signatures:

Chair, Facility Animal Care Committee:	Kathryn Hall	**Date:** Oct 9/02
University Veterinarian:	Rob Zatt	**Date:** Nov. 21 '02
Chair, Ethics Subcommittee(as per UACC policy):		**Date:** 11/21/02
Approved Period for Animal Use	**Beginning:** Nov. 1, 2002	**Ending:** Oct. 31, 2003

☒ This protocol has been approved with the modifications noted in Section 13.

ENTERED DEC 13 2002

OCT 17 2002

Centre universitaire de santé McGill
McGill University Health Centre

BIOHAZARD CONTAINMENT CERTIFICATION

The Health and Safety Committee of the Research Institute of the McGill University Health Centre (MGH site) has examined the following research project entitled:

"Immunomodulation of Renal Cell Carcinoma"

submitted by: **Dr. Simon Tanguay**

and certifies that the proposed research will be carried out under Level 2 containment conditions (Laboratory Rooms R1-113, R1-121) as specified in the Medical Research Council of Canada's Laboratory Biosafety guidelines (1990) and in compliance with all relevant governmental agencies' regulations.

Jean-Marie Chavannes
Secretary,
Health and Safety Committee

Certificate effective date: October 11, 2002
expiry date: October 11, 2003

Applicant

FORMS/bio